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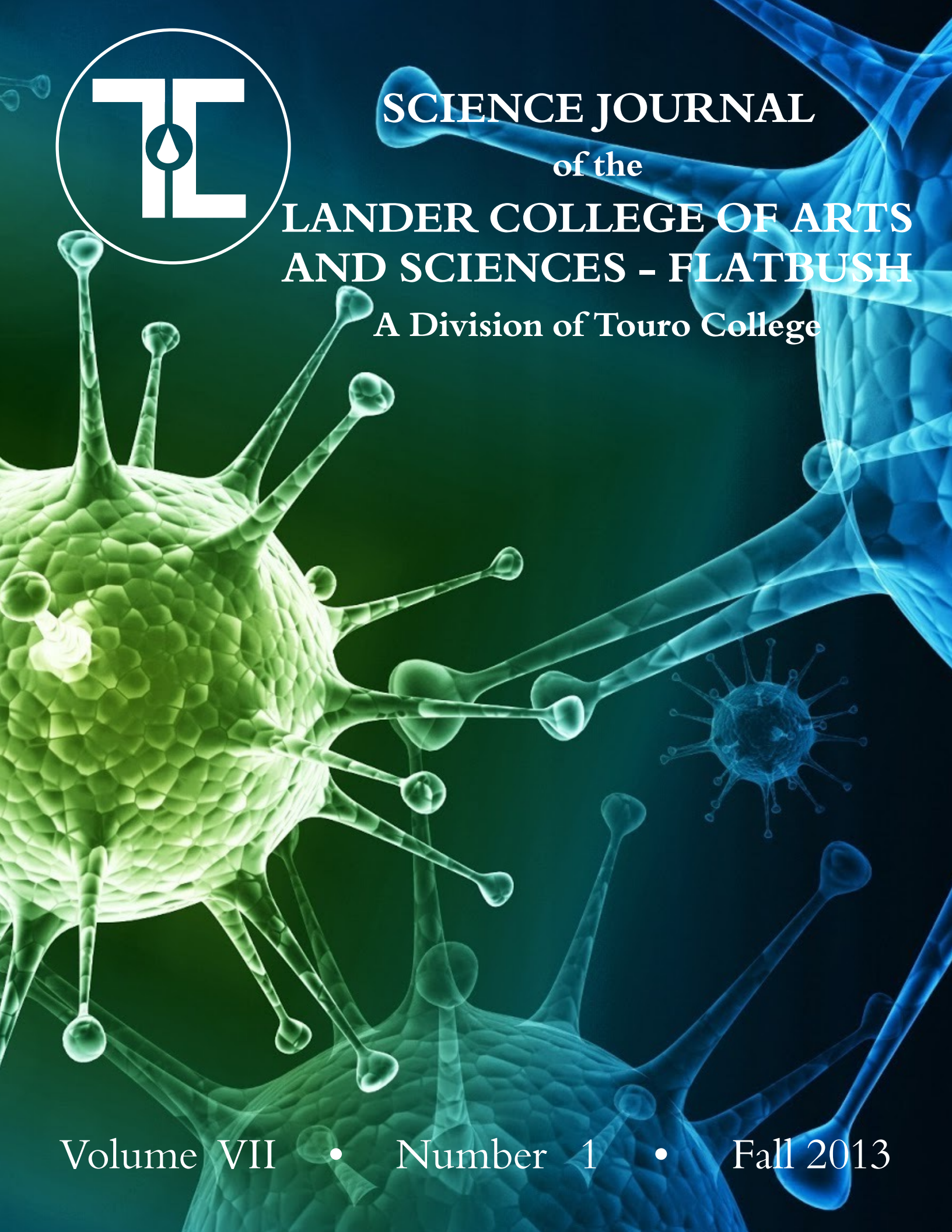

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The Lander College of Arts and Sciences at Touro in Flatbush

Throughout its 36-year history, Touro's Lander College of Arts and Sciences in Flatbush (with separate men's and women's schools) has provided cohorts of aspiring high school graduates from well-regarded yeshivas and seminaries with a foundation of academic excellence for professional career growth, in an environment that is supportive of the religious values of its students. Graduates have assumed leadership roles and continue to strengthen Jewish communities throughout the world.

Lander College of Arts and Sciences-Flatbush offers more than 25 majors and preprofessional options, and three joint undergraduate/graduate degree programs in occupational, physical therapy, and physician assistant studies with the School of Health Sciences. Honors tracks in biology, the health sciences, political science and psychology are currently offered.

Students are also required to complete a carefully designed core curriculum that emphasizes the development of communication skills, critical thinking and analytical competencies, computer literacy, and quantitative reasoning. Enrollment in science courses, notably biology and chemistry, continues to increase, reflecting the career interests of premedical and health science students. Faculty members continue to earn recognition for outstanding achievements, including Joshua November, Assistant Professor of Languages and Literature, who was selected as a finalist for the Los Angeles Times Poetry Book of the Year Prize in 2011; Karen Sutton, Assistant Professor of History, whose significant Holocaust analysis, "The Massacre of the Jews of Lithuania, 1941-44", was published in 2008; and Atara Grenadir, Assistant Professor of Art, whose works were displayed at the Art Expo 2011 show in New York City.

Notable alumni distinctions of Touro's Lander College of Arts And Sciences in Flatbush include; David Greenfield (JD, Georgetown), elected to the New York City Council (44th District) in 2010; Dr. Israel Deutsch (MD, Einstein) appointed as Director of Brachytherapy at New York-Presbyterian Hospital/Columbia University; Yossi N. Heber (MBA, Wharton), President, Oxford Hill Partners; Dr. Haim Mozes (PhD, NYU), Associate Professor Graduate School of Business, Fordham University; Vivian Schneck-Last, Managing Director, Goldman Sachs; and Sara Grossman-Weiderblank, who published her fourth novel, "Pass or Fail", in 2010. Alumni have published articles in the New York Law Journal, Bloomberg Law Reports, Institutional Investors Journal and other peer reviewed journals.

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Contents

What Is The Role of Incretin Mimetics In The Treatment of Type 2 Diabetes?

Isaac Silberstein

Abstract

Type 2 diabetes mellitus (DM) is an intricate disorder defined by insulin resistance, impaired insulin secretion, hyperglycemia, and both microvascular and macrovascular complications. Standard antidiabetic agents like metformin, insulin, sulfonylureas and thiazolidinediones are often insufficient at glucoregulation and do not address the decline in beta cell function that characterizes type 2 diabetes. Moreover, the adverse effects of some of these pharmaceuticals, such as hypoglycemia and weight gain, are disappointing and further limit their clinical utility. Research demonstrates that the actions of two potent incretins, Glucagon-like peptide 1 (GLP-1) and gastric inhibitory polypeptide (GIP), address these concerns as they stimulate beta cell activity, increase insulin secretion and decrease glucagon secretion both in a glucose dependent fashion, and increase satiety which results in weight loss. These effects have attracted increasing interest and excitement in the scientific literature as incretin mimetics have been introduced for patients in which first line therapy is unsatisfactory. Three drugs that mimic the actions of endogenous GLP-1 have been introduced---Exenatide, Exenatide LAR, and liraglutide---and this paper will focus on the role and efficacy that these novel treatment options play in the management of type 2 diabetes as clinicians are shifting away from traditional therapy.

Introduction

Type 2 Diabetes Mellitus (DM) is characterized as a chronic metabolic disorder disrupting the delicate balance of glucose homeostasis. Hyperglycemia, which is the most common hallmark of the disease, is closely linked with alterations in protein and lipid metabolism. Long term complications such as myocardial infarction, stroke, neuropathy, nephropathy, and retinopathy occur due to derangements in glucose metabolism. Most cases of diabetes are divided into two categories: Insulin-dependent or type 1 and Non-insulin dependent or type 2 diabetes mellitus. Type 1 diabetes comprises approximately 10% of the diagnosed population, and is usually caused by an autoimmune response of the body against the pancreas. Clinically, the pancreas is unable to secrete insulin and patients require insulin replacement in the form of subcutaneous injections in order to survive. Individuals with type 1 diabetes are usually less than 30 years of age, have no functional reserve in the pancreas, and display symptoms of polyuria, polyphagia, and polydipsia. In contrast, persons with type 2 DM make up approximately 90-95% of the diabetic population. Type 2 patients exhibit insulin resistance, manifested by a positive family history and obesity and display hypertension and dyslipidemia. The onset is often gradual and often goes undiagnosed for years. Treatment includes diet and exercise, in combination with medications. The prevalence of type 2 DM increases dramatically with age and obesity. Due to modern science, both men and women have increased longevity, which predisposes them to diabetes because of an aging pancreas. The risk of diabetes has increased fourfold among the elderly, who have increased amounts of abdominal fat (American Diabetes Association, 2009). There are also differences among ethnic groups, with the highest incidence found among American Indians and Alaskan Natives, followed by African Americans, Hispanics, and non-Hispanic whites. The health care costs of managing diabetes are staggering, with a total cost of about 170 billion dollars. Approximately, 55% of all medical expenditures spent on diabetes comprise the elderly. Physician visits, hospital and nursing home admissions, and medication, all place an excessive toll on overloading our already fragile health care system (Centers for Disease Control and Prevention, 2011). With both early prevention and diagnosis, type 2 diabetics can reduce long term complications and the burden on our nation's health care. This paper will, therefore, analyze the increased role of novel pharmaceutical agents called the incretin mimetics as a treatment approach to type 2 diabetes.

Methods

The author located the relevant information for this paper using the PubMed search engine in order to select and extract the most appropriate journal articles pertaining to the role of incretin mimetics in the treatment of type 2 diabetes.

Discussion

The pathogenesis of type 2 Diabetes Mellitus is extremely intricate, and research has uncovered several important factors contributing to the disorder. One of the main features of type 2 diabetes is insulin resistance during which the body cells are no longer sensitive to the actions of insulin. This unresponsiveness leads to reduced glucose uptake, rising blood glucose levels, and the utilization of both proteins and fat for energy (Peterson, Shulman, 2006). In addition, insulin deficiency is another factor leading to hyperglycemia, caused by defective secretion or a reduction in beta cell mass. Research has shown that insulin secretion is directly related to the amount of beta cells present in the pancreas. In fact, most patients with type 2 diabetes mellitus have lost about 50% of their beta cell function at the time of diagnosis, and this decline continues over time. With a steady loss of beta cells, the natural progression of the condition results in rising HbA1c levels, indicating poor glycemic control (Wajchenberg, 2007).

Additionally, impaired insulin secretion results in proteins being broken down into amino acids, which are then converted to glucose by a process known as gluconeogenesis. This new glucose released into circulation further aggravates hyperglycemia. Ultimately, this protein catabolism may even lead to organ dysfunction and muscle weakness (Guillet, et. al. 2012). Another factor contributing to hyperglycemia is hepatic

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glucose output resulting from non-inhibition of glucagon. This results in the degradation of glycogen to glucose by the liver and leads to elevated blood glucose levels (Dunning, Gerich, 2007). There is also growing evidence that adipose tissue plays a significant role in the pathology of type 2 diabetes. Under normal circumstances, insulin inhibits lipolysis, preventing an increase in free fatty acids in circulation. With impaired insulin secretion, however, hormone sensitive lipase breaks down triglycerides in adipose tissue, allowing high levels of Free Fatty Acids (FFA's) to accumulate in the blood. In the liver, the FFA's are converted into triglycerides. The excess triglycerides stored in the hepatic organ results in a fatty liver, which can eventually lead to cirrhosis. Moreover, the increased levels of FFA's stimulate hepatic glucose output from the liver, further aggravating the current hyperglycemic state (Bayes, et. al. 2004).

Type 2 Diabetes is usually diagnosed during a routine blood test. A fasting plasma glucose level greater than 126mg/dl on 2 consecutive occasions is highly suggestive of the disorder. The HbA1c level is a significant determinant in allowing the clinician insight into the patient's blood glucose regulation for the previous 60 to 90 days. The result of this test will determine whether one will develop long term complications such as neuropathy, nephropathy and retinopathy. The American Diabetes Association recommends that an HbA1c value below or equal to 7% is preferred for most type 2 diabetics (ADA, 2007). Another essential diagnostic tool is the C-peptide level test during which an amino acid is released from the beta cells in combination with insulin. This test is an indirect measure of insulin secretion and is important in determining the need for insulin in type 2 diabetes patients. The normal C-peptide level is between 0.17 to 0.83 umol/liter. Sinking below this level will alert the physician that some deterioration of beta cell function has occurred and that exogenous insulin may be required as a treatment option. Finally, another test known as the fructosamine assay measures glycation of albumin and affords the clinician critical insight into glycemic control over the previous 2 to 3 weeks (Van Cauter, et. al. 1992). The fructosamine assay demonstrates the most diagnostic utility when patients are afflicted with medical conditions, such as hemolytic and iron deficiency anemia, or blood loss transfusions, rendering the previously mentioned HbA1c test inaccurate. Therefore, the fructosamine assay would reveal glycemic control over the previous three weeks (Goldstein, et. al. 2004).

Treatment Strategy

The American Diabetes Association, the American College of Endocrinology, and the National Institutes of Health, have all published guidelines for the selection of medications that should be incorporated into the type 2 diabetes treatment plan. All three organizations agree that newly diagnosed patients should begin lifestyle changes such as diet and exercise, along with the drug metformin (Inzucchi, et. al. 2012). There is a disagreement, however, among the organizations concerning drug selection as an add-on to metformin when the patient doesn't reach their HbA1c target goal within a 3 month period. If the HbA1c value is still elevated after this period of time, then a second drug that has a different mechanism of action is added to metformin monotherapy. If after another 3 months, the HbA1c goal is still not reached, then a third drug or insulin injection may be incorporated in the antidiabetic treatment regimen. For example, insulin will usually be required when the HbA1c levels are above 10%, or when fasting blood glucose levels are greater than 250mg/dl, or in individuals who display symptoms such as polyuria or ketosis in the blood Therefore, the choice of which drug to select, coupled with metformin, is dependent on several factors: 1) Weight loss 2) Aversion to injection 3) Fasting and/or postprandial hyperglycemia 4) Cost of medications 5) Hepatorenal function 6) Pre-existing edema or heart failure 7) Erratic eating patterns 8) Osteoporosis (Inzucchi, et. al. 2012). All these factors will be further elucidated as each drug class is discussed at length.

Overview of Traditional Oral Therapies

To begin, metformin is an anti-hyperglycemic agent which has been shown to suppress hepatic gluconeogenesis. It also improves the sensitivity of both skeletal muscles and the liver to insulin, lowering insulin resistance (Kirpichnikov, et. al. 2002). Since metformin's mechanism of action does not involve insulin secretion, the drug does not pose significant issues with hypoglycemia and weight gain. Metformin can be used safely together with all other anti-diabetic agents, both oral and injectable, and has been recommended to treat pre-diabetes from developing into overt diabetes. The main adverse effects are gastrointestinal in nature, such as abdominal discomfort, cramping, nausea, and diarrhea. These side effects can be minimized by taking the medication with food and slowly titrating upward toward a maximal dose of 2000mg/day (Campbell, et. al. 1996). Chronic use of the drug may lead to malabsorption of folate and vitamin B12, so periodic blood tests are required to measure these levels (De Jager, et. al. 2010). Importantly, the use of metformin provides benefits, such as a significant reduction in of HbA1c levels and decreasing fasting blood glucose levels about 50-70 mg/dL (Kirpichnikov, et. al. 2002). It has an excellent safety profile, does not cause weight gain or hypoglycemia, and can be combined with other antidiabetic medications. All these advantages make metformin the physician's first choice in the treatment of type 2 diabetes (Inzucchi, et. al. 2012).

Another treatment option is the collection of Thiazolidinediones (TZD's), which are drugs that counteract insulin resistance by making skeletal muscle, adipose tissue, and liver cells more responsive to insulin. TZD's stimulate a peroxisome proliferator activated receptor-y (PPAR-Y), which in a diabetic is downregulated due to insulin resistance. Once triggered by the drug, this unique receptor activates genes which affect glucose and lipid metabolism. For instance, the enzyme Glut-4, which is the chief glucose transporter activated by PPAR-Y, enables the blood glucose to enter the cells and reduce hyperglycemia. TZD's have a slow onset of action with a maximum effect only visible after about 2 months. Fasting blood glucose is reduced by about 60-70 mg/dl, with an HbA1c decline between 0.5% and 1.4%. They are usually given in

combination with other agents, rather than monotherapy (Yki-Järvinen, 2004). The side effect profile and the high association with bladder cancer and bone fractures, has relegated these drugs almost extinct. For example, a study involving the TZD known as pioglitazone, and its prevention in macrovascular events, showed that patients receiving the drug had a higher incidence of bladder cancer compared to placebo (Dormandy, et. al. 2005). Additionally, French health officials found that patients who used pioglitazone had a 22% increased risk of bladder cancer, and that high doses and prolonged usage greater than 2 years placed patients at a high risk for the disease (Nuemann, et. al. 2012). A recent study confirmed that women who took TZD's had a higher fracture rate of about 25% due to a reduction in osteoblastic activity and an increased excretion of calcium in the urine (Bazelier, et. al. 2012). TZD's cause both significant edema and weight gain and, therefore, must be cautiously prescribed to patients with cardiovascular disease (Yki-Järvinen, 2004). For example, heart failure can be induced when TZD's are given to patients with pre-existing edema (Giles, et. al. 2008). Although TZD's target insulin resistance, their side effects and safety profile limit their use.

Yet, another drug option is the insulin secretagogues known as the sulfonylureas (SU). Originally, these drugs were considered first line agents in the treatment of type 2 diabetes until they were replaced by metformin. Their mechanism of action is blocking the outflow of ATP sensitive potassium channels and allowing the inflow of calcium intracellularly, which leads to increased insulin secretion (Panten, et. al. 1996). Therefore, these medications are only effective in patients with adequate beta cell reserve. The SU's reduce HbA1c levels of about 1.5%, with a reduction of fasting blood glucose similar to metformin. There are several major concerns when prescribing these medications. First, most type 2 diabetics are obese, with insulin resistance already present at the time of diagnosis. Since sulfonylureas cause insulin secretion, patients must eat to avoid hypoglycemic episodes, causing excess pounds to accrue, further aggravating insulin resistance (Porta, Trento, 2007). Second, these agents exhibit a primary failure rate ranging between 15% to 25%, and a secondary failure rate of 5% to 7% per year. Whether this failure is due to this drug class, or to the declining progression of the disease, remains unclear (Donath. et. al. 2005). Third, besides weight gain, hypoglycemia is an adverse effect, particularly in the elderly. In this population of individuals, large doses of a long acting SU preparation, in conjunction with renal dysfunction and reduced carbohydrate intake, are prime factors in causing hypoglycemia (Halter, Morrow, 1990). In addition to the SU's, but similar in action, are the non-sulfonylureas known as meglitinides. Like SU's, these drugs cause an increase in insulin secretion and require the patient to possess an adequate beta cell reserve. Their main function is in lowering postprandial glucose levels. They must be given approximately 30 minutes before meals, and if a meal is skipped, the dose should not be taken as the risk for hypoglycemia is increased. Due to their mechanism of action of insulin secretion, hypoglycemia and weight gain are to be expected (Koda-Kimble, et. al. 2013). The utilization of sulfonylureas has declined, mainly due to the increased number of hypoglycemic events, along with weight gain, which further exacerbates the resistance to insulin. Newer drugs, such as incretins, are replacing SU's because they either cause weight loss or are weight neutral, and do not induce hypoglycemia.

A New Paradigm of Treatment

In the 1960's, scientists wanted to test whether the route of glucose infusion in the body would produce a marked effect on insulin secretion. Indeed, when both oral and intravenous glucose infusions were offered to the participants of the study, the researchers found that even though equal amounts of glucose were infused through both routes, the oral glucose load produced a greater insulin response (Perley, Kipnis, 1967). A similar effect has been proven in other studies as well in regards to the amount of postprandial C-peptide released, as opposed to insulin, during an intravenous and oral glucose challenge (Nauck, et. al. 1986b). This remarkable effect has come to be known as the incretin effect, in which gut hormones, known as incretins, stimulate the secretion of insulin in response to food ingestion. The incretin effect has been well established to be present in both diabetics and nondiabetics. In type 2 diabetic patients, however, the incretin effect has been shown to be substantially diminished (Nauck, et. al. 1986a).

So what exactly are the incretins? There are mainly two incretins that have been identified: Glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). GIP is synthesized and secreted from the enteroendocrine cells, known as the K cells, the majority of which are located in the duodenum and proximal jejunum. GIP is released in response to ingestion of carbohydrates and fats and binds to specific pancreatic beta cells. Once bound, it carries out a number of interesting effects on glucose regulation. In the pancreas, GIP has been shown to increase glucose-dependent insulin secretion. It has also been shown to increase beta cell proliferation and mass even in an environment rich with chemicals toxic to beta cells, such as streptozotocin. And GIP has antiapoptotic and neogenetic effects on the beta cells. Although GIP has a multitude of effects of on the beta cells, it does not affect the alpha cells in any way. In addition, it does not influence gastric emptying or satiety. Even though GIP has numerous beneficial effects on carbohydrate metabolism, GLP-1 is secreted in much higher concentrations and lowers glucagon release (Baggio, Drucker, 2007). Most importantly, for reasons not well understood, studies have shown that GIP has lost a significant portion of its insulinotropic activity, which renders the hormone not very useful as a pharmacological agent (Meier, et. al. 2001). Therefore, the remainder of this paper will explore the other significant incretin: GLP-1.

GLP-1 is a peptide hormone synthesized and secreted from the L cells of the lower small intestine, specifically the distal ileum and colon (Baggio, Drucker, 2007). Once released, it exerts a number of glucoregulatory effects. For example, in a 1993 study, researchers examined the effect of GLP-1 in type 2 diabetic patients exhibiting hyperglycemia while in a fasting state. On the first day, the placebo subjects received an

administration of intravenous saline and their blood glucose levels decreased at a slow rate. On the following day, however, the participants received a GLP-1 infusion and their blood concentrations fell at a significantly faster rate. Based on this experiment, the investigators concluded that GLP-1 is effective at dramatically lowering glucose levels by stimulating insulin secretion. More interestingly, the researchers noted that GLP-1 stimulates the beta cells to release insulin in a glucose-dependant manner. That is, as the glucose concentrations fell to homeostatic levels, the ability of GLP-1 to stimulate insulin was slowly becoming diminished. This is an extremely vital characteristic of GLP-1: it will stimulate insulin secretion only under hyperglycemic circumstances, not when glucose levels are below normal; this action prevents the occurrence of hypoglycemia. Similarly, this study indicated that GLP-1 exerts a glucose-dependent effect on the alpha cells as well. GLP-1 will decrease glucagon secretion but only to an essential minimum. Thus, if glucose levels start to fall to the normal range, glucagon will stop decreasing and start to rise in concentrations, demonstrating its glucose-dependent action (Nauck, et. al. 1993).

In addition to acting as an insulin secretagogue and suppressing glucagon in a glucose dependent fashion, GLP-1 has been found to slow gastric emptying in type 2 diabetics (Wettergren, et. al. 1993).This finding is important because the gastric emptying rate has been observed to be accelerated in type 2 diabetics, which explains why their postprandial glucose levels are elevated (Smith, 1996). By delaying this process, GLP-1 ensures a more gradual release of glucose into the bloodstream, which can now be properly balanced by the delayed or reduced insulin response found in type 2 diabetics. This results in a slower rise in blood glucose after a meal (Wettergren, et. al. 1993). The mechanism of this action is as follows: high density receptors for GLP- 1 are located in the central nervous system, specifically the medullary structure called the area postrema (Yamamoto, et. al. 2003). After GLP-1 binds to its receptor in this area, it activates the efferent projections of the vagus nerve which ultimately decreases the smooth muscle contractions of the stomach (Nauck, et. al. 2002). As a consequence of lowering gastric activity, GLP-1 reduces appetite and, in turn, decreases food intake, which ultimately leads to weight loss (Parker, et. al. 2013).

GLP-1 also works by expanding beta cell mass, particularly beta cell proliferation and growth by activating the phosphatidylinositide 3-kinases. Although the precise mechanism has yet to be elucidated, it has been proposed that perhaps it is carried out by transactivating the epidermal growth factor receptor (Buteau, et. al. 2003). GLP-1 has been shown to decrease beta cell apoptosis in isolated human islets (Farilla, et. al. 2003) and increase neogenesis. The combination of beta cell proliferation and antiapoptosis results in beta cell mass expansion (Baggio, Drucker, 2007).

With all of the advantageous effects of native GLP-1 in type 2 diabetes, exogenous GLP-1 would nevertheless act as an ineffective pharmaceutical agent due to its very short plasma half-life of less than 2 minutes. This is because GLP-1's last two amino acids of its N-terminal, arginine and glycine, are immediately cleaved by the enzyme dipeptidyl peptidase-IV (DPP-IV). Following proteolysis, inactivation of the molecule occurs, rendering it therapeutically useless (Vilsbøll, et. al. 2003). Through what means then have researchers circumvented the challenge posed by the DPP-IV system? The answer has unexpectedly taken a turn to the southwest, where venomous lizards called Gila monsters reside. Analyzing their saliva, investigators have discovered a peptide molecule called exendin-4. Even though researchers are unaware of what function this molecule serves for the reptile, they are aware that Exendin 4 is resistant to degradation by DPP-IV because of its unique amino acid sequence (Holst, 2006). However, it nonetheless shares 52% amino acid homology with native GLP-1 (Chen, Drucker, 1997), and because of this similarity in structure, it binds to the GLP-1 receptor and mimics its glucoregulatory effects. From this knowledge, researchers have developed an antidiabetic drug, using synthetic exendin-4, called exenatide which is administered to patients in the form of a twice daily subcutaneous injection. Since this incretin mimetic is a peptide, it cannot be orally infused as it would be degraded by gastric secretions (Holst, 2006).

Exenatide has been found to mimic many key actions of GLP-1. First, it increases glucose dependent insulin production (Egan, et. al., 2002) and decreases glucagon secretion after meals in type 2 diabetic patients suffering from postprandial hyperglycemia (Kolterman, et. al. 2003). Exenatide has also been shown to reduce gastric emptying (Nielsen, et. al. 2004) and decrease food intake which results in weight loss (Szayna, et. al. 2000). Furthermore, exenatide has been shown to both enhance the first and second phase insulin response when the beta cells are exposed to infused glucose (Fehse, et. al. 2005) and increase islet cell growth (Tourrel, et. al. 2001). Another incretin mimetic is the GLP-1 agonist exenatide long acting release (LAR). In this unique form, exenatide is encased in microspheres to lengthen its interval of action. Unlike twice daily exenatide, the long acting formulation is more convenient as it is administered as a once weekly subcutaneous injection. The GLP-1 agonist escapes from the microspheres over a period of 6 to 10 weeks but exerts its complete effect after 9 weeks. Because of this, the clinical effectiveness of exenatide LAR cannot be determined immediately (Krause, Kirwin, 2010). Clinical trials comparing the effectiveness of exenatide with its long acting counterpart demonstrated that exenatide once weekly produced a significantly greater decline in A1C and fasting blood glucose levels. In fact, the HbA1c value for the exenatide LAR group dropped 0.7% greater than the twice daily exenatide group from baseline, and the fasting blood glucose value declined by a difference of 23mg/dL between the two groups (Blevins, et. al. 2011).

In addition to exenatide, once daily liraglutide was introduced as yet another incretin mimetic. This analogue has a 97% structural identity with endogenous GLP-1 by the substitution of the amino acid arginine for lysine near the tail of the molecule and the linkage of a C16 fatty acid to lysine at position 26. This acylation allows liraglutide to bind with serum

albumin and protect it from the proteolytic effects of DPP-IV (Agersø, Vicini), greatly increasing the biological half- life of the drug to approximately 12.6 hours (Agersø, et. al. 2002). As with exenatide, liraglutide has been shown to exhibit similar effects like glucose dependant insulin production, glucagon suppression, beta cell growth, decreased gastric emptying time, and satiety, all of which lead to weight loss (Wajcberg, Amarah, 2010). In a 2009 study, researchers formed two groups of participants who were on metformin, a sulfonylurea, or both. One group received liraglutide and the other was treated with twice daily exenatide. This trial demonstrated that liraglutide lead to a modest decline in HbA1c (-1.12%), as opposed to exenatide (-0.79%) but was nevertheless a superior glycemic controller (Buse, et. al. 2009). Furthermore, patients who replaced their exenatide treatment with liraglutide showed an even further decline in HbA1c (-0.32%) (Buse, et. al. 2010).

There are a number of safety considerations and adverse effects that clinicians should keep in mind before prescribing an incretin mimetic. For example, nausea was by far the most common complaint experienced by those receiving treatment exclusively with liraglutide or exenatide. Clinical trials have demonstrated that such gastrointestinal disturbances, however, subsided the longer the treatment progressed. In addition, increasing the dose of exenatide from 5-µg to 10-µg increased the risk of gastrointestinal related side effects. Moreover, although the risk of a hypoglycemic episode is minimal while on incretin mimetic therapy due to its glucose dependant action, it can nevertheless occur if a GLP-1 agonist is combined with a non-glucose dependent drug like a sulfonylurea. In order to prevent hypoglycemia, therefore, antidiabetic treatment should begin with a small dose of sulfonylurea while on an incretin mimetic. Another factor for clinicians to take into account is that there have been some reports of acute pancreatitis while only on liraglutide or exenatide therapy. Patients with type 2 diabetes, however, have a higher risk of developing this severe condition (Noel, et. al. 2009) so the evidence focusing on the relationship between incretins and pancreatitis is inconclusive. But it is advised that doctors take a cautionary approach when prescribing incretins to type 2 diabetes patients.

Let us now focus on three recent clinical trials that have proven to be pivotal in providing evidence that incretin mimetics are superior to other treatments in a number of crucial parameters. First, in 2012, an open label, European Exenatide (EUREXA) study was conducted by Gallwitz and colleagues who wished to determine whether a GLP-1 agonist or a traditional treatment agent is more effective at reaching target HbA1c levels, when monotherapy with metformin failed. The researchers compared twice daily exenatide versus once daily glimeperide, an oral sulfonylurea, in addition to metformin therapy. A total of 1,029 participants were arbitrarily assigned to either exenatide or glimeperide as an add-on to metformin, with baseline HbA1c values between 7% and 9%. After a four year evaluation, the results in table 1 demonstrate that fewer subjects in the glimeperide group reached the HbA1c level of less than 7% as compared to the exenatide group. Furthermore, while taking exenatide, fewer patients succumbed to treatment failure and considerably more met their target HbA1c goal. Moreover, those receiving the incretin mimetic lost weight, whereas those in the glimeperide group gained weight, and hypoglycemic events were more commonly reported in those taking glimeperide. As can be seen, however, although a limited number of patients in the glimeperide group both reached their target HbA1c and blood glucose levels, nevertheless, its side effect profile decreases its usefulness. Therefore, these results indicate that the GLP-1 agonist exenatide is more efficacious than glimeperide in a number of crucial factors of successful diabetes treatment (Gallwitz, et. al. 2012).

	Exenatide	**Glimepiride**
Dosage	5-10mcg 2xday	1-8 mg/day
Population	515	514
Treatment Failure: HbA1c > 7%	54%	54%
Target HbA1c < 7%	44%	31%
Mean Weight	Lost 10 lb	Gained 5 lb
Hypoglycemia	186 Patients	338 Patients

Table 1: Results of the EUREXA trial (Gallwitz, et. al. 2012).

In 2010, another study that was 1.5 years in duration was conducted to determine whether exenatide LAR was more likely than insulin glargine to reach target HbA1c goals. Enlisted in this randomized trial were 456 participants who suffered from chronic type 2 diabetes. These patients were already taking oral first line treatment like metformin or sulfonylurea therapy. However, based on the average HbA1c value of 8.3%, their current treatment was deemed ineffective. The results of the trial showed that the average HbA1c value for the incretin mimetic group was 7.1% and 7.3% for the insulin glargine group. Even though there was not any profound clinical difference, this is nevertheless a statistically significant result. And this illustrates the notion that statistical significance does not always translate to clinical significance. In terms of weight, exenatide patients lost about 5lbs and insulin glargine subjects gained about 10lbs. Moreover, hypoglycemic episodes occurred with much greater frequency in those taking insulin glargine. Therefore, these findings demonstrate that once weekly exenatide is recommended for patients who favor convenience, weight loss, and have a high risk of hypoglycemic episodes (Diamant, et. al. 2012).

Finally, in 2012, a randomized, placebo controlled DURATION-4 trial was conducted by Russell-Jones and colleagues to compare the safety and efficacy of exenatide LAR with monotherapy treatment options for type 2 diabetes such as metformin, pioglitazone, and sitagliptin in drug naïve patients. This 26 week study enlisted 800 type 2 diabetes

patients who lacked glycemic control evidenced with a mean HbA1c level of 8.5%. The results of the study showed that metformin, exenatide, and pioglitazone shared a similar reduction in HbA1c of 1.5% and exhibited superior glycemic control than sitagliptin with a decline of 1.2%. Furthermore, those taking metformin and exenatide showed the greatest weight loss of 4.4lbs. In addition, major hypoglycemic episodes were not reported with the use of any of the aforementioned monotherapies (Russell-Jones, et. al. 2012). Taken together, these trials demonstrate that incretin mimetics are effective at positively addressing multiple diabetic factors like weight, hypoglycemia, and dysglycemia.

Conclusion

Type 2 diabetes is a progressive disease that clinicians have had profound difficulty in properly managing. Traditional pharmaceutical agents such as sulfonylureas, for example, have resulted in weight gain and risk of hypoglycemia which has unfortunately defined a typical diabetic's glucose lowering regimen. Now, however, with the introduction of incretin mimetics such as exenatide and liraglutide, patients can take advantage of this novel treatment approach. Counteracting the many pathophysiological mechanisms involved in the disease, coupled with the expansion of beta cell mass, is a significant advancement in the treatment of type 2 diabetes. In the past, clinicians were fighting an uphill battle against this condition, but with incretin based therapies, and recent high profile studies clearly demonstrating their efficacy and safety, we are moving one step closer to properly managing this multifaceted disease.

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Efficacy of Probiotics *Lactobacillus Rhamnosus GG* and *Saccharomyces Boulardii* in the Treatment of Antibiotic-Associated Diarrhea and *Clostridium Difficile* - Associated Disease

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Abstract

Antibiotic therapy may cause serious side effects. Two disturbing effects of antibiotic administration are antibiotic–associated diarrhea and *Clostridium difficile*-associated disorder. Antibiotic-associated diarrhea occurs as a direct result of the normal flora destruction due to the antibiotics – which do not discriminate against pathogens or healthy forms of bacteria. *C. diff* disorder also occurs as an indirect result of antibiotic administration, because the destruction of the normal flora prevents people from having healthy bacteria to prevent disease. There have been studies conducted to determine if replacing the destroyed normal flora with probiotics, or beneficial microorganisms will prevent or treat these conditions. Studies have been conducted to show that the bacterium *Lactobacillus rhamnosus GG* has shown great promise in the treatment of antibiotic-associated diarrhea as there have been positive results achieved in many heterogeneous studies. Treatment of *Clostridium difficile*-associated disorder with the yeast, *Saccharomyces boulardii* remains controversial as different medical researchers struggle to prove or disprove its effectiveness and safety.

Introduction

After being admitted to hospitals, most people expect their health to improve. They believe that the hospitals' sanitary conditions will surely keep them from contracting any illnesses in their weakened states. However, infections can spread rapidly in hospitals and people can also suffer from the side effects of the very treatments intended to help them recover. Both *Clostridium difficile*-associated disease and antibiotic–associated diarrhea are examples of these growing healthcare concerns (Wistrom, 2001). Antibiotic-associated diarrhea involves the onset of diarrhea following the administration of antibiotics. The resulting diarrhea is not linked to a previous disorder or condition (Bartlett, 2002). About 10% to 20% of cases which are attributed to antibiotic-associated diarrhea are also caused by *Clostridium difficile* toxin (Bartlett, 2002). *Clostridium difficile*-associated diarrhea or *Clostridium difficile*-associated disease (CDAD) refers to the diarrhea and other gastrointestinal complaints that result from a *Clostridium difficile* infection (McFee, 2009). It is possible that the percentage of diarrhea cases attributed to *Clostridium difficile* toxin is slightly inaccurate as the *Clostridium difficile* bacteria present in the stools of those diagnosed with antibiotic-associated diarrhea may be benign. Whether the antibiotic-associated diarrhea resulted from the ingestion of antibiotics or from the presence of *Clostridium difficile* toxin, which people who have been immunocompromised are more susceptible to, the results remain the same. People are suffering gastrointestinal distress and hospitals are spending their limited cash resources on dealing with these issues. It is difficult to eliminate the sources of these infections, because the antibiotics used to treat infections in, but not limited to, hospital patients are ineffective against toxin and spore-forming *Clostridium difficile* bacteria. In addition, it is almost impossible to stop the bacterial growth and spread of CDAD within hospitals, as antibacterial cleaning products do not destroy spores from the hands of health care workers and hospital sinks and toilets (McFee, 2009). Medical researchers have been looking for an alternative method to treat AAD and CDAD.

Diarrhea results when the balance of normal intestinal flora is disturbed. In order to restore the normal intestinal flora to its healthy state, medical researchers are turning to probiotics, or live microorganisms which benefit their hosts, (Santosa, 2006) to replace the salutary microorganisms that normally inhabit the gastrointestinal tract (Avadhani, 2011). The intestinal microflora normally play a number of roles in gastrointestinal health. These include: strengthening the layer of epithelial cells in order to prevent the movement of pathobiotics, or microbes that are harmful to their hosts, competing with pathobiotics for positions on the epithelial lining, the production of compounds which will inhibit pathobiotic growth, and the enhancement of the immune response to pathobiotics (Patwary, 2012). Twentieth century Russian scientist Eli Metchnikoff first recognized the health benefits of probiotics when the life span of Bulgarian peasants who consumed fermented milk which contained lactic acid bacteria was longer than expected (Culligan, 2009). The first mechanism he proposed was that the ingested probiotics replace the pathobiotics that have taken up residence in the gut (Surawicz, 2003). As written above, this is still an accepted hypothesis of medical researchers.

Two characteristics that probiotics share with the normal flora that they are intended to replace are their ability to survive the acidic conditions and the enzymatic activity of the human gastrointestinal system (Singhal, 2009). These characteristics allow probiotics to mimic the behavior and functions of normal flora in the gut making it possible for probiotics – microorganisms normally present outside of the gut – to be considered for use as alternative treatments for numerous gastrointestinal problems. Both L*actobacillus rhamnosus GG*, a bacterial microbe, and *Saccharomyces boulardii*, a yeast, have been studied to determine their efficacy and safety in the treatment of antibiotic-associate bacteria and *Clostridium difficile*-associated disorder, respectively. This review will explore the published literature to shed light on

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these health claims.

Methods

Literature searches were conducted using the health science related databases of the Touro College Online Library: MEDLINE, Proquest Medical Library (Health and Medical Complete), EBSCO multi-search, and PubMed. Both the Touro QuickSearch option and Google Scholar were also utilized. The following keywords were searched: probiotics, probiotics and gastrointestinal health, probiotics and gastrointestinal disorders, probiotics and antibiotic-associated diarrhea, probiotics and *Clostridium difficile*-associated disorder, efficacy of probiotics in the treatment of *a*ntibiotic-associated disorder, efficacy of probiotics in the treatment of *Clostridium difficile*-associated disorder, antibiotic-associated diarrhea and *Clostridium difficile*-associated disorder, probiotic safety, *Lactobacillus rhamnosus GG* and antibiotic-associated diarrhea, *Saccharomyces boulardii* and *Clostridium difficile*-associated disorder, and fungemia and *Saccharomyces boulardii.* Several of the sources listed in the articles found by using these keywords were also used as references where appropriate. Additionally, only articles published in scholarly peer-reviewed journals after the year 1995 were included.

Results and Discussion

Antibiotic-Associated Diarrhea

Although there have been several studies done using different probiotics in the treatment of antibiotic-associated diarrhea, the probiotic which seems to be the most efficacious is *Lactobacillus rhamnosus GG.* It is also a strain which has been well-researched (Hawrelak, 2005). For this reason, Hawrelak and his colleagues conducted a review of six trials which involved the study of the effects of *Lactobacillus rhamnosus GG.* The specific requirements for inclusion were that the studies must concern human clinical trials and investigate the effects of probiotics on antibiotic-associated diarrhea. In addition, the probiotic in question needed to have been *Lactobacillus rhamnosus GG.* He did not discriminate by age, however, and utilized studies which were conducted on both adults and children (Hawrelak, 2005). In addition, Hawrelak included research articles which varied in probiotic dosage. The dosages of colony forming units that were administered for each study ranged from 250 ml LGG yogurt with no CFU count provided to 2 X 1010 CFU capsules twice daily. Despite these inconsistencies, the overall consensus was that the subjects who were receiving *Lactobacillus rhamnosus GG* in any form during each study suffered from diarrhea for a shorter duration (Hawrelak, 2005). Due to the lack of heterogeneity, the results of these studies could not be combined into one and the overall statistical efficacy could not be determined (Hawrelak, 2005). As with Hawrelak's systemic review, the common thread among the studies conducted regarding *Lactobacillus rhamnosus GG* as a potential treatment for antibiotic-associated diarrhea is that *Lactobacillus rhamnosus GG* was used in some form, alone or in conjunction with another probiotic, on a person, of any age, suffering from antibiotic-associated diarrhea.

Hawrelak's review is cited by a study conducted in the University Hospital of North Norway where Wenus and his colleagues studied the possible prevention of antibiotic-associated diarrhea by a fermented probiotic milk drink (Wenus, 2008). The multistrain probiotic milk drink included *Lactobacillus rhamnosus GG* as well as *Lactobacillus acidophilus La-5* and *Bifidobacterium Bb-12* (Wenus, 2008). This study was limited to patients aged 18 years and over and excluded patients with immune deficiency disorders, those who had diarrheal episodes in the past, and those who had taken fermented probiotic drinks as dietary supplements two weeks prior to the study (Wenus, 2008). The final study included 87 adults, 41 of which were included in the placebo group and 46 of which were included in the probiotic group. At the conclusion of the study, 63 patients were available for evaluation. Of those treated with the fermented probiotic milk drink, 5.9% still developed antibiotic-associated diarrhea. However, 27.6% of subjects in the placebo group developed antibiotic-associated diarrhea.

According to Sherwood L. Gorbach, M.D. of Tufts University School of Medicine, the heterogeneity of the studies which include the treatment of antibiotic-associated diarrhea with *Lactobacillus rhamnosus GG*, does not diminish the proof of its effectiveness. In fact, it indicates its versatility. Whether consumed in a fortified milk product or in lyophilized powder form, *Lactobacillus rhamnosus GG* will boost the gastrointestinal tract's defense mechanisms (Gorbach, 2000) .

Although considered technically well-researched by Hawrelak and Gorbach, the effects of *Lactobacillus rhamnosus* on patients affected by antibiotic-associated diarrhea need to be studied further. There needs to be some uniformity in the studies conducted. For example, the elderly should be given yogurt fortified with 2 x 1010 CFUs of *Lactobacillus rhamnsous GG* twice daily and the same study should be conducted on children. Medical researchers are limited if the elderly are given capsules of 1.2 x 1010 CFUs of both *Lactobacillus rhamnosus GG* and *Lactobacillus acidophilus* daily while children are given milk fortified with *Lactobacillus acidophilus La-5* and *Bifidobacterium Bb-12*. It becomes difficult to determine if the bacterial strain, dosage, or medium through which the probiotic is administered caused the patients to improve. A specific ratio of all three may be determined if further research is conducted.

Gorbach's description of the potential health benefits of LGG has led other medical researchers to conduct studies to test the effectiveness of LGG in AAD prevention. Gorbach's conclusions were tested with a study conducted on children. (Vanderhoof, 1999). A group of 202 children with a median age of four years were recruited to participate. These children, who were prescribed oral antibiotics at a primary care pediatric practice, were divided into two groups. One group of children was given inulin placebo pills during the course of antibiotic treatment, while the second group received LGG in capsule form. Children weighing less than 12 kg were given one pill which contained 10 billion colony-forming units of LGG and those who weighed more than 12 kg received a double dosage. Parents were told to document the stool consistencies of their

children. Of the 202 recruits, 188 were evaluated at the end of the study. The subjects who received the LGG capsules were less affected by AAD. Only 7% of those who were administered the LGG capsules suffered from diarrhea and 26% of the children in the placebo group were affected (Vanderhoof, 1999).

Vanderhoof's study shows that LGG minimizes the effects of antibiotics on the gut in children prescribed oral antibiotics. However, the researchers were relying on the cooperation of the children and parents to administer the LGG or placebo capsules and determine if the children's stools were loose enough to be considered diarrhea or not. If this study were conducted under the supervision of the researchers, human error would be minimized. This does not diminish the fact that a significantly smaller percentage of children suffered from AAD after having taken the LGG capsules.

Clostridium Difficile-Associated Disorder

As an alternative treatment of the hospital "superbug" *Clostridium difficile*-associated diarrhea, the yeast *Saccharomyces boulardii* has shown some promise. The Journal of the American Academy of Nurse Practitioners published a meta-analysis of the efficacy of probiotics in the treatment of CDAD. Two of the studies included discussed the efficacy of the nonpathogenic yeast, *Saccharomyces boulardii* (Avadhani, 2011). One of the studies conducted in Gulhane Military Medical Academy, Department of Infectious Diseases and Clinical Microbiology, included 151 patients between the ages of 25 and 50 receiving antibiotic-therapy who were administered *S. boulardii* or a placebo in capsule form twice daily. The stools of those suffering from antibiotic-associated diarrhea were assayed for the presence of *Clostridium difficile* toxin A. In the group receiving the placebo, two patients' stools contained toxin A, while the stool of the one patient in the treatment group suffering from AAD did not (Can, 2006).

The second study included by Avadhani and Miley discussed the lack of therapeutic effect of *S. boulardii* on patients suffering from AAD which resulted from the *Clostridium difficile* infection (Lewis, 1998). This study was limited to elderly patients who had been prescribed antibiotics within the preceding 24 hours. Seventy-two patients were randomly selected for inclusion in either the placebo group or the group that received 113 mg of *S. boulardii* twice daily. In addition, their stool samples were evaluated by the nursing staff to determine whether their stools were loose enough to be considered diarrhea. Whether hard or loose, all stools samples were sent to be tested for *Clostridium difficil*e toxin. Of the 33 people evaluated in the active group, five people were found to have *C. difficil*e toxin present in their stools. In the placebo group, *Clostridium difficile* was found in the stools of 7 people. There was no visible improvement in the group who was administered the S. boulardii. In the discussion section of the study, the researcher, S.J. Lewis, mentions how previous researchers such as G.W. Elmer and L. V. McFarland showed that there was a benefit in taking *S. boulardii*, as opposed to taking a placebo. However, they were unable to repeat these results in later well-designed studies (Lewis, 1998). In response to Lewis' evaluation of previous studies, Elmer and McFarland commented on Lewis' study and claimed that the small trial failed to prove that *Saccharomyces boulardii* is ineffective and that Lewis should have followed up with patients after they had stopped receiving antibiotics. Lewis' study took place over 6 to 11 days. Elmer and McFarland felt that a 6 to 8 week follow up period should have been conducted (Elmer, 1998). Elmer and McFarland also commented on Lewis' point that later studies by McFarland, et. al. (McFarland, et. al., 1995) fail to prove the efficacy of *Saccharomyces boulardii* to treat CDAD. McFarland states that Lewis took the results out of context, because McFarland's study took a follow up period into account and Lewis left those results out of the evaluation of McFarland's work (Elmer, 1998). Lewis replied by pointing out that Elmer and McFarland are biased, because they are associated with Biocodex - the company that manufactures *S. boulardii.* Lewis also explains that he tried to match up the parts of the studies that were comparable in order to present an accurate review of the studies (Lewis, 1998b).

It is clear from this exchange why the use of probiotics to treat disorders is still considered an alternative care method. It is difficult to compare studies that only share a few characteristics in common. The common thread may be that *S. boulardii* was used to treat CDAD, but the dosages, patient-types, and study-length vary. There are researchers who use McFarland's studies and reviews and use them as a basis for their research regarding probiotics and human gastrointestinal health and evidence for the efficacy of *Saccharomyces boulardi*i (Guslandi, 2006) and there are those who remain skeptics (Miller, 2009). In the context of a later meta-analysis conducted by McFarland and published by the American Journal of Gastroenterology, McFarland recognized that the use of probiotics in the treatment of *Clostridium-difficile* disorder remains controversial (McFarland, 2006).

Mark Miller discussed the probiotic movement and described it as a mass hysteria, because people are desperately trying to minimize the after-effects of antibiotic use and cure all gastrointestinal ills. People are placing store in an alternative methods that do not have sufficient evidence to prove their health benefits (Miller, 2009). Miller also notes that McFarland's 2006 meta-analysis states that *Saccharomyces boulardii* is an effective treatment for CDAD when there have been previous meta-analyses to the contrary (Miller, 2009).

In addition to possibly being ineffective in the treatment of CDAD, *S. boulardii* may be harmful to those who ingest it. The use of *Saccharomyces boulardii* may not be appropriate for those who are immunocompromised or immunosuppressed. *Saccharomyces boulardii* was marketed as a dietary supplement that improves gastrointestinal health. However, dietary supplements are not regulated by the FDA's strict regulations and while this yeast may improve the gastrointestinal health of individuals who are not immunocompromised, those who are not in good health may suffer from fungemia, or the presence of fungi in the blood (Venugopalan, 2010). There have been five documented cases of fungemia in patients who were receiving

S. boulardii as treatment for CDAD (Miller, 2009). As with other infections, babies, young children, and the elderly are at a greater risk of contracting CDAD. It appears then that the very people *Saccharomyces boulardii* is intended to treat – those who are immunocompromised due to age, illness, and antibiotic treatment - may not be the people who can benefit from its probiotic properties.

Conclusion

After a review of the available literature discussing efficacy of probiotics *Lactobacillus rhamnosus GG* and *Saccharomyces boulardii* in the treatment of antibiotic-associated diarrhea and Clostridium difficile – associated disorder, respectively, it can be determined that *Lactobacillus rhamnosus GG* has a role in the prevention of AAD. However, it appears that *Saccharomyces boulardii* has not shown itself to be a safe and effective probiotic for those who are ill and should not be used to treat those who suffer from CDAD until more rigorous testing has been done.

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Guilty or Not Guilty: Can DNA Help Prove Guilt or Innocence?

Suzanne Eckstein

Abstract

Throughout our history, science was always on the front lines for discovery and exploration. Science is used as an investigative tool by the human race to figure out all the mysteries of the universe. The discovery of DNA was tremendous, providing each human being with their own unique genetic identity - no longer would an individual be genetically confused with another. DNA fingerprinting, in particular, has changed the world. In the 1980's the legal system began using DNA fingerprinting to help establish the guilt of an indicted criminal. DNA (besides for fingerprints) is the only way to confirm scientifically if the individual was at the scene of the crime. Over the years, many methods for forensic DNA testing have emerged. Polymerase chain reaction is a method used to amplify the smallest amounts of DNA, creating thousands of copies which can be analyzed. Restriction fragment length polymorphism looks for variations in homologous DNA. Short tandem repeat technology looks for repeated sequences in the bases of the DNA sample. Mitochondrial DNA analysis tests the mitochondrial genome which is highly polymorphic between individuals. Finally, Y-Chromosome analysis is used for males, and usually accompanies PCR or RFLP. DNA is now commonly used in criminal investigations and is often the most substantial piece of evidence. In recent years, DNA fingerprinting is also being used for exonerations. People who have been languishing in prison for years for crimes they did not commit are being released due to the breakthrough of forensic DNA and DNA fingerprinting establishing their innocence.

"The blood or semen that (the perpetrator of the crime) deposits or collects- all these and more bare mute witness against him. This is evidence that does not forget. Physical evidence cannot be wrong; it cannot perjure itself; it cannot be wholly absent. Only human failure to find, study and understand it can diminish its value."

(Paul Kirk, Crime Investigation, 1953)

Introduction: Forensic DNA

Forensic DNA is an identification system that allows DNA typing to be performed on an extremely minute amount of organic human matter. The DNA can be extracted from bloodstains, hair, saliva, debris from fingernail, teeth, dandruff, epidermal cells, fingerprints, personal items, and more. Forensic analysis of DNA is a commonly used - though relatively recent method - of helping to identify the victim or perpetrator in criminal investigations. In modern crime investigation, once a crime is committed, forensic protocols swing into action, with police and specialized teams that analyze and comb through all available evidence. Upon discovering possible DNA evidence, it is collected with the greatest importance given to keeping it sterile and untainted. Once all the DNA has been collected, it is brought to a lab that will determine which method of DNA typing will be performed, based on the quantity of DNA collected.

DNA evidence, as it is now used, is a very powerful investigative tool when at a crime scene. DNA is strong, concrete evidence which can help link a suspect to the crime, or, in the alternative, prove that a certain individual was not present at the scene of the crime. Because of this, the combination of forensic science utilizing the properties of DNA is taking up an ever-increasing role in the investigation of crimes. DNA is collected routinely, and is many times the key investigative evidence sought after and used by the authorities. Although DNA testing can take anywhere from one week to 3 month to obtain results, its high rate of accuracy is well worth the wait. The process of comparing DNA linked to a crime is simple; one sample is taken from the suspect, and one sample is taken from the crime scene. The DNA from both samples is studied and compared, and if the DNA matches, then there is near complete certainty that the tested individual was present at the crime scene.

While it may seem to be a boon for prosecutors looking to put criminals behind bars, DNA evidence is also being used with great success to clear individuals of a previous convicted guilt. Many cases that were brought to trial when DNA evidence testing was not a viable criminal justice method have been retried based on evidence obtained through DNA testing. Evidence of DNA at the crime scene, however, is not absolute proof. While DNA testing establishes with almost complete certainty the presence of an individual's DNA to the crime scene, there still remains the possibility for sample error. For example, a person's DNA could have been at the crime scene before the crime had been committed, or an accused person can have DNA nearly identical to a relative, such that the sequencing may be an almost exact match. Due to this uncertainty, there is significant debate over the level of weight DNA evidence should be granted in courtroom proceedings.

What is DNA?

Deoxyribonucleic acid, or DNA, is a very powerful molecule present in each human being. Furthermore, DNA is unique to each individual, so that no two people (besides identical twins) share the same precise DNA combination. The rainbow of different human attributes, from physical to mental traits, is due to the unique DNA we all carry. The structure of DNA was first discovered by Watson and Crick in 1953. They found that DNA had two parallel strands that took the shape of a double helix. The parallel strands, each made up of four nucleotides (adenine, thymine, guanine and cytosine) chained together in a specific sequence. The backbone of DNA is composed of alternating sugar and phosphate residues (figure 1). The sugar in the

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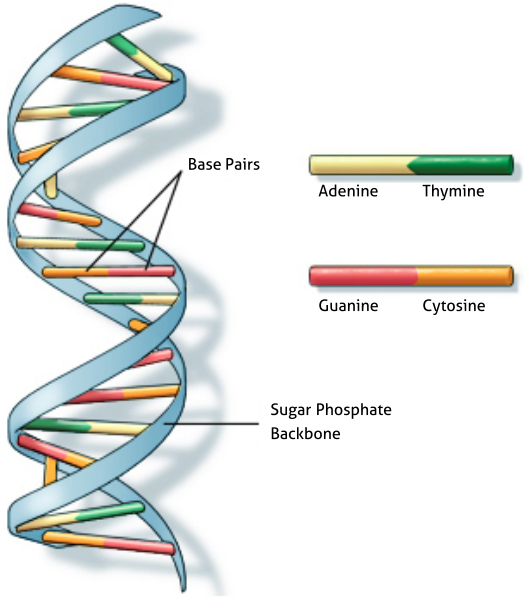

Figure 1: DNA Helix
Source: U.S. National Library of Medicine

backbone is a 5 carbonsugar (pentose) which is called 2-deoxyribose. Phosphate groups are what hold the sugar molecules together, and provide the strand of DNA with a direction. DNA is anti-parallel; the directionofone strandof nucleotides is the opposite direction of their bonded strand. There is a 5 prime end which has a phosphate group and a 3 prime end which has a hydroxyl group. So while one strand is going from 5 prime to 3 prime, the second strand is going from 3 prime to 5 prime. DNA sequence is virtually the same in every single cell within a person's body. However, while all humans share 99% of their DNA with everyone else it is a mere 1% difference that differentiates one person from the other. There is only 1 difference in every 1,200 to 1,500 nucleotides. But that one difference is found in sufficient quantity to allow for tremendous variety.

The difference from one individual's DNA to another's is in their genome, which is the complete set of DNA, including all of its genes. Genome variations are differences in a person's DNA sequence. Generally, most genome differences are simple, only involving variations in a few bases. Each of these sequence difference is called a DNA polymorphism. In addition to the genome variation of different bases, there is another DNA polymorphism involving the number of repetitions of a particular sequence of nucleotides.

DNA within the nucleus of each cell encodes the genetic instructions for all development and functioning of each being. It is the building block for each individual's genetic make up. DNA is one of the major macromolecules essential for all known forms of life. Genetic information is encoded as a sequence of the four nucleotides. DNA is different from one person to the next, but within each person their DNA is the same in every cell within their body.

Collection and Extraction of DNA

The process of DNA analysis begins with the extraction of the DNA. The DNA must be separated from from all other cell components. "There are various possible DNA extraction methods and when dealing with crime scene samples the type of evidence and the amount of DNA it contains will help determine the extraction method used. Common forensic DNA extraction methods include the use of chelex beads" (Kobilinsky, 2011). Chelex beads are ion- exchange resins that protect DNA by binding to magnesium ions. This process inhibits magnesium from destroying DNA. DNA is released from the cell, after the cells have been broken open by boiling the cellular material in the presence of chelex beads. After this process, the cells are placed in a centrifuge and all cellular material - including the chelex beads - fall to the bottom of the tube, while the liquid in the tube contains the extracted DNA. The liquid is usually transferred to a new tube, where it is frozen at -20 or -80 celsius until it is used for analysis. Another method for DNA extraction is Organic Extraction. This involves adding chemicals to the DNA sample. "First sodium dodecylsulfate (SDS) and proteinase K are added to break open the cell wall, and to break down the proteins that protect the DNA molecules while they are in chromosomes. Next a phenol/chloroform mixture is added to separate the proteins from the DNA. The DNA is more soluble in the aqueous portion of the organic-aqueous mixture. When centrifuged, the unwanted proteins and cellular debris are separated away from the aqueous phase, and double stranded DNA molecules can be cleanly transferred for analysis" (Butler, 2005).

Before being analyzed in the lab, the amount of human DNA must be measured. This is due to that fact that all kinds of DNA are collected from a crime scene, not only human DNA. So the DNA Advisory Board standards require human specific DNA quantitation. The most common process is called the "slot-blot" procedure. This test is specific for human DNA. On a nylon membrane with addition of a human specific probe, genomic DNA is captured. It is a measurement of the comparison between the unknown samples to a set of standards.

Methods for Forensic DNA Profiling

Polymerase Chain Reaction

There are many methods of forensic DNA testing used to analyze the evidence. The one most commonly used today is Polymerase Chain Reaction (PCR). It is the most commonly used method due to its practicality - only a small sample is required, and it can be done on samples that have not been recently collected. In fact, PCR can be performed on old samples of DNA many years later. It was developed by Kary Mullis in 1983. In the PCR test, biochemical technology is used to amplify a small sample of nuclear DNA to millions of copies of a particular DNA sequence. There are several steps in this procedure. First the DNA has to be denatured. Denaturation separates the complementary strands of DNA held together in the duplex by hydrogen bonds. Thus, samples are heated to 94°-96° Celsius for one to two minutes until the DNA is separated into single

strands. This works because the strands are bonded together with a weak hydrogen bond, as the sugar and phosphate backbone is bonded together with a strong covalent bond. Next, in the annealing step, the temperature is lowered to 50º-65º Celsius, and primers bind to the DNA. "A primer is a single stranded sequence of nucleotides known as an oligonucleotide. Each primer is complementary to one of the original DNA strands to either the left 5 prime side, or right 3 prime side of the sequence of interest." (Schochetman, Ou, Jones. 1988) The primer binds to the primer template and acts as a starting point for DNA formation. Two primes are involved in PCR, one for each strand. Next is the extension step where new DNA strand is synthesized complementary to the DNA template. At the end of this cycle, there are two new DNA strands identical to the original target sequence. These DNA strands are called Amplicon. The extension step can vary in time and cycles. It depends on DNA polymerase used, and the length of the DNA fragment to be amplified. After a few cycles of this, the target sequence of the original DNA strand is amplified.

Restriction Fragment Length Polymorphism

The second method is Restriction Fragment Length Polymorphism (RFLP). It was first discovered in the 1980's by Alec Jeffreys. This was the first method in testing forensic DNA. Jefferys was working on DNA profiling (DNA fingerprinting). He used the difference in length of nuclear DNA regions created by variations of numbers in repeated sequence to distinguish between individuals. Restriction enzymes recognize specific sequences of nucleotides in DNA called restriction endonuclease recognition sites. "The enzymes that are commonly used for restriction fragment length polymorphism analysis require 4-6 base pair recognition sequences. Cleavage frequency can be estimated by making the assumption that each of the different nucleotides occur randomly and in equal amounts for a given DNA sequence" (Bernatzky, 1988). The enzyme cuts the DNA in a process known as restriction digest. DNA's restriction sites, and distances between the sites differ from one person to the other. These differences are called restriction fragment length polymorphism. By using a restriction enzyme to cut a DNA sample, different lengths are obtained. The resulting pieces of DNA are passed through Agarose gel electrophoresis, which sorts out a pattern of bands by length that is unique for the particular DNA being analyzed. These repeated regions of DNA are called Variable Number Tandem Repeats. The fragments of DNA are transferred to a sheet of nitrocellulose which is exposed to a radioactive probe. After, a photographic film is laid on top of the sheet to expose an image corresponding to the DNA fragments. RFLP occurs when the detected length varies between individuals." RFLP analysis has been used for a variety of purposes. Since restriction sites are actual samples of nucleotide sequence the variation for the presence of sites has been used to estimate genetic divergence of individuals" (Bernatzky, 1988). Each fragment length is considered an allele, and has genetic property to it. This method is not used very frequently for forensic DNA because a fairly large sample is needed; a sample of 100,000 cells or more. Another downside to RFLP is that the DNA sample needs to be "fresh" from the crime scene, and as a result, this method cannot be performed on old DNA samples. Although PCR is used more often, RFLP is considered a more accurate test.

Short Tandem Repeat Technology

The third method is Short Tandem Repeat Technology (STR), which is also referred to as Microsatellites, or Simple Sequence Repeats (SSR). It was introduced in the late 1990's. It's used for the analysis of specific regions found in nuclear DNA. STR's is a type of polymorphism where short sequences of tetra or penta nucleotide repeats of DNA are repeated and the repeated sequences are adjacent to each-other. The pattern can range from 2 to 10 base pairs, and is typically in the non-coding intron region, making the DNA unimportant. STR's are not considered so important because they do not code for a protein. By looking at many STR loci and counting how many specific repeats there are, it is possible to create a unique genetic profile for individuals. Once a STR has been found, the PCR process is often used to amplify that specific sequence. Once these sequences have been amplified, they are put through gel electrophoresis. After, the DNA is placed under a fluorescent dye to be visualized.

Mitochondrial DNA Analysis

Another method for testing forensic DNA is called Mitochondrial DNA Analysis. It is used when the DNA evidence is not suitable for PCR, STR, or RFLP. This mitochondrial DNA is present in the mitochondria of every human cell. It is very different than nuclear DNA. Mitochondrial DNA is useful for forensic purposes because it has two properties. Firstly, the mitochondrial genome is highly polymorphic, which is very helpful when is comes to human identification. Secondly, It's genes exist in a high concentration even though mtDNA only makes up for only 1% of the DNA within a cell. This is very useful for old or degraded DNA that needs to be tested and lacks nuclear DNA. In addition, mtDNA is strictly inherited from the maternal side. Therefore all siblings have the exact same mtDNA in the absence of a mutation. It comes in handy in a missing persons investigation, but it has a down side to it. There is no differentiation between mother and all her offspring.

Y-Chromosome Analysis

The last method for discussion is called Y-Chromosome Analysis. This method usually performed as an adjunct to one of the other discussed tests. In particular, the Y-Chromosome analysis is useful in cases involving sexual or paternal allegations. The Y-Chromosome is passed directly from father to son, which can provide determinative biological evidence involving multiple male contributors. Although this may be somewhat useful in a crime case, it usually used when trying to find familial relationships.

DNA Under the Law of Scientific Evidence

With the new technologies for forensic DNA, the courts have applied many standards to make sure the reliability of the evidence is true. There are two types of regulations. The Frye

rule requires all scientific evidence to be "generally accepted" by the scientific community before being admitted into the courtroom. The second standard is the Federal Rules of Evidence (FRE), which the Supreme Court ruled superseded the Frye standard. The FRE requires the scientific evidence to be helpful and relevant.

Ever since DNA has been admitted into the courtrooms, there have been more guilty verdicts. DNA evidence is extremely helpful and useful in solving a crime and finding the guilty party, but it not completely sufficient yet. Forensic DNA evidence is nearly, but not 100% accurate. Yet as the years pass, new technology is being introduced to achieve the 100% standard for scientific evidence. Eventually the technology will compel complete acceptance of DNA evidence.

Case Study: The O.J. Simpson Murder Trial

On June, 13 1994 Nicole Brown Simpson and Ronald Goldman were found dead outside Brown's house. All the evidence collected from the scene led the police to suspect that O.J. Simpson was the person who committed the murder. At this point, forensic DNA was relatively new, and not always accepted or believed as concrete evidence. "Simpson's lawyers are expected to mount a vigorous assault on the validity of forensic DNA evidence in an effort to convince Judge Lance to keep it out of court'" (Norwak, 1994). There was a great amount of forensic evidence that proved O.J. Simpson was indeed the murderer.

There was a great amount of strength and weakness of the DNA evidence against O.J. Simpson. The prosecution found that O.J. cut his hand during the murder, and left a trail of blood from the murder, to his car and into his house. There was also Nicole's blood found on bottom of his sock. There was also a glove found in Simpson's house that was covered in Nicole and O.J.'s blood. During the trial, Barry Scheck, a lawyer who specializes in forensic DNA, spent eight full days questioning the forensic evidence that was collected and tested. During this cross-examination, several aspects were brought to light about the collection of the DNA evidence that created doubt as to the accuracy of the sample obtained. The defense was able to debunk all of this evidence. They stated that the glove which had Nicole and O.J.'s DNA on it was contaminated at the LAPD laboratory. LAPD lab criminalist Collin Yamauchi admitted that the glove was indeed contaminated, and that he accidentally spilled a vial of O.J.'s blood on the glove. "The criminalists were poorly trained with respect to sample handling, were not following a written protocol, did not understand the purpose and importance of precautionary measures, such as changing gloves and made serious errors when attempting to demonstrate proper sample collection and handling techniques" (Thompson, 1996). The defense alleged that the DNA evidence was indeed tampered with, or not processed correctly. It was found that Andrea Mazzola had collected a blood DNA sample from O.J. Simpson, but had let that sample sit in her lab coat pocket the entire day before returning it to the lab for testing. Barry Schenck was able to convince the jury that the forensic evidence was not handled correctly, and that there was a reasonable doubt that it could be relied upon in proving Simpson's guilt.

The O.J. Simpson trial was one of the first trials that concentrated much of it's efforts in the areas of forensic fingerprinting. While ultimately the DNA evidence was not accepted by the jury, the publicity of the trial created a far greater awareness of the methodology and its great power as evidence.

Project Innocence: Exoneration

"In New Jersey, March of 1988, Byron Halsey was convicted for the brutal rape and murder of a seven year old girl and an eight year old boy. The evidence used to convict Halsey was his supposed confession, which he gave after over thirty hours of interrogation and sleep deprivation. Halsey had to "guess several times" before he could correctly describe to police how the crime occurred and other key factors...They jury convicted him using that evidence. After nineteen long years in prison, newly analyzed DNA test results proved Halsey's innocence and implicated the actual killer" (Sophia Chang, 2009).

Aside for the conviction purposes, recently DNA has been widely used for exoneration purposes. It is true that DNA has so much power that it can send someone to prison. But it also possesses the same amount of power in setting a man who was wrongly convicted free.

The innocence project was founded in 1992 by Barry Schenck and Peter Neufeld at the Cardozo School of Law at Yeshiva University. They came up with this idea to assist people who can be proven innocent through proper DNA testing. Before they take on a case, they do extensive screening to see if there is proper DNA to be tested. "DNA testing has opened a window into wrongful convictions so that we may study the causes and propose remedies that may minimize the chances that more innocent people are convicted" (The Innocence Project). To date there have been nearly 300 prisoners in the United States that have been exonerated because of DNA testing.

Conclusion

Science is in constant state of evolution. The first big break in forensic evidence was fingerprinting, which was discovered over 100 years ago. The next big discovery for forensic evidence was DNA fingerprinting. Since the development of forensic DNA testing in the early 1980's it's sophistication and accuracy has continuously improved, so that it is now considered a fundamental part of any investigation. The methods for analyzing DNA evidence are quite varied, with unique advantages and disadvantages to each. Yet, they are all really about one thing-the cataloging in DNA of the unique attributes of every person. By utilizing this method, there is a far greater likelihood of the investigations leading to the actual perpetrator. One needs to look no further than the many exonerations due to the Innocence Project to see how relatively primitive previous investigative methods are in comparison to DNA forensics. The careful study of the mechanics of DNA and

its attendant forensic methods will likely yield every greater scientific results in the future.

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The Common Allergic Mechanism of Rhinitis and Asthma

Esther Feinstein

Abstract

Allergic rhinitis and asthma are closely linked diseases which are very prevalent within the population, affecting millions. They are both characterized by chronic airway inflammation. They are often present in the same patients and rhinitis is even considered an independent risk factor for asthma. Treating allergic rhinitis can reduce the severity of asthma. Mechanisms connecting the two have been researched for many years. Studies show that they have a similar allergic mechanism that is mediated by the same cells. The allergic inflammation is characterized by the presence of eosinophils and is mediated by T-helper type 2 lymphocytes. The circulation of Th2 cells and the cytokines it releases, including IL-4 and IL-5, are important factors in airway inflammation. When their levels are reduced, the condition is improved. Controlling the production and circulation of these cells can potentially help treat allergic rhinitis and asthma.

Introduction

Allergic rhinitis is a common disorder, affecting over 600 million people worldwide. The disease involves an IgE mediated inflammation of the nasal membranes due to allergen exposure. Allergic rhinitis negatively impacts the patient's sleep, work, cognitive abilities and social life. Allergic rhinitis is also noted as a risk factor for asthma. (Bousquet,et. al. 2008) The association between the two disorders has been studied for many years. Numerous studies have been done to identify the mechanism and strength of the connection. In a survey done by the European Community Respiratory Health Survey, 13.4% of participants with rhinitis reported asthma and 71% of subjects with asthma reported rhinitis. (Leynaert, 2004) In a study done on college students, 56%-86% of asthma cases were associated with allergic rhinitis. Eighteen to twenty-one percent of the students with allergic rhinitis also had asthma. In a 12 year follow-up of college students without asthma, 10.5% of those originally diagnosed with allergic rhinitis later developed asthma. Only 3.6% of students without allergic rhinitis developed new asthma. (Settipane and Settipane, 2000) It has also been shown that treating allergic rhinitis can improve asthma symptoms. In one study, patients treated for allergic rhinitis were seen to have a significantly lower incidence of emergency room visits or hospitalizations than the asthmatics whose rhinitis symptoms were not treated at all. (Crystal-Peters, et. al. 2002) Another study treated asthma patients with medications directed at the nose, such as nasal steroids and antihistamines. The number of emergency department visits decreased among these patients compared to the control group. (Adams, et. al. 2002) It can be concluded that asthma and allergic rhinitis are intrinsically connected and should be considered one syndrome within the united airway. Much work has been done to determine the mechanism in which the two affect each other. Knowledge of how they are linked can help in the prevention, diagnosis and treatment of these airway disorders. Several hypotheses have been proposed, yet none have been conclusively proven.

Methods

The NCBI PubMedCentral database, the Touro College library database, and Google Scholar were the search engines used to find information. A search for articles containing the key words "asthma" and "allergic rhinitis" resulted in several review articles discussing this topic. The review articles provided an idea of what the current hypotheses and research are. Their references were used to find original research papers. In addition, more key words important to the topic were discovered in the review articles. A search for "epidemiology" with the words "allergic rhinitis" or "asthma" was conducted to find information regarding the prevalence of the disease. In order to find out about the common allergic mechanism, several searches were tried varying the terms "asthma", "allergic rhinitis", "mechanism", "Th2", "allergic response", "cytokines", "upper airway", "lower airway" and "unified airway". Articles that were marked as similar to the ones found in searches and articles that cited or were cited by pertinent articles were also used.

Results

Many of the researchers on the topic of asthma and allergic rhinitis discuss the allergic response mechanism involved in allergy. Exposure to an allergen leads to a series of cellular events concluding in an inflammatory response. (Benson, et. al. 2001) When a person is first exposed to an allergen, the allergen is recognized by T-helper lymphocytes (TH). The TH lymphocytes cause the B-cells to produce IgE that is specific to that allergen and primed to respond to it. The IgE then binds to the surface of mast cells. The next time the immune system is exposed to the same substance, the allergen is recognized by the IgE already on the surface of the mast cells. The mast cells are activated, releasing mediators like histamine and leukotrienes. The mast cells also release cytokines that can recruit other leukocytes like eosinophils, neutrophils and Th2 lymphocytes. This cascade ultimately leads to typical allergic symptoms, including sneezing, runny nose, nasal obstruction or airway constriction. (Cirillo, et. al. 2009)

The upper and lower airways share many anatomical and physiological properties. The majority of both the upper and lower respiratory tract is covered in ciliated epithelial cells. There are also many goblet cells associated with the epithelium, although their presence decreases closer to the lungs. The entire airway is also characterized by a dense network of blood vessels and nerves. Although each portion of the respiratory tract has its own task to fulfill, like humidification, filtration,

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warming, olfaction and gas exchange, the airways are united by a common purpose: the passage of air. (Ciprandi, et. al. 2012) Furthermore, the nose and lungs are susceptible to the same irritants and have similar reactions to them. Some agents that can trigger a response in the upper and lower airways are infections, viruses, pollutants, certain drugs, cigarette smoke, allergens, and sometimes physical exercise. (Caimmi, et. al. 2012) Allergic rhinitis and asthma are two common respiratory disorders. Although they affect different parts of the airway, they have similar causes and inflammatory processes and are characterized by chronic airway inflammation.

Allergic mucosal inflammation is identifiable by high levels of eosinophils in body tissue. (Braunstahl, 2001) This characteristic is often used to judge reaction levels in allergic subjects. A study was done to compare inflammation in the upper and lower airways in asthma and allergic rhinitis. The results showed increased eosinophil and mast cell levels in the mucosa of the bronchi and the nose. Surprisingly, the subjects with allergic rhinitis and asthma showed similar levels of inflammatory cells in the upper and lower airways as the patients with allergic rhinitis only. Although the rhinitis subjects exhibited no clinical signs of asthma, their lower airways showed the presence of inflammatory cells. (Braunstahl, et. al. 2003) The inflammation was present all along the airway, regardless of asthmatic symptoms. This supports the idea of a unified airway, the hypothesis that the upper and lower airways are connected and should be considered as one entity.

Lymphocytes are white blood cells that play a significant role in allergy and immunity. There are two categories of lymphocytes, T-cells and B-cells. The T-cells are divided into killer T-cells and T-helper cells. There are two types of T-helper cells. Type 1 T-helper cells (Th1 cells) are "infection fighters" and type 2 T-helper cells (Th2 cells) are "allergy promoters". Th2 cells and Th1 cells can inhibit each other's functions. Th1 reactions lead to a delayed immune response by activating macrophages. (Settipane and Settipane, 2000) A Th2 reaction releases certain key cytokines involved in inflammation; particularly interleukins (IL) 4 and 5. The cytokine IL-4 causes an overproduction of IgE. IL-5 causes an influx of eosinophils. The allergic reactions in rhinitis and asthma have been attributed to an immune pathway mediated by Th2 cells (Benson, et. al. 2001).

To determine the role of Th2, one study used Glucan, an immunomodulator that stimulates a Th1-mediated anti-tumor response. The study was completed using 24 subjects with allergic rhinitis. The test subjects received a treatment of Glucan pills and the control group was given a placebo. Under the influence of Glucan, allergens that normally would stimulate Th2 cells activate Th1 cells. Levels of cytokines in nasal lavage fluid were tested before and after nasal allergen challenge. Due to the decrease of Th2 mediated reactions caused by the Glucan, levels of the Th2 secreted cytokines IL-4, IL-5 were significantly decreased in the nasal fluid of the test subjects compared to the controls. The Th1 secreted cytokine IL-12 was found to be increased in the experimental group. The higher than usual Th1 levels inhibited the Th2 cells, lowering the concentration of the cells and cytokines responsible for the inflammatory response. (Kirmaz,et. al. 2005) This supports the idea that it is specifically the Th2 cells that are involved in allergic rhinitis.

In asthma, Th2 is thought to be very important in regulating the disease. The levels of theTh2 triggered cytokines IL-4 and IL-13 were found to be elevated in the lungs of asthmatic patients. A study comparing the bronchioalveolar lavage fluid of asthma patients to control subjects found a significantly greater concentration of Th2 associated cytokines in the lungs of the asthmatic subjects. (Robinson, 1992) One study used Toll-like receptor ligands to bias the immune system toward a Th2 response. The results in the test subjects showed that Th2 cells can aggravate asthma. (Redecke, et. al. 2004) Th2 is clearly a common factor in asthma and allergic rhinitis and is highly involved in allergic response.

A 2010 study set out to determine the mechanism in which the upper and lower airways interact. Using mice, they created a model of allergic rhinitis. OVA sensitized mice were used as controls. Controlling for Th2 cells allowed the researchers to determine its role. When the mice were subjected to a lower airway challenge of the allergen, the allergic rhinitis group showed a much higher concentration of eosinophils in the brochoalveolar lavage fluid than the control group. Using anti-CD3 antibodies to deplete Th2 cells before airway challenge significantly reduced lower airway inflammation, as shown by reduced numbers of eosinophils, dendritic cells and Th2 cells. The researchers then injected cultured Th2 cells into mice before the airway challenge. The mice that had received the OVA specific Th2 cells exhibited much higher levels of IL-5 and eosinophils than the controls. The presence of these Th2 cells was sufficient to induce inflammation upon challenge. Next, while developing allergic rhinitis in the mice, they were treated with FTY720, which prevents recirculation of lymphocytes. This did not stop the mice from developing allergic rhinitis. Later, when challenged with the allergen OVA, the AR mice treated with FTY720 did not display the eosinophilic inflammation that was expected and had very reduced levels of T cells in the bronchoalveolar lavage fluid. This suggests that blocking the Th2 cells from circulating prevents lung inflammation upon challenge, even in the presence of allergic rhinitis. This study concludes that allergic rhinitis models are much more susceptible to lower airway inflammation than controls and that circulating Th2 cells are necessary for the interaction of the upper and lower airway during allergic inflammation. (Kleinjan, et. al. 2009)

Discussion

The connection between asthma and rhinitis is well known and accepted. (Caimmi, et. al. 2012) Studies have been conducted for many years in an attempt to elucidate the mechanism in which they relate to each other. Several hypotheses have been proposed over the years. Researchers suggested a possible nasal-bronchial reflex involving sensory nerve endings. Although this reflex can be observed in animals there is no conclusive data proving its existence in humans.

Another hypothesis is that mouth breathing due to nasal blockages in people with allergic rhinitis impaired nasal humidification and filtration of air. Unconditioned air would enter the lungs and cause problems there (Togias, 1998). However, the most tested and observable connection is the similarity of the allergic response. Inflammation of the upper and lower airways share many features and are characterized by the prevalence of specific cells.

Allergic rhinitis and asthma have many similarities. One frequently studied factor in inflammatory airway diseases is the role of Th2 lymphocytes. Several studies have been conducted to bring evidence that the cytokines released in a Th2 mediated immune response are greatly involved in inflammation in both allergic rhinitis and asthma. Experiments that reduced the levels of Th2 and its associated cytokines by a variety of methods had similar results. Most importantly, reducing Th2 was enough to lower inflammation. Increasing Th2 and its cytokines aggravated the disease. Another important discovery was the fact that airway inflammation is not localized to only the nose or only the lungs. Patients with allergic rhinitis or asthma have shown signs of allergy in the opposite parts of their respiratory tract. The lungs in people with allergic rhinitis are also much more easily affected by allergen challenge than people without any airway inflammation at all. Once present in the airways, inflammatory cells evidently circulate, potentially affecting the entire tract. Blocking the Th2 cells in the nose from reaching the lungs reduces the sensitivity of the lungs to allergens. This may mean that if scientists can find a way to target Th2 or specific cytokines that lead to allergic reactions, allergic diseases like rhinitis or asthma can be controlled or even prevented. In many cases, allergic rhinitis seems to be leading to asthma. It is possible that through blocking Th2 cell recirculation in allergic rhinitis, the inflammatory cells can be contained within the upper airway and not spread to the lungs.

Conclusion

The inflammatory response in allergic rhinitis and asthma is important to understand in order to prevent and treat these common and debilitating diseases. The upper and lower airways make up a continuum and should be treated as one entity. Disorders in both areas should be treated by the same specialists. When diagnosing one, the physician should check for the other. The airway must be considered as a whole, with the realization that they share an allergic mechanism that can affect the entire respiratory tract. Additional research must be done to determine a course of treatment that could practically and effectively control the inflammatory cells. This could prevent people with allergic rhinitis from developing asthma; a phenomenon that is very widespread. It could also help in the treatment and diagnosis of these diseases and perhaps enable sufferers to lead more pleasant lives.

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Is There an Alternative Way of Treating Drug Resistant Epilepsy? The Effects of the Ketogenic Diet in Children with Intractable Epilepsy

Chaya M. Weinberg

Abstract

Many children with epilepsy experience seizures that cannot be resolved with medication. Since surgical intervention is not always an option, the ketogenic diet (KD), a high fat, low carbohydrate and protein diet, offers a chance for seizure reduction and in some cases freedom from seizures and medication. Side effects do exist, although none are serious. Efficacy has been proven through many studies. The mechanism of the KD's effectiveness is still unknown, although several hypotheses exist, including the theory that ketone bodies themselves are anticonvulsant, and the hypothesis that glucose restriction stops seizures. Adenosine A1 receptors are also thought to have a role in seizure reduction. Additionally, some researchers believe that ketone bodies provide the brain with energy to withstand seizures, although there are contradictions to this theory. Finally, the KD may play a neuroprotective role in the treatment of epilepsy.

Introduction

Epilepsy is a disorder characterized by recurrent seizures (Greenberg et al., 2012) which are caused by transitory disturbances of cerebral function due to abnormal paroxysmal firings by neurons in the brain (Aminoff, Kerchner, 2013). In the United States alone, over 300,000 children under the age of fifteen are affected by the disorder (epilepsyfoundation.org). Epilepsy has a great impact on a child's quality of life, psychosocial functioning, and cognitive functioning. These children experience social stigmatization and isolation from their peers. Standard treatment of seizures involves anti-epileptic drugs, of which many are available today, as are infinite ways in which drugs and dosages can be combined. Many children suffer from intractable epilepsy, which is defined by seizures that cannot be treated adequately despite optimal efforts using anti-epileptic drugs (Papandreou et al., 2006). Medical treatment options for intractable epilepsy are scant; in fact, the only choices are implantation of a vagus nerve stimulator or brain surgery (PubMed Health).

The Ketogenic Diet, a high-fat, adequate protein, and low carbohydrate diet that aims to biologically mimic the fasting state (Huffman, Kossoff, 2006) by producing a controlled ketonemia, is a non-invasive way of treating epileptic seizures (Papandreou, et al., 2006). Contrary to popular belief, the KD is not a "holistic" or "alternative" therapy; rather, it is a medical treatment which has been carefully studied and proven to be successful (Freeman et al., 2007a). The present paper will review the ketogenic diet and its effects on children with epilepsy as well as some proposed mechanisms of the diet's actions.

Seizures: A Brief Overview

A seizure is caused by abnormal excitation of neurons in the brain. Hyperexcitability can occur due to increased excitatory synaptic neurotransmission, decreased inhibitory neurotransmission, or alterations in ion flow or voltage-gated ion channels (Bromfield, et al.,2006).

Poor compliance with an anti-epileptic drug can lead to status epilepticus, a medical emergency classified by an occurrence of two or more convulsions without recovery of consciousness between attacks, or a seizure that lasts over 30 minutes. Status epilepticus can result in mental impairment or death (Papadakis, McPhee). It is therefore of utmost importance that seizures be controlled. When two medications fail, the KD should be considered. It should not be used as a last resort (Freeman et al., 2007a).

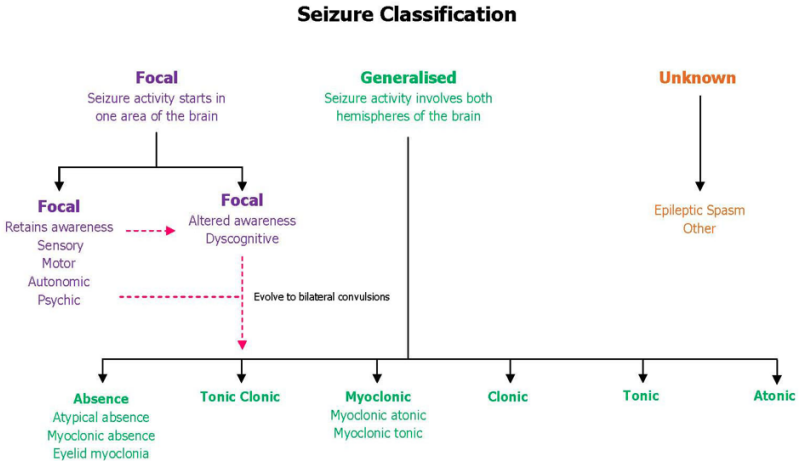

Figure 1: Classification of seizures
Source: http://www.epilepsy.org.au/about-epilepsy/understanding-epilepsy/seizure-types-classification

History of the Ketogenic Diet

Fasting as a cure for epilepsy can be traced back to as far as Hippocrates, who prescribed it to an epileptic patient as a means of purging the body of "polluted humors" (Huffman, Kossoff, 2006). Although not commonly known to many, the KD has been in use since its inception in 1921 (Martinez et al., 2007). The KD retained its novelty until 1938, when the anti-epileptic drug phenytoin (Dilantin) was discovered. Until then, pharmacological treatment of epilepsy could only be achieved with phenobarbital and bromides, both of which had severe sedative adverse effects. Thus the KD was lost in the sea of emerging anti-epileptic drugs, and encouraged by drug companies, physicians looked toward drugs as a primary method of treatment for epilepsy (Freeman, et al., 2007b). However, regardless of increased availability of new drugs, approximately one third of patients have seizures that resist

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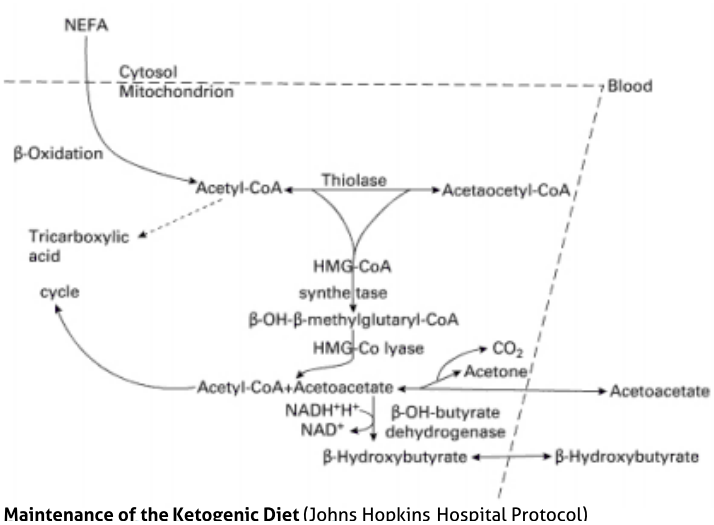
even these anticonvulsants (Noh, et al., 2008). The resurgence of the KD occurred in the mid-1990's, largely due to attention from the media. Since then, there has been a significant worldwide increase in the KD's use (Freeman, et al., 2007b).

Methods

Research for this paper was conducted by evaluating a variety of peer-reviewed journal articles from online databases. Databases include Proquest, Medline, and Pubmed. Access Medicine was used for medical information. In addition to journal articles, diet information was obtained from a book written by Johns Hopkins Hospital M.D.s and dietitians.

The Diet

The KD is so named due to the ketonemia it causes in patients. The diet is comprised mostly of fat and is low in carbohydrates and protein, usually in a 4:1 or 3:1 ratio of fat to carbohydrates and protein (Hartman, Vining, 2007). Calories are restricted to 75-80% of the recommended daily allowance and fluid intake is reduced to 80% of usual amount. This deviation from a normal diet leads to production of ATP from fatty acid metabolism rather than from the usual glucose metabolism. Essentially, the KD has the same physiological effects as starvation. Under normal conditions, aerobic oxidation of glucose yields energy for the brain. In the absence of glucose, fatty acids are ß-oxidized in the liver, generating ketone bodies which can be used as an alternative energy source (Papandreou, et al., 2006). Ketone body levels in blood are usually maintained at ~0.3 mM, but can rise up to ~10 mM with the diet (Juge, et al., 2010). The main ketone bodies are ß-hydroxybutyrate and acetoacetate. Decarboxylation of acetoacetate yields acetone, a minor volatile ketone body (Papandreou, et al., 2006) which vaporizes in the lungs and gives the characteristic "ketone breath" odor (Wheless et al., 2001). Figure 2 shows the steps of ketogenesis in the liver.



Maintenance of the Ketogenic Diet (Johns Hopkins Hospital Protocol)

Figure 2: Ketogenesis in the liver. NEFA, non-esterfied fatty acids. HMG, β-hydroxy- β-methylglutaryl. NAD, nicotinamide adenine dinuclueotide. Source: Papandreou et al., 2006

It is of utmost importance that the KD be administered under close medical supervision (Freeman, et al., 2007a). Initiation of the diet typically occurs in a hospital setting, where the patient can be closely monitored in the event that there are complications. Patients must be accepted into the program to ensure that they are proper candidates (Casey, et al., 1999).

Initiation of the diet involves a 4-day hospital stay (Casey, et al., 1999). The KD normally begins with a fast of 36-48 hours (Freeman, et al., 2007a). Blood glucose is monitored and checked every 6 hours. Although glucose levels may fall very low (25-40mg/dL), they need not be treated unless the patient is hyperemetic or is extremely lethargic (Hartman, Vining, 2007). Introduction of substantial nutrition begins when ketone bodies begin to appear in the urine (Papandreou, et al., 2006). Calories are administered gradually in the form of "eggnog"; 1/3 of planned caloric intake is given on day 1 of feeding, followed by 2/3 and full caloric intake on days 2 and 3 respectively (Hartman, Vining, 2007). During the fasting period, parents attend daily classes on diet management.

After the initiation period, the child begins the actual ketogenic diet. Each child's diet is created to provide optimum seizure control while maintaining adequate nutrition for growth. Anthropometric measurements, activity status, and present medications are considered when calculating a diet. The diet is fine-tuned to give the child a high level of ketosis. A sample KD is shown in Table 1.

A Sample Ketogenic Diet for a 3-Year-Old Boy With No Medical Problems Other Than Intractable Epilepsy

Wt. 15.9 kg.	1035 calories daily/ 4:1 ketogenic ratio	19 grams protein daily
Ht. 42.5 in.	3 meals daily: 305 calories per meal	7 grams carbohydrate daily
65 cal/kg	1 snack daily: 120 calories	103.35 grams fat daily

Breakfast	**Snack**
22 grams egg	Peanut butter cup:
10 grams of applesauce	7 grams creamy Peanut butter
18 grams of butter	10 grams butter
30 grams of 36% cream	(mix together, roll into a ball and chill)
5 grams of bacon	
Lunch	**Dinner**
18 grams American cheese	14 grams chicken
28 grams cucumber	15 grams green beans
15 grams butter	22 grams butter
35 grams 35% cream	35 grams 36% cream

Table 1: Source Casey, et al., 1999

Breakthrough seizures can occur if the diet is not strictly maintained. Since the calorie restriction of the diet causes a loss of almost all body fat, seizures can occur when the body has no fat to burn and begins to break down protein to obtain glucose. Therefore, a snack should be given before bedtime, when the body will not have sufficient fat to metabolize for a prolonged period of time (Casey, et al., 1999). Parents must also be aware of hidden carbohydrates, as they may cause seizures. Even sugar alcohols such as sorbitol can cause seizures and can

be hidden in products such as suntan lotion and toothpaste. Certain medications can also contain starches which can cause breakthrough seizures (Freeman et al., 2007a).

Efficacy of the Ketogenic Diet

The KD offers a greater chance for seizure control than any of the anti-epileptic drugs developed recently (Freeman, et al., 2007a).There have been many studies that prove the efficacy of the diet. Effectiveness is generally not correlated with seizure type (Murphy 2005). Overall, about 10% of children become seizure-free on the KD (Martinez, et al., 2007) and about 50% have a 50% or greater improvement (Neal, et al., 2008). Freeman, et al., (2007a) prospectively studied 150 children on the KD. Before starting the diet, these children averaged over 600 seizures a month and had been on an average of 6 medications. After a year on the KD, 27% of children had a >90% reduction in seizures (Table 2). In a prospective study,

Number initiating And diet status	Seizure control	Time After Starting the Diet 3 Months	6 Months	12 Months	3-6 Years
Total N=150	Seizure-free	4 (3%)	5 (3%)	11 (7%)	20 (13%)
	>90% seizure reduction	46 (31%)	43 (29%)	30 (20%)	21 (14%)
	50-90%	39 (26%)	29 (19%)	34 (23%)	24 (16%)
	<50%	36 (24%)	29 (19%)	8 (5%)	18 (16%)
Continued on Diet		125 (83%)	106 (71%)	83 (55%)	83 (55%)

Table 2. Outcomes of the Ketogenic Diet-Johns Hopkins 1998 (Adapted from Freeman et al., 2007a)

150 children with medically refractory epilepsy who averaged 410 seizures a month were treated with the KD. After a year, 83 children remained on the diet, and almost all had a >50% reduction in seizures. Forty-one (27%) of the 150 had a >90% reduction in seizures. After 3-6 years on the diet, 13% of the original 150 were seizure free and an additional 14% had a >90% improvement (Hemingway, et al., 2001).

A more recent randomized controlled trial (Neal, et al., 2008) studied 145 children between the ages of 2 and 16. These children had daily seizures that had failed to respond to at least two medications. Seventy-three children were assigned to the KD group, and 72 to the control group. In both groups, seizure frequency was recorded during a 4 week baseline period. The diet group then started the KD for 3 months while the control group underwent no changes in epilepsy treatment (the control group had the opportunity to initiate the diet after the 3 month period was over). Data for 103 patients were available for analysis; 54 on the KD and 49 in the control group. Results showed a 62% mean drop in seizures in the KD group, and a 137% increase in seizures in the control group. This surprising increase in seizures was due to 3 outliers; when their data were removed, the percent of seizure increase in the control group went down to 12. Although these results are not as drastic as those of other studies, they are still very significant as they show a direct comparison between children on the diet and children being treated unsuccessfully with medication (Table 3).

	Diet Group (n=73)	Control Group (n=72)
>90% reduction in seizures	5 (7%)	0 (0%)
>50% reduction in seizures*	28 (38%)	4 (6%)
<50% reduction in seizures†	45 (62%)	68 (94%)

Percentages based on numbers allocated to each intervention.
* Includes patients who reported >90% reduction.
† Includes 71 patients with data and 42 unknown (16 did not receive treatment, 16 with no data)
Table 3: Number of children in each group who achieved 50% and 90% seizure reduction at 3 months. (adapted from Neal et al., 2008)

Along with reducing seizure frequency, the KD also has been shown to slightly improve overall developmental functioning and motor skills, as well as attention and social problems in children who remained on the diet for at least a year (Pulsifer, et al., 2001). Additionally, medications can be lowered in dosage or in some cases eliminated completely. Although there are side effects to the KD (see below), most parents preferred these consequences to the sedation and cognitive dulling that result from anti-epileptic drugs (Groesbeck, et al., 2006).

Children who benefit from the diet usually remain on it for 2 years, or until they have successfully stopped medication for a year. They are then slowly weaned off the diet, going from a 4:1 ratio to a 3:1 ratio for 6 months. If a child remains seizure free, the ratio can be lowered to 2:1 for another 6 months, after which the child can return to a normal diet (Freeman et al., 2007a). A retrospective study (Martinez, et al., 2007), reviewed 557 children who were treated at Johns Hopkins Hospital. Sixty-six (12%) discontinued the diet after becoming seizure-free. Ninety-two percent of these 66 children were also medication-free. Thirteen (20%) children had their seizures recur after about 2.4 years off the diet, yet 7 of the 13 became free of seizures their second time on the KD, 4 with anticonvulsant therapy. Thus, children who are seizure-free on the KD have a 20% chance of recurrence. This is significantly lower than the 30-50% rate of seizure recurrence in children who stop medication. However, it is important to note that this is the only study of its kind.

Side Effects and Disadvantages of the Ketogenic Diet

Kidney Stones

As with all medical treatments, the KD has some side effects and disadvantages. In a retrospective study of 195 children on the KD for a median of 12 months, 13 (6.7%) developed kidney stones. Fortunately, this did not result in termination of the diet for any of these children. A few factors put children on the diet at risk of nephrolithiasis. The KD causes a general acidosis which can lead to bone demineralization and hypercalciuria. It also causes hypocitraturia; since citrate usually solubilizes free calcium in the urine, a shortage of it will leave more calcium available to form stones. Uric acid is also less soluble at a low pH and can form crystals that attract

calcium. Stones can form due to the fluid restriction of the diet (Sampath, et al., 2007). Family history of nephrolithiasis is taken before initiation of the KD. Patients at risk are treated prophylactically with oral citrate salts (Hartman, Vining, 2007).

Dyslipidemia

Dyslipidemia (abnormal amounts of lipid in the blood) due to the high fat content of the diet was also found in children on the KD. Interestingly, total cholesterol levels were found to decrease in patients over time, suggesting that eventually, their bodies can better metabolize cholesterol and fat (Nizamuddin, et al., 2008). Additionally, since the high fat is accompanied by an overall restriction of calories, changes in blood levels of lipids, cholesterol, LDL, and lipoproteins are slight (Freeman, et al., 2007a, 117).

Lack of Growth

The KD seems to have an effect on long term growth in children. In a study of 28 children on the diet, 14 were below the 10th percentile for height before initiation. Follow-up measurements showed that this number had increased to 23. Height and weight percentiles remained proportionate to each other (Groesbeck, et. al., 2006). Other studies have also confirmed the KD's slowing effect on growth (Kim, et al., 2013, Williams et al., 2002). A child's growth is constantly monitored on the KD. If the child is not growing normally, the diet ratio can be lowered to allow more protein (Freeman, et al., 2007a, 116).

Noncompliance

The KD is a very stringent diet. Food must be weighed for every meal and the child must eat everything on the plate to ensure that a correct ratio of fat to carbohydrates and protein is received. Many children discontinue the diet for non-medical reasons. In a study of 46 children on the KD, there were 9 such cases. Non-compliance was more common in older children (Lightstone, et al., 2001). Reasons for discontinuation in this study are shown in table 4.

Table 4. Reasons for Discontinuation of the Ketogenic Diet

	All		Neurological Status		SES[a]		Age (years)		
	Number	Percentage	Normal	Abnormal	Low	High	<6	6-12	>12
Total Number of Children Initiating Diet	46		8	38	7	39	25	15	6
Number Remaining on the Diet at 6 Months	27	59%	5	22	2	25	14	11	2
Medical Reasons for Discontinuing Diet	10	22%	1	9	2	8	9[b]	1[b]	0[b]
Lack of efficacy	8	17%	1	7	2	6	7	1	0
Complications	1	2%	0	1	0	1	1	0	0
Acute hospitalization (unrelated)	1	2%	0	1	0	1	1	0	0
Nonmedical Reasons for Discontinuing Diet	9	20%	2	7	3	6	2[c]	3[c]	4[c]
Caregiver issues	5	11%	0	5	2	3	2	1	2
Too regimented/ could not prepare diet in a timely fashion	2	4%	0	2	1	1	0	0	2
Overwhelming anxiety re: food preparation and measurement	1	2%	0	1	0	1	1	0	0
Perception of too little	1	2%	0	1	1	0	0	1	0
Refusal of caregiver to follow the diet	1	2%	0	1	0	1	1	0	0
Patient Issues	4	9%	2	2	1	3	0[b]	2[b]	2[b]
Patient refused to eat diet foods	2	4%	0	2	1	1	0	2	0
Patient cheated on the diet	2	4%	2	0	0	2	0	0	2

[a]SES= socioeconomic status
[b] $p < .05$
[c] $p < .01$

Table 4: Reasons for discontinuation of the Ketogenic Diet. Source: Lightstone, et. al, 2001

Mechanisms of the Ketogenic Diet

Although many studies prove KD's efficacy, its exact mechanism of action remains unknown. However, many theories have been hypothesized as a result of experimentation using animal models. The following are the some of the proposed mechanisms of the KD.

Ketone Body Hypothesis

It seems evident that a high concentration of ketones in the blood is responsible for the anticonvulsant effects of the KD. After comprehensive research on the subject, it is not yet clear as to whether this is the case. Still, there have been some significant correlations between ketone bodies and seizure reduction (Masino, Rho, 2011). In humans on the KD, seizure control often does not peak until after 2 weeks, when ketone levels are at their highest (Bough, Rho, 2007). Moreover, blood levels of ß-hydroxybutyrate seem to be related to the degree of seizure control in children on the KD (Masino, Rho, 2011). However, when carbohydrates are abruptly reintroduced to the diet, breakthrough seizures and loss of ketosis can occur. Yet, overall seizure resistance waned gradually in patients who discontinued the diet. This indicates that a breakthrough seizure does not reflect complete loss of ketosis; ketone levels are still high after introduction of carbohydrates. Thus a certain degree of ketosis is necessary, but is not sufficient to control seizures (Bough, Rho, 2007).

Although the KD has been successful in many age groups, it seems to be more effective in infants and children. This is another reason why ketones are thought to have an anticonvulsant effect (Wheless, et al., 2001). Ketones pass across the blood-brain barrier by means of monocarboxylate transporters. Studies have indicated that a KD can increase the expression of monocarboxylate transporters in the brain of adult rats. However, this enhancement was found to be far greater in suckling rats (Papandreou, et al., 2006). Prior to weaning, a rat's blood level of ketone bodies is high due to the fatty composition of rat milk (Morris, 2005), and young rats' brains are more accustomed to using ketones as an energy source (Papandreou, et al., 2006). In fact, the blood-brain barrier's permeability to ß-hydroxybutyrate has been shown to increase by a factor of 7 during the suckling period in rats, and decrease after weaning (Morris, 2005). A child is able to extract ketones from the blood and transport them to the brain four to five times as efficiently as in adults. (Wheless, et al., 2001). However, some studies have indicated that the KD is as effective in adults as it is in children (Morris, 2005).

ß-hydroxybutyrate is the most prevalent ketone body in the blood. Although levels of plasma ß-hydroxybutyrate are raised in a KD patient, it has not been proven to have anticonvulsant effects (Masino, Rho, 2011). However, when cultured glutamatergic neurons metabolized

ß-hydroxybutyrate, their glutamate content decreased (Lund, et al., 2009). Metabolism of this ketone body in the place of glucose may reduce the availability of glutamate, an excitatory neurotransmitter, and thereby have an indirect anticonvulsant effect (Masino, Rho, 2011). ß-hydroxybutyrate also has structural similarities to gamma-aminobutyric acid (GABA), an inhibitory neurotransmitter (Morris, 2005).

The other ketone bodies, acetoacetate and its decarboxylated product, acetone, have prevented seizures in animal models. In one study, acetoacetate was found to inhibit vesicular glutamate transporters, which are needed for exocytotic release of glutamate (Masino, Rho, 2011). Cl^- acts as an allosteric activator and regulates these transporters. When neurons derived from rat hippocampus were stimulated with KCl, considerable amounts of glutamate were released. Addition of acetoacetate to the culture medium inhibited glutamate exocytosis, and this inhibition was fully reversed upon removal of acetoacetate. (Juge, et al., 2010). Acetoacetate competes with an anion-dependent regulatory site on presynaptic vesicles, thus decreasing the amount of glutamate and excitatory neurotransmission (Masino, Rho, 2011). This may explain why sudden ingestion of carbohydrates can cause an immediate seizure. Ketosis suppresses glutamatergic neurotransmission through inhibition of vesicular glutamate storage (Figure 3). Acetoacetate levels decrease upon introduction of carbohydrates, and vesicular glutamate transporter action is turned on, leading to an influx of glutamate into synaptic vesicles (Juge, et al., 2010). However, this still does not explain why seizures occur despite the fact that ketone levels remain high after carbohydrate introduction. Acetone may also contribute to the KD's anticonvulsant properties. In one study, magnetic resonance spectroscopy showed the presence of acetone in the brains of five out of seven patients successfully treated by the KD (Bough, Rho, 2007). There was no evidence of ß-hydroxybutyrate or acetoacetate in the spectra even though they were present in these patients' urine. Acetone may be the principle intracerebral intracellular ketone amassed in response to the KD (Seymour, et al., 1999).

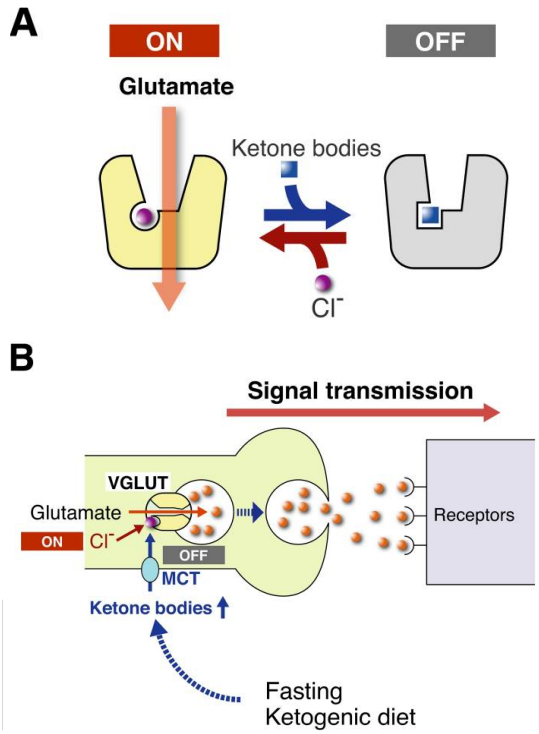


Figure 3: Proposed mode of action of ketone bodies on VGLUT-mediated suppression of glutamatergic neurotransmission.
VGLUT: vesicular glutamate transporter
MCT: monocarboxylate transporter
Source: Juge et al., 2010

Additionally, Increased amounts of ketone bodies lead to increased levels of α-ketoglutarate, part of the tricarboxylic acid cycle. α-ketoglutarate is also a component of the GABA shunt; if elevated, increased input into the GABA shunt may occur. This may have an increasing effect on GABA, an inhibitory neurotransmitter, in local areas of the brain (Wheless, et al., 2001). In humans, cerebrospinal fluid levels of GABA were found to be higher during the KD than before the diet, and the best responders to the diet had the highest levels (Hartman, et al., 2007).

Glucose Restriction Hypothesis

The flip-side to the ketone body hypothesis is that glucose restriction is responsible for the anticonvulsant effects of the KD. As ketonemia develops, glucose levels in the blood are reduced simultaneously. The hypoglycemia may just work to stabilize ketosis, but some studies suggest that the lack of glucose itself can reduce seizures (Bough, Rho, 2007). One study reported that during epileptic seizures, uptake of glucose is high and lactate, the precursor to glucose is also increased (Papandreou, et al., 2006). Greene, et al. (2001) hypothesized that calorie restriction reduces the amount of energy from glycolysis and restricts a neuron's ability to obtain the high levels of energy needed for epileptogenesis.

Another hypothesis involves the effect of low glucose on ATP-sensitive potassium (KATP) channels. KATP channels are ligand gated receptors found in neurons and glia throughout the central nervous system. These channels sense fluctuating levels of ADP and ATP and cell membrane excitability changes accordingly (Bough, Rho, 2007). Although overall levels of ATP in the brain are elevated during the KD, the oxidative metabolism of ketone bodies causes a reduction in brain glucose utilization. ATP derived from glycolysis may play a prioritized role in controlling processes at the cell membrane, including regulation of KATP channels and fueling of ATP-driven sodium pumps (Yellen, 2008). As intracellular glycolytic ATP concentration falls during the KD, KATP channels open to hyperpolarize the cell. When ATP levels rise in the presence of glucose, KATP channels close. As such, KATP channels may regulate seizure threshold (Bough, Rho, 2007). These channels are predominant in the GABAergic projection neurons of the substantia nigra pars reticulata, the region of the brain thought to be responsible for regulating seizure threshold; (Yellen, 2008) therefore, they are in the ideal position to regulate many

threshold; (Yellen, 2008) therefore, they are in the ideal position to regulate many different types of seizures. Genetically engineered mice that exhibited an overexpression of the sulfonylurea subunit of the KATP channel were substantially more resistant to seizures than wild type mice. (Bough, Rho, 2007). Similarly, KATP channel knockout mice exhibited grand mal seizures and death following brief hypoxia, while wild-type mice all recovered from the same stimulus (Yamada, et al., 2001).

Other Hypotheses

There are several other theories as to why the KD works. A recent study showed that increased activation of adenosine A1 receptors suppresses seizures in mice. Adenosine has been found to be a powerful anticonvulsant, and the KD elevates its levels in the brain by reducing expression of adenosine kinase. Overexpression of adenosine kinase has been linked to seizures, and can reduce adenosine A1 receptor activation. In transgenic mice, the KD stopped spontaneous seizures caused by deficiencies in adenosine metabolism if adenosine A1 receptors were intact. Seizure activity was reduced by 50% in mice that had half the amount of receptors, and unaltered in mice that lacked adenosine A1 receptors. Western blot analysis showed that the KD reduced amounts of adenosine kinase. Likewise, brain tissue of humans with intractable epilepsy showed increased levels of adenosine kinase, signifying possible adenosine deficiency (Masino, et al., 2011).

Others say that ketone bodies provide more energy per unit of oxygen to the brain. This may help to enhance a neuron's ability to endure metabolic challenges (Hartman, et al., 2007) and resist hyperexcitability (Rho, Sankar, 2008). However, this contradicts the hypothesis that glucose restriction results in reduced availability of energy for epileptogenesis.

Other hypotheses include the KD playing a neuroprotective role by reducing the amounts of reactive oxygen species in mitochondria, and enhancing glutathione, an antioxidant (Rho, Sankar, 2008).

Conclusion

Many parents of children with medically refractory epilepsy have given up hope of their child becoming seizure-free and leading a normal life. However, when medication and surgery are not options, the KD can effectively reduce seizures in many cases. The KD has shown its success both in studies and in individual patients. It is both a cheaper and less toxic treatment than drugs or surgery. It can increase mental clarity and improve motor functioning in children. As with all medical treatments, the KD has side effects, although not as severe as those of medications.

The diet's mechanism of action is still unknown, but scientists are still researching the possibilities. Many hypotheses currently exist, yet most of these are based on animal models. Further research may need to be done on human subjects for scientists to discover the real mechanism. However, this may be impossible as many of the animal studies would be considered inhumane if implemented in humans. Discovery of a mechanism may eventually lead to a drug that works differently than those that are currently available. Still, the fact that so many hypotheses exist may indicate that the real mechanism is a combination of many of the possibilities. Regardless of what is known about the KD, it is still a miracle cure for many patients who suffer from epilepsy.

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Huntington's Disease and its Effect on the Brain

Shaina Rivkin-Drizin

Abstract

Huntington's disease is a neurodegenerative disease that leads to gradual extensive brain damage, especially in the striatum and the cerebral cortex. Initial symptoms are cognitive difficulties, loss of motor control, and sudden mood imbalances. Cognitive function slowly declines into dementia, coupled with behavioral and psychiatric problems. Sufferers die within 20 years due to illness complications: a fall, pneumonia, or heart disease (Walker, 2007). This paper reviews the principle biological cause of the disease, its effect on the brain, diagnosis, and treatment.

Huntington's Disease

Huntington's disease is a severe neurological disorder that strikes about 1 person in 10,000 in the United States. It is also called Huntington's chorea, as in choreography, because sufferers' involuntary writhing sometimes resembles dancing. It causes widespread and pervasive damage throughout the brain, starting in the striatum and extending to many cortical areas. The most visible symptom is the lack of motor control, however, there are many cognitive and emotional deficits associated with the Huntington's: apathy, depression, irritability, psychosis, anxiety, obsessions and compulsions (Kingma, et al., 2007). The disease most often appears between the ages of 30-50, although it can occur in early childhood too. Huntington's is caused by a combination of known heredity factors and some unknown environmental factors. There is no cure yet for Huntington's disease. Currently, health care professionals treat the symptoms of the disorder with drugs; physical, occupational and speech therapy; and counseling.

Genetics

The gene that predicts Huntington's disease is autosomal dominant. (A recessive mutation leads to the loss of a desired function, while a dominant mutation creates an undesired function. The undesired gain here is the elongation of the Huntingtin protein.) The gene is shown in figure 1 and is located on the short arm of chromosome 4; It codes for the Huntingtin protein (Htt). In healthy individuals, the gene has a sequence of bases C-A-G (cytosine, adenine, guanine) repeated 11-24 times. Beyond normal threshold, the gene will code for a mutant elongated polyglutamine tract close to the N-terminus of the Huntingin protein (mHtt). Pathological indications begin when the gene has 35-38 repeats and predicts an elevated risk for late-onset of the disease, and/or of passing the elongated gene onto one's children. When the gene is passed on maternally, CAG tend to shorten; when passed paternally, CAG may elongate causing Huntington's to spontaneously appear in offspring, or decreasing the age of onset in offspring. The greater the number of C-A-G repeats, the earlier the onset of the disease and the more rapid the deterioration.

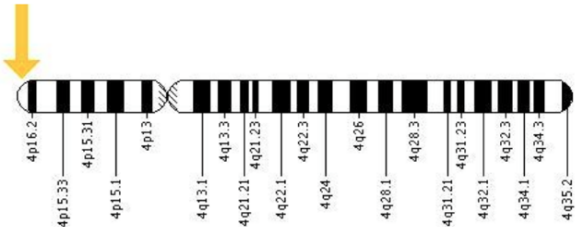

Figure 1: Chromosome 4 (U.S. National Library of Medicine, 2013)

Huntingtin Protein

The function of the Huntington protein is not entirely understood, but scientists have discerned that it plays a role in cell signaling, intracellular transport, and transcription. It is also crucial to neurological development of the fetus. The protein is found all over the body with the highest concentration in the brain and testes, and moderate amounts found in the liver, heart, and lungs (Walker, 2007).

Mutant Huntingtin Protein

Pathologically, abnormally long mutant Htt proteins break up into smaller, toxic segments that bind together and accumulate in the neuron. The inclusions present a mechanical blockage to neurotransmitters because the synaptic vesicles can no longer move through the cytoskeleton, as shown in figure 2. It is grossly detrimental in the striatum due to the presence of the striatal protein, Rhes (Subramananiam, et al., 2009). Rhes proteins induce sumolyation. Sumolyation [by Small Ubiquitin-like Modifier (or SUMO) proteins] is the modification of post-translational proteins that are involved in nuclear- cytosolic transport, transcriptional regulation, apoptosis, response to stress, and progression through the cell cycle (Hay, 2005). By this process, Rhes proteins prevent the aggregation of mHtt: this produces a soluble form of mHtt that is cytotoxic. Additionally, the cell's involvement in degrading the extra protein comes at the expense of other vital cell functions. Mutant Htt impinges on transcription regulation, apoptosis, tumor supression, mitochondrial function, and vesicle transport. Normally, when a neuron is recognized as a contributing, functional unit, it is nourished by

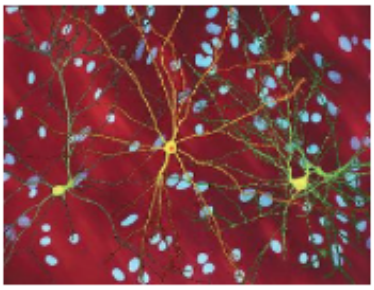

Figure 2: Medium spiny nueron (yellow) with nuclear inclusion (orange) (Huntington's Diesease 2013)

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nearby neruons and glial cells with brain-derived neurotrophic factor. However, due to mHtt interference, the neuron does not receive support and nourishment from nearby glial cells and neurons and the cell eventually dies (Walker, 2007).

Repercussion to the Brain

The Striatum

The striatum (figure 3) is the largest part of the basal ganglia, a sub cortical collection of nuclei. It is made up of two grey areas-the caudate nuclei and the putamen- and separated by the internal capsule. The internal capsule contains ascending and descending tracts between the cerebral cortex and medullary pyramids. This area is involved in modulating the afferent information from the cortex. Seventy-seven percent of the striatum is composed of medium spiny projection GABA-ergic neurons that receive converging stimulation from many areas of the cortex and feed selected signals to the prefrontal cortex. GABA is an inhibitory neurotransmitter and its release allows the activation of motor activity. Activation of this area correlates with expectations of rewards and consequences, and affects decision-making (Stocco, et al., 2010).

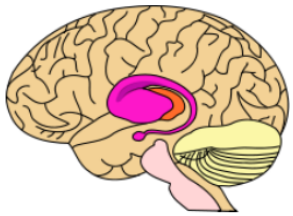

Figure 3 The striatum, colored magenta, is most damaged by Huntingon's (Huntington's disease, 2013)

The striatal medium spiny neurons of the basal ganglia are most vulnerable to Huntington's disease harm. Those that contain enkaphalin and project to the external globus pallidus are involved in circuits known as basal ganglia-thalamocortical circuits. The circuits project to the motor cortex and direct movement. For that reason, their damage predicts a lack of motor control (Walker, 2007).

Effect on Learning

The striatum also negotiates interactions between pairs of cortical areas. These associations create stimulus-response relationships that assist in learning skills and developing habit. There are several models that explain how this occurs. This paper describes the SPEED model. SPEED- Subcortical Pathways Enable Expertise Development- is designed to account for how automaticity is acquired (Ashbey, et al., 2007). The SPEED model asserts that the striatum mediates a conversation between a stimulus and a task-dependent motor response. When the correct response is achieved a burst of dopamine is released. Consequently, the cortex prefers the motor response associated with positive feedback, and in a mechanism known as Hebbian learning, the brain will strengthen the response tract and depress surrounding competing areas. These tracts are impaired in Huntington's patients and they are unable to learn new skills (Stocco, et. al., 2010).

Other Regions Effected by Huntington's

Other regions effected by the disease include the substantia nigra, cortical layers 3, 5, and 6, the Ca1 region of the hippocampus, the angular gyrus in the parietal lobe, the Purkinje cells of the cerebellum, the lateral tuberal nuclei of the hypothalamus, and the centromedial parafascicular complex of the thalamus (Walker, 2007).

The substantia nigra is part of the motor system. It receives stimulation from the motor cortex, premotor cortex, caudate nucleus and putamen. It sends information to the striatum and thalamus. It has a high dopamine level and is involved in planning and initiating movement, and reward.

The Ca1 region of the hippocampus is involved in long term potentiation for memory and learning and also indicated in mood regulation (Macey, et al., 2009) A dysphoric mood was associated with increase of hippocamal activity in Huntington's sufferers.

The angular gyrus in the parietal lobe is involved with number processing, spatial recognition, memory retreival, attention, theory of mind, and language. The angular gyrus has a substantial projection to the caudate nucleus and the diseased suffer considerable loss in this area (Macdonald, et al., 1997).

The Purkinje cells of the cerebellum, with an elaborate tree-like network of dendrites, are some of the largest cells. These cells play a crucial role in coordinating movement. HD is associated with reduced Purkinje cell density (Jeste, et al., 1984).

Results from post-mortum analysis of patient's with motor disturbances also display neuronal loss in the NTL (Lateral Tuberal Nucleus of the hypothalamus) (Kremer, et al., 1990). In humans, the NTL appears to control body weight and thermoregulation.

The centromedial (CM) and parafascicular complex (Pf) of the thalamus each contribute excitatory input to the striatum. The CM projects to the entire sensorimotor area of the striatum and the Pf provides complementary input to the associative region (Sadikot & Rymar, 2009). They also receive input from the motor and associative limbic system.

Diagnosis

Before being diagnosed, the patient may experience small imperceptable changes to personality, cognitive abilities, and motor control. Multi-tasking becomes difficult, the individual becomes irritable, forgetful, and unreliable, leading to anxiety. Diagnosis is usually sought once chorea and saccadic eye movement set in. Cognitive degeneration impairs planning, judgement, self-care, and organising and delays new-motor skill attainment. Mental degeneration is also significant; depression and suicidal ideation are common. Some patients also become manic and\or psychotic (Walker, 2007). A diagnosis is obtained by testing the patients ability to maintain a voluntary muscle contraction and fine motor execution. The patient may be

asked to stick out his or her tongue (HUNTINGTONS 3, 2009) or to carry out a finger-tapping rhythm.

Genetic Testing

Huntington's disease is a model for the opportunities and challenges of genome testing. Conclusive genetic testing is possible and can be helpful for people who want to be informed before they choose a career and start a family. However, fewer than 5% of those at risk seek testing. Individuals may want to avoid any discrimination associated with the diagnosis. Testing is also risky because it sometimes leads to suicide. Prenatal testing and preimplantation genetic testing are also optional, although many parents decline in the hope that by the time their children reach the age of risk there will already be a remedy in place (Walker, 2007).

Treatment

Medicine has yet to find a cure for Huntington's disease. Currently, patients can rest from the constant involuntary movements by taking antichoreic drugs like tetrabenezine or neuroleptics to control psychotic symptoms. Patients benefit from support groups, family support, and counseling. Physical, Occupational, and Speech therapy all help patients to maintain functioning for as long as possible (Walker, 2007).

Research

Research conducted along various avenues yield hopeful possibilities for the future. One potential therapy is RNA interference which aims to reduce the expression of the Huntingtin gene. The therapy was conducted on the Rhesus Macacque and established that a 45% reduction of the gene does not significantly affect motor function in healthy primates (McBride, et al., 2011).

Also in progress is research in cell therapy which aims to protect vulnerable neurons and replace dysfunctional cells through use of fetal or stem cells and through dispensing neurotrophic factors to support the brain (Clelland, et al., 2008).

Conclusion

Although HD's initial damage occurs almost exclusively in the striatum of the basal ganglia, much like a moldy strawberry that ruins the entire basket, in due course the spoil is far-reaching and extensive. The striatum is centrally located in the brain and projects widely. By associating with this defective spot, additional areas of the brain lose their functional capacity.

This paper is a detached, technical review of scientific review and research articles. Although it describes the clinical symptoms that HD presents, it hardly captures the heartache and distress the disease inflicts on the sufferers and their families. A diagnosis is a sure death sentence, prolonged and full of suffering. It is a twenty-year decline into certain doom- dementia and loss of motor function. It is humbling and difficult to confront an illness where sometimes the best one can do to help its victims is to validate their loss.

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Are Oncolytic Viruses a Cure for Cancer? A Look at Reovirus, Adenovirus, and HSV-1 in Cancer Treatment

Yehuda Rosenberg

Abstract

This paper aims to evaluate the option of utilizing Oncolytic Viruses as a viable treatment in fighting cancer. However, due to the broad nature of the subject, a more limited purview is necessary. With that in mind, the focus will be on a few of the more researched ones: Reovirus, Adenovirus, and HSV-1. In each case, we will examine what makes each of these potential options. This will include an examination of each ones tumor-specificity. Cancer and viral physiology will be discussed as necessary to examine the distinct protein expressions in tumor cells, so that the virus's method of battling the host's cell defense is only effective for cancer cells. In addition, its strength and weaknesses in terms of battling metastasized cancers, overall efficacy, as well as its capability to be used in tandem with other treatments will be discussed. Included in this analysis is the current prognosis of OV as demonstrated in several clinical trials. Finally, we will summarize several current obstacles to OV and some suggested solutions.

Introduction

Cancer is one of the most feared and deadliest diseases that afflict people. Current treatments for cancer surgery, radiotherapy, and chemotherapy have little success in treating metastasized cancers. Scientists continue to search for new potential treatments and cures. One prominent approach is virotherapy, or using viruses to treat cancer. Since the early nineteen hundreds, scientists have noted the correlation between viruses and, at least, the temporary remission of certain cancers. Further understanding of cancer and viruses in the mid-1900's, sparked new interest in the possibility of using virus for cancer treatment. However, those early attempts proved mostly unsuccessful because of the immunogenic nature of viruses. The 1990's ushered in advances in technology, better understandings in the fields of Virology and Cancer biology, and with it renewed interest in Oncolytic Viruses (OV). These viruses are, generally, genetically altered to differentiate between healthy cells and cancer cells, and then as viruses, replicate and lyse cancer cells. It should be noted that most if not all viruses can be altered to have OV-tendencies. However, obviously discussing every type of virus for every type of cancer is not feasible. Instead, a look at the two most researched OV: HSV and Adenovirus-based OV, as well as the naturally occurring Reovirus OV, which practically is easier to study than engineered OV will be the focus. This paper aims to explain the methods of attaining tumor selectivity, current obstacles OV face, and the progress made. In addition, this paper will attempt to project realistic hopes for the future of Oncolytic Viruses, and its impact on cancer treatment.

Cancer and its current prognosis

Cancer is a class of disease in which damaged and physiologically-altered cells replicate uncontrollably. It occurs when the genes regulating replication are altered, usually due to mutation. These mutations either cause hyper-expression of oncogenes, which promote cell growth, or under-expression of tumor-suppressor genes, which limits replication of damaged cells. Currently, the most successful treatment for cancer is surgery; however, its efficacy is limited to instances when the cancer is in a single spot. If the cancer metastasized, through the lymph nodes or bloodstream, surgery's effectiveness is limited. Radiation and chemotherapy are also used to fight cancer. However, the toxicity and side-effects – some serious such as infertility, pain, the possibility of it causing other forms of cancer, and death – and the limitations of these treatments, especially against metastasized cancers, makes further study of cancer necessary.

Virotherapy

Using viruses (virotherapy), as a possible cure for cancer is currently being investigated and has progressed to the level of Phase III clinical trials. Viruses are, obviously, generally regarded as pathogens, although out of the millions of different species only a relatively small number are dangerous to humans. A virus is made up of nucleic acid, either DNA or RNA, a protein coat called a capsid, and sometimes has an envelope. Inherently non-living, viruses must infiltrate and hijack a host cell to replicate. Most viruses lyse the host cell after replication, though some are latent. Scientists think viruses with, little or no pathogenicity to people, are attracted to cancer cells, they can replicate and lyse cancer cells and may be a viable cancer treatment. Additionally, viruses may be used as vectors, — to transport proteins that stimulate host immune response to tumors. These viruses that prefer cancer cells and have little or no toxicity to healthy cells are called OV and achieve this selectivity through different mechanisms.

Criteria for use as an Oncolytic Virus

Viruses need to be able to exhibit certain attributes to be viable oncolytic options. Although a virus doesn't necessarily need all of the forthcoming features, many are needed in general, and some are needed under certain circumstances. Viruses that are pathogenic and infect humans are generally poor choices, since the host's previous exposure to the virus increases the probability that the host has built-up immunity to the virus. The immune system can hamper viral activity and effectiveness. Another concern is safety: viruses are first regarded as a parasitic threat, their capability of hijacking healthy cell metabolism or producing dangerous toxins must be monitored simultaneously with their ability to fight cancer. The ability to kill out-of-control dangerous viruses via antiviral drugs is another aspect of selecting a safe choice. Another

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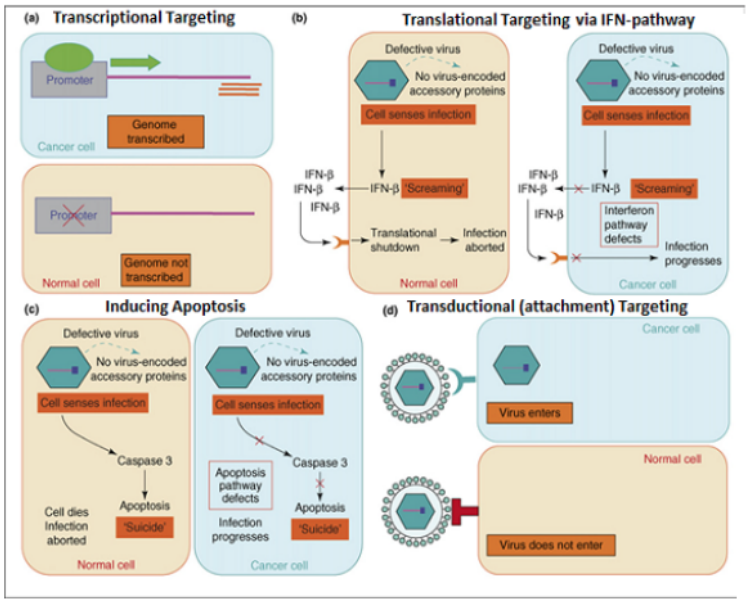
drugs is another aspect of selecting a safe choice. Another safety concern is the possibility that the virus may mutate into a dangerous virus. To limit this, viruses that cannot enter the host cell's nucleus or that cannot undergo recombination with host genes are recommended. Viruses with certain characteristics are better suited to fight cancer than others. Viruses with rapid life cycles that replicate, lyse, and spread to other cancer cells quickly are more suited as OV than slower viruses. Another critical feature for OV is its requirement to be tumor-selective rather than targeting both healthy and tumor cells indiscriminately. Often, the Oncolytic virus by itself is not powerful enough to wipe out the cancer. Its effectiveness may be increased when used together with conventional treatments – radiation and chemotherapy. In a similar way, OV can be combined with genes that also fight cancer, using the virus as both vector and oncolytic agent. Therefore, the ability to easily manipulate the viral genome to insert these genes, as in the case of adenoviruses, is an important feature. Finally, to counter metastasized cancers, the ability to deliver it intravenously is critical to efficiently spread the OV. (Kelly & Russell, 2007)

Methods of Tumor Selectivity and Tumor Cell Death

Oncolytic viruses achieve tumor-selectivity in four distinct ways: targeting transcription, targeting attachment, IFN-signaling, and cell apoptosis (Figure 1). Some viruses can be altered with a tissue-specific-promoter in their genome to regulate genes essential for viral replication. Thus, only in tumor cells is the factor that is required for replication available. An example of this method is Adenovirus 7870, engineered so that its E1B is under the control of prostate cancer-specific promoter (Small et al, 2006). A second method is by targeting viral attachment. To understand this, some knowledge of tumor physiology is needed. As stated earlier, cancer cells are mutated cells that are out of control. As such, they often result in distinct proteins that are either overexpressed or mutated. Viruses can be engineered to bind to the proteins, thereby becoming tumor-selective. Coxsackie A21, from the picornovirus family, utilizes this method by binding to intercellular adhesion molecule-1 (ICAM-1) and decay accelerating factor (DAF) which are overexpressed in cancer cells (Shafren et al, 2011). Third, many cancer cells have defective IFN-pathways. Viruses can be altered so that their defense to block IFN-pathways is removed, leaving them extremely vulnerable in normal cells to interferon. However, tumorous cells with defective pathways are unable to carry out the pathway and, therefore allow viral replication. Examples of this method are demonstrated by the wild-type Reovirus and the ICP-34.5-null HSV-1-based OncoVEX GM-CSF, which will be explained in detail later. Finally, the last method involved apoptosis – programmed cell death. In response to infection, host cells carry out apoptosis as a virus-limiting mechanism, facilitated by tumor suppressor protein, p53. Tumor cells commonly lack expressed p53 genes because it also carries out cell death in response to uncontrollable growth. These p53-null cells are unable to effect cell death when infected , allowing viral replication. Adenovirus Onyx-15 uses this technique through deletion of its E1B gene, though as explained later this is not a precise explanation. Once inside the tumor cell, virus brings about cell death by normal viral replication and lysis. Additionally, viruses may induce apoptosis, enabling the increase of viral progeny. Moreover, viruses attract the host's immune system; in turn, an activated immune system eliminates cancer cells, through natural killer and other cells.

Figure 1: Methods of Tumor-Selectivity Source: Russel & Peng 2007



Reovirus

There are several viruses that are naturally oncotropic, possessing an affinity for tumor cells. One such species is the Reovirus, a virus that structurally has no envelope and contains double stranded RNA. Short for Respiratory Enteric Orphan virus, it usually infects the respiratory system and intestines. The reovirus also has several factors that make it a viable option as an OV. First, it's very common. Additionally, it has minimal pathogenicity to adults, and does not typically cause symptoms. Reovirus's oncotropic nature is linked to its replication mechanism through the overexpressed EGFR\RAS-pathway.

Reovirus: Ras-pathway Selectivity?

There are two prevalent theories for reovirus replication. (Strong et al, 1998) The first possibility is that reovirus binds to EGFRs, or Epidermal

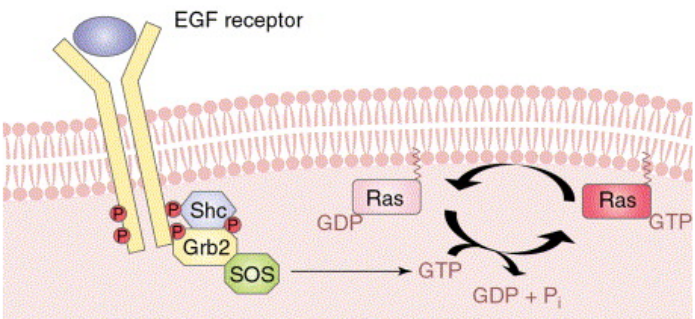


Figure 2: Ligand-Mediated activation of EFG receptor, stimulates Ras-Pathway by phosphorylation. The pathway regulates activation of membrane effectors. Source: Norman et al., 2005

growth factor receptors, which in turn, activates tyrosine kinase, triggering a chain of cell signaling leading to the subsequent steps of the infection process. Alternatively, reovirus may take advantage of a signal transduction pathway previously activated by EGFR in the host cell. The latter possibility implies a correlation, albeit indirect, between reovirus replication and EGFR stimulation. Thus, having established a connection between the two, the tumor-specifity can be explained. In the case of healthy host cells, virus replication phosphorylates double stranded RNA-activated protein kinase (Bischoff & Samuel, 1989). This leads to intermolecular transphorylation (Thomis & Samuel, 1993), activating the protein kinase. This in turn phosphorylates the alpha subunit of eIF-2 which inhibits viral translation (Panniers & Henshaw, 1983). However, in tumerous cells, PKR phosphorylation is inhibited by an overactive Ras-pathway –common oncogenes prevalent in about half of all cancers (Strong et al, 1998)—allowing viral RNA translation to occur (Figure 3). An obvious defiecency, then, in utilization of Reoviruses as OV is its dependence of cancers with compromised Ras pathways.

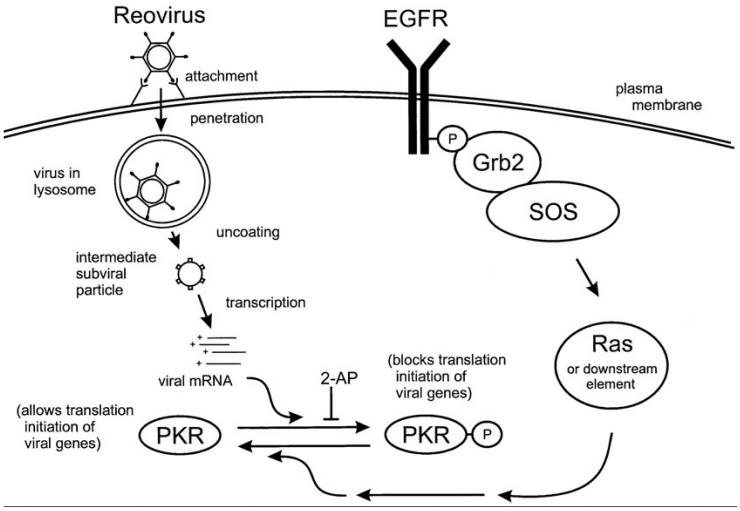


Figure 3: The molecular basis of reovirus oncolysis: usurpation of the host cell Ras signaling pathway. For both untransformed (reovirus-resistant) and EGFR-, Sos- or Ras-transformed (reovirus-susceptible) cells, virus binding, internalization, uncoating and early transcription of viral genes all proceed normally. In the case of untransformed cells, secondary structures on the early viral transcripts inevitably trigger the phosphorylation of PKR, thereby activating it, leading to the phosphorylation of the translation initiation factor eI F-2α, hence the inhibition of viral gene translation. In the case of EGFR-, Sos- or Ras transformed cells, the PKR phosphorylation step is prevented or reversed by Ras or one of its downstream elements, thereby allowing viral gene translation to ensue. The action of Ras (or a downstream element) in promoting viral gene translation (and hence reovirus infection) in the untransformed cells can be mimicked by deletion of the Pkr gene or by blocking PKR phosphorylation with 2-aminopurine (2-AP). (Source: J. Strong et al, 1998)

Clinical Trial for OV Reolysin

Reolysin, the commercial Reo-OV owned by Oncolytics Biotech Inc., has undergone several trials. The reovirus has been studied for a variety of cancers: melanoma, pancreatic, non-small cell lung, ovarian, colorectal, and head and neck cancers. In human non-small cell lung cancer (NSCLC), Reovirus type 3 Dearing strain was tested in vitro when combined with the chemical paclitaxel. ReoT3D, alone, demonstrated lytic activity in 7 of 9 NSCLC cell lines examined. The combination of ReoT3D and paclitaxel showed increased poly (ADP-ribose) polymerase (PARP) cleavage and caspase activity relative to just the Reovirus alone, indicating a higher rate of apoptosis (Shizuko et al,2009). Similarly, in June 2012, the NCIC began a Phase II trial of intravenous Reolysin for patients with advanced or metastatic breast cancer. The aim of the study is to evaluate the difference between paclitaxel and the combination of paclitaxel and Resolysin. Approximately 50 patients will be in each arm of the trial (Clinicaltrials.gov).

Adenovirus

The Adenovirus is a popular candidate and therefore is one of the most well- researched OV. Structurally, it is a DS-DNA, which makes it easily susceptible to genetic manipulation.. The most famous adeno-OV is the China-approved H101, the only currently approved OV, adenovirus for head and neck cancer (Garber, 2006).

Mechanism for Tumor Selectivity

The H101, and the similar Onyx-15, gain their selectivity by deletion of the E1B gene – the gene which produces proteins to delay host-cell lysis. In healthy cells, the E1B-defecient virus's replication is blocked by the host's cell tumor suppressor protein p53. However, cancer cells lacking p53 would be unable halt viral replication and host cancer-cell lysis. Although this strategy is clever, it was proven incorrect by first the virus's targeting of healthy p53-containing cells. Moreover, wild-type E1B Onyx-15 viruses have had as much success in some trials as the genetically manipulated E1B-deficeint. Nevertheless, although the exact mechanism for selectivity is unknown, the H101 in Phase III trials, "reported a 79% response rate for H101 plus chemotherapy, compared with a 40% for chemotherapy alone." (Garber, 2006) On the other hand, the Phase III trial, admittedly, failed to study patient survival rate, so the ultimate effectiveness is unknown. Additionally, its complementary success with chemotherapy, instead of its stand-alone efficacy, as well as the inability to deliver the OV intravenously limits the potential of fighting metastasized cancer. Thus, the success of the OV is only moderate.

Methods of Improving Adeno-OV

Adenovirus Combined with siRNA Gene Regulation

siRNA – small interfering RNA – is a small double-stranded RNA consisting of aproximately 20 base pairs. Biologically, its most significant function is its involvement in gene regulation. More specifically, for our discussion, is the importance of its role in RNA interference by shutting off a gene. siRNA is phosphorylated to separate into single strands. One RNA strand becomes part of the (RISC) RNA-induced silencing complex. There it guides the endonuclease (which breaks nucleotide backbone) Argonaute to cleave mRNA. siRNA was considered

(and still is potentially) a promising way to cure cancer by inhibiting translation of oncogenic proteins caused by mutated genes in tumor cells. However, it has faced several obstacles in its utilization. Problems included incidental activation of the innate immune system (WBC) and interferon induction. Also, off-targeting may occur if the siRNA can bind to different genes other than those intended. The possibility of joining OV and also using the adenovirus as a vector for RNA delivery has been altered to target tumors mitigates the potential of off-targeting occurring and also lessens the risk of RNA activating the innate immune system (Choi et al, 2012). Mortalin, a protein which is overexpressed in cancer cells and plays a role in inhibiting tumor suppressor protein p53, is one gene that can be targeted by shRNA -small hairpin RNA. In an experiment, the Ad-ΔB7-shMot was injected into breast cancer tumors caused by overexpressed mortalin which were xenografted into mice. The adeno-OV demonstrated enhanced apoptosis, substantiating interest in this method (Yoo et al, 2010). Another strategic use of siRNA is to target VEGF, vascular endothelial growth factor, a signal protein that stimulates angiogenesis. Specifically, U6 promoter (RNA Polymerase III promoter) was used to control shRNA expression. This adenovirus, designated Ad- ΔB7-shVEGF, in mice, demonstrated increased anti-tumor activity and increased duration time when compared to just OV alone. It also justified the theory that the adenovirus with VEGF-targeting shRNA has an anti-angiogenesis affect by the reduction in tumor vessels. Additionally, the combination of the two worked better than Ad- ΔE1-shVEGF, the viral vector (without replication capabilities), demonstrating that the combination works better than each alone (Yoo et al, 2007).

Oncolytic Adenovirus Armed with Suicide genes

Scientists are able to engineer oncolytic adenoviruses to add a transgene. This bolsters Adeno-OV efficacy by killing both the infected cell and neighboring tumor cells. Prodrug activating- genes, also called suicide genes, is a separate possibility in treating cancer. However, it may be possible to combine the two in order to enhance treatment. The HSV thymidine kinase gene, when combined with the prodrug gancyclovir is the most prominent suicide gene. Procedurally, HSV-tk phosphorylates gancylovir, which is further phosphorylated by other kinases into gancyclovir triphosphate. This activated form is toxic to both viral and cellular DNA synthesis and can spread to other tumor cells through gap junctions. Several experiments with divergent results question the effectiveness of this technique. In an experiment in mouse models for Retinoblastoma (Xunda et al, 2009), Colon Cancer (Wildner et al, 1999), Hepatic cancer (Zheng et al, 2009) and in malignant gliomas (Nanda et al, 2001) (figure 4) it was found that the HSV-tk adeno-OV showed promise. On the other hand, improvement was not found in treatment combined with GCV of peritoneal carcinomatosis which metastasized from colon cancer (Wildner and Morris, 2000). Furthermore, in several Cancer cell lines, mesothelioma, lung cancer, and cervical carcinoma and an intraperitoneal tumor model, HSV-th adeno-viruses and GSV didn't reduce tumor size. The effectiveness of this approach may be limited because the toxicity towards viral replication may stop further spreading of OV and outgain its cancer toxicity (Lambright et al. (2001). Moreover, Aghi et al, (1999) proposed the theory that in some tumors, the number of gap junctions can be low, hindering secondary effects of the toxic molecule and shifting the scale towards inhibiting viral growth. However, both the number of studies and the currency of some of the studies in favor should indicate that perhaps the experiments that reported no gain had technical problems such as the duration of the experiment or the viral dosage. Another altered OV is the AdFGR-adenovirus, which utilizes a double-suicide transgene. The adenovirus lacks its E1B-55kD and contains the cytosine deaminase thymidine kinase fusion and the HSV-tk gene. Chemically, cytosine deaminase converts 5-flourocytosine into 5-flourouracil, a molecule enzymatically converted into pyrimidine antimetabolites which are anti-tumoral (Duarte et al., 2012). Further, when combined with intravenously supplied glancyclovir, 5-flourocytosine, and radiation therapy boosts potency. In Phase I clinical trials for patients with prostate cancer, this regimen led to greater delay of tumor growth and an 80% complete response to treatment, in patients with either newly diagnosed intermediate or high-risk prostate cancer (Rogulski et al, 2000). In a different experiment, Zhang and Huang (2006) found that the double suicide gene wasn't harmful to human epithelial and fibroblast cells and also increased potency with respect to lung cancer cell lines.

HSV OV: A Case Study of OncoVEX GM-CSF

Another class of well-researched OV is the HSV1-based viruses. The most famous example is called OncoVEX GM-CSF. Previous examples of OV have demonstrated that the vast majority work best in conjunction with conventional treatments. However, OncoVEX is an exception – and an optimistic outlier – of what OV may be able to do. HSV has double stranded DNA, an icosahedral protein capsid, and a lipid bilayer envelope. The HSV is an excellent candidate for study because it is easily manipulated genetically. Additionally, it has a versatile ability to infect many types of cells and a rapid replication cycle which increases the rate of cellular lysis.

Method of Tumor-Selectivity

OncoVEX GM-CSF has both the deletion of its ICP 34.5 and ICP47 genes which engenders tumor-selectivity. ICP34.5 codes for a protein that is thought to prevent the host cell's attempt to block translation by activating PKR (Smith et al, 2006); ICP47 blocks the translocation of the TAP-dependent peptides, leaving the MHC I in the ER, and ensuring that CD8 cytotoxic T cells are unable to recognize the infected cell(Galocha et al, 1997). The OV, in addition to the ICP34.5 and ICP47 gene deletions, is also inserted with the gene that makes the protein granulocyte macrophage-colony stimulating factor. GM-CSF is a cytokine usually secreted by white blood cells to stimulate other WBC to grow and move to the infection site. In this case, the OV would provide a secondary anti-cancer benefit by bringing WBC to fight the cancer cells. (Liu et al, 2003)

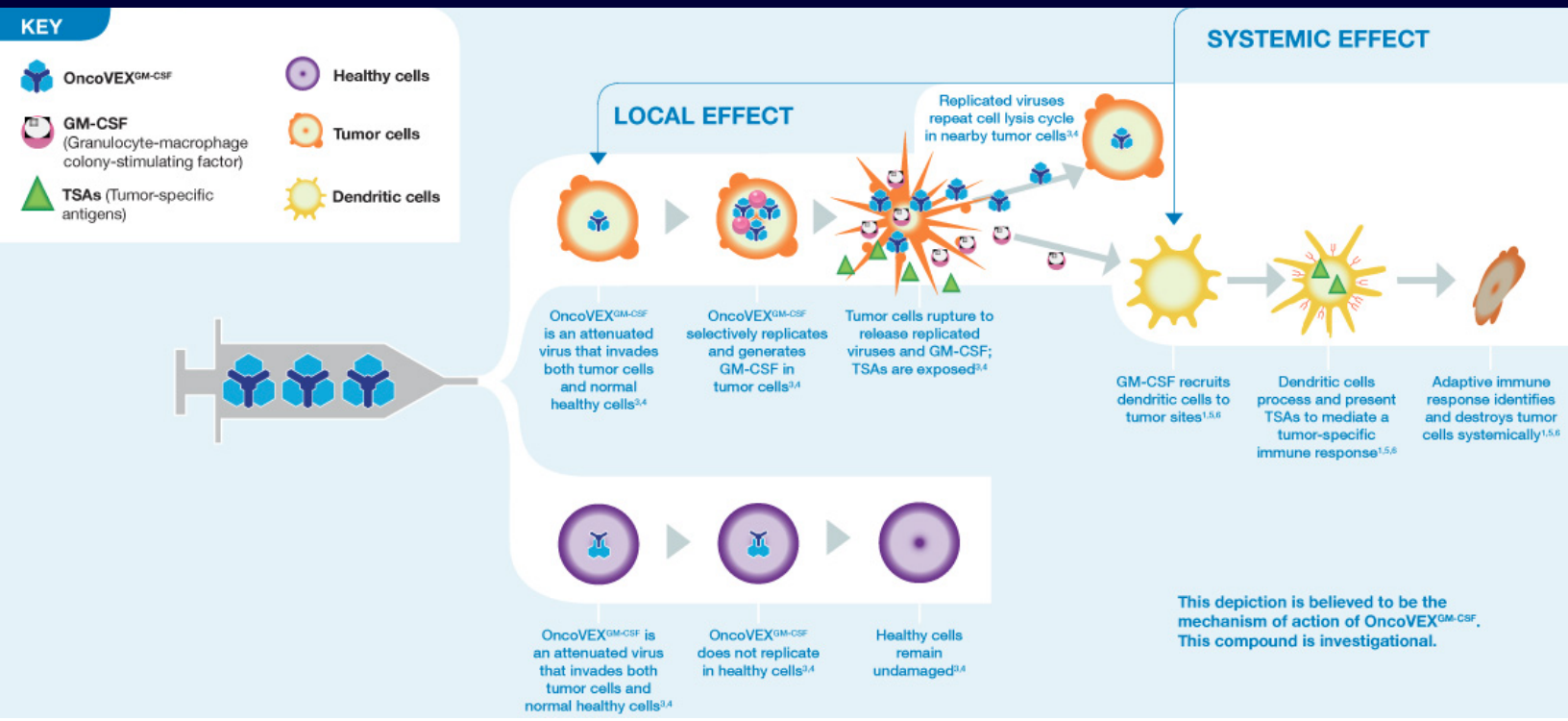


Figure 4: Source: www.amgenoncology-international.com

Clinical Trial for OncoVEX GM-CSF for Late-Stage, Stage III or IV Melanoma

Stage IV Melanoma, does not currently have many viable treatment options. Currently, the most effective treatment is high-dose of IL-2 with a survival rate of approximately 15-20%, with only a small number of patients with long-term benefits. The toxicity of IL-2 coupled with its mediocre benefit makes this treatment problematic. To test OncoVEX GM-CSF in a Phase II clinical trial for late stage melanoma, patients with unresectable, malignant late Stage III or IV melanoma were treated with 106 plaque forming units/ml of up to 4ml, depending on their exact stages of melanoma. Then, after 3 weeks, the regimen changed to 108 PFU/ml for two more weeks. In all, 50 patients were enrolled in the trial, and they received an average of 6 injections. The OV did cause flu-like symptoms. However, there was a 28% response rate, including 8 complete responses – 16% -- and 5 partial responses. Twelve out of the 13 of these had these responses sustained for 7-31 months; Overall 2-year survival rate was 52% (26/50) (Senzer et al, 2009). Additionally, several of these patients who participated in the trial had tumor samples evaluated for immunological activity. An increase of MART-1-specific lymphocytes in the tumor environment, in both injected and untreated tumors in addition to the diminished number of regulatory T cells and myeloid-derived-suppressor cells indicates increased anti-tumor immunity. Based on these results, a Phase III clinical trial is underway, initiated in late 2009, in a similar structure to the Phase II, except on a larger scale, with more patients and a more precise aim to see exactly how effective OncoVEX GM-CSF can be against late-stage melanoma (Cancerresearch UK, 2009).

Several Obstacles to OV and Possible Methods of Overcoming Them

Antibody Neutralization and Complement Activation of OV

Because of the pathogenic nature of viruses, people have developed immune responses to them. In the case of OV, antibodies which neutralize OV are an obstacle to its success. The vaccinia virus, named after its use as a smallpox vaccine, has made human immune systems resistant to the Vaccinia OV. Another example is in the case of Reovirus OV. Since reoviruses are very common, many people have been exposed to them, which has caused a built-up immunity towards it. Over time, researchers have come up with a number of methods to overcome antibodies. One way is to deliver serotypes or chimera virus, a similar virus but with enough variants that it would not attract the antigen-specific antibodies (Zhang et al, 2011). Another strategy is what is termed the "Trojan horse." Similar to what occurred in the mythical story of Troy, cells – for instance, dendrocytes – are extracted from host, are ex vivo injected with OV, and reinserted into the host, effectively disguising viruses in host cells (Yotnda et al, 2004). The complement system is related to antibody neutralization, by helping antibodies and phagocytes clear pathogens from the host organism. The vaccinia virus naturally secretes a virulence factor, vaccinia complement control protein, which binds to complement molecules C3b and C4b (Girgis et al, 2008). Also, it reduces the number of CD4 and CD8 cells by the infection site, raising the possibility of using the regulatory protein to inhibit complement system (Pushpakumar et al, 2011); in our case in combination with OV. In the case of Adeno-OV, in vivo pre-clinical studies indicate that complement system activity can be reduced by the addition of the masking agent polyethylene glycol, which limits protein-protein interaction

(Tian et al, 2009). Second, Adeno-OV may be able to induce the Protectin protein which inhibits complement binding.

Antiviral Cytokines and Physical Barriers

Cytokines play an important role in host immune defense against viruses. IFNs 1, 2, and 3 promote apoptosis in host cells infected by virus, stopping viral replication, and antiviral cellular resistance in uninfected cells. Although an important positive feature in host versus virus in the case of pathogenic viruses, in the case of OV the Interferon systems are a problem hindering the spread of the virus. To overcome this obstacle, the previously mentioned Trojan horse strategy is implemented. In this case, Adeno-OV is injected into mesenchymal stem cells, which hide the OV and suppress activated T-cells (Ahmed et al, 2010). Another strategy is via pretreatment with histone deacetylase inhibitors which block the protein that expresses cytokine related DNA . Treating glioma with an HSV-based OV, Otsuki et al. (2008) employed valproic acid before injecting the OV. It reduced host ability to activate IFN-stimulated genes, and therefore increased potency. Another hindrance is that the liver and spleen absorb many viruses, removing them from the bloodstream. Kupffer cells – macrophages located in the sinusoids of the liver- absorb the vast majority of Adenovirus-type-5. Several strategies have been proposed to counter this issue. The anticoagulant warfarin depletes the number of Kupffer cells, thereby preventing Liver uptake of subsequent Adeno-OV. A second possibility raised by Zhang et al. (2011) involves blood coagulation factor X. The protein factor X cleaves prothrombin, activating it into thrombin. This factor is involved in liver uptake because it binds to the hexon protein of the virus (coat protein in Adenovirus); therefore, a hexon-chimeric Adenovirus, or an altered adeno-OV which only weakly binds to factor X has demonstratively less liver uptake.

Conclusion

The use of viruses as an anti-tumor agent is a complex topic. Oncolytic viruses are effective against cancer as demonstrated in clinical trials performed with OncoVEX GM-CSF, Adenovirus-based Onyx-15, and Reolysin, as well as other viruses. Based on these studies, the utilization of oncolytic viruses in combination with chemo and radiation therapy as conventional treatment is a promising possibility for the near future. However, there is little evidence that oncolytic viruses will play a large role in cancer treatment by supplanting current treatments. Second generation viruses such as increasing oncolytic potency by arming it with anti-tumor genes, and other methods, and third generation may improve viral effectiveness, but it is unrealistic to anticipate such techniques transforming OV as a reliable, independent cure for cancer.

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Are Epidermal Barrier Defects Responsible for the Underlying Pathology of Atopic Dermatitis?

Naomi Davis

Abstract

Atopic dermatitis, often referred to as eczema, is a chronic inflammatory skin condition frequently seen in young children. It is a complex disease involving environmental factors, genetics and immune dysregulation. There is currently no cure with conflicting opinions from physicians regarding treatment and management. A clearer understanding of pathogenesis of atopic dermatitis can hopefully lead to new and improved treatment options for patients. Current evidence seems to support epidermal skin barrier defects as the cause of this disease. This paper seeks to investigate if this out-in hypothesis can be responsible as the sole pathogenesis of atopic dermatitis. To write this paper a systematic search of online databases was conducted to find relevant studies. Through analysis of the research it can be concluded that although epidermal skin barrier defects definitely play an important role in the pathogenesis of atopic dermatitis, further research will most likely blend existing theories.

Introduction

Atopic dermatitis (AD) is the most common childhood skin disorder in the United States and developed countries. The pathogenesis of atopic dermatitis is complex and not fully understood. Until recently, researchers believed in underlying immune system abnormalities. Current evidence seems to support the hypothesis that epidermal barrier defects may be responsible for the underlying pathology.

According to the American Academy of Family Physicians, the diagnosis of atopic dermatitis is dependent on the presence of three essential features; pruritus, eczema, and chronic or relapsing history. Eczema can be acute, chronic, or subacute with typical morphology and age-specific patterns. Examples provided are facial, neck, and extensor involvement in children, flexural involvement in any age group, and sparing of groin and axillary region. In addition to these nonnegotiable aspects, there are important features and associated features. Important features that support the diagnosis are onset at early age, personal or family history of atopy or immunoglobulin E reactivity, and xerosis (dry skin). Associated features that can help suggest the diagnosis are atypical vascular responses such as facial pallor and delayed blanch response; keratosis pilaris, hyperlinear palms, and ichythosis; ocular or periorbital changes; other regional findings such as perioral changes; and perifollicular accentuation, lichenification, and prurigo lesions. (Buys, 2007)

An alternative definition of atopic dermatitis relies on the UK refinement of the Hanifin and Rajka Diagnostic Criteria. In order to be considered AD, the patient must have an itchy skin condition in the last twelve months plus three or more of the following: onset before two years of age (not applicable in child under four years), history of flexural involvement, history of generally dry skin, history of other atopic disease (or history in first degree relative if child is under 4 years), and visible flexural dermatitis (Brown & Reynolds, 2006).

The term atopic dermatitis is often used interchangeably for eczema. Eczema is a type of dermatitis that can be subdivided into atopic and non-atopic dermatitis (Brown & Reynolds, 2006). Atopy is associated with IgE antibody sensitization, with a prevalence of 80% for IgE sensitization in infants with AD (deBenedictis et al., 2009), although more recent finding show inconsistent results in up to two thirds of individuals with eczema (Flohr et al. 2010). The atopic triad consists of asthma, allergic rhinitis, and atopic dermatitis (Hall, 1999). Up to 70% of patients with AD have a family history of asthma, hay fever, or eczematous dermatitis. Individuals with non-atopic eczema exhibit the same cutaneous manifestations without atopic features. Response to treatment has not been shown to differ, but prognosis for atopic dermatitis is worse (Habif, 2005). Those with AD are more likely to suffer from asthma later in life and their skin condition has a higher likelihood of persisting into adulthood (Flohr et al., 2010). The atopic march refers to the tendency of individuals with atopic dermatitis to develop asthma and allergic rhinitis later in life (Elias, 2010).

In light of the current evidence, is it possible that a defective skin barrier is the number one culprit in the pathogenesis of atopic dermatitis? Many studies claim factors are multifactorial and too complex for simplification but some point to an underlying genetic basis that undermines the skin barrier and leads to the development of the atopic triad. The importance of understanding the pathology of this skin condition is in its implications for treatment guidelines and recommendations. During recent years parents have been receiving conflicting advice from physicians, including highly specialized allergists and dermatologists, due to the disparities in the literature. Obviously, there is a need to hone in on the evidence to enhance treatment based medicine.

Methods

Relevant research concerning epidermal barrier dysfunction in atopic dermatitis was identified by searching online databases. Databases were searched for publications from 1999 through 2013 and were limited to full text, peer reviewed articles. Preference was given to original articles published in the last two years. Key articles were obtained primarily from EBSCO, Proquest Central, PubMed and MEDLINE using search terms, "atopic dermatitis", "eczema", "skin barrier", "atopic march", "pathogenesis", "immunoregulation",

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filaggrin", and "epidermal barrier defects" and "treatment".

Discussion

A large number of children are affected by atopic dermatitis as reported in epidemiological surveys. Studies of epidemiological trends in AD demonstrate increasingly higher prevalence, especially in developed countries. The Diepgen, 2000 most recent study reported a national prevalence rate of 10.7% with a wide range of 8.7 to 18.1%. Highest prevalence was reported for the East Coast states as well as Nevada, Utah, and Idaho. Eczema prevalence in New York was calculated at 11.75% and 13.14% for New Jersey (Shaw et al., 2010). Other localized studies conducted in the state of Oregon and Italy report prevalence rates on the higher end of the spectrum, 17% in school aged children and 18.1% among three to five year olds, respectively (Laughter et al., 2000; Peroni et al., 2008).

The pathogenesis of atopic dermatitis is complex and not fully understood (Peroni et al., 2007). Until recently, researchers believed in underlying immune system abnormalities (Habif, 2005). Current evidence supports the hypothesis that epidermal barrier defects are responsible for the underlying pathology (Cork & Danby, 2009; Elias, 2010; Palmer et al., 2006). The most superficial layer of the skin, the epidermis, is mainly comprised of keratinocytes which migrate upwards and differentiate over time into a dead end-product, the stratum corneum (Saladin, 2012; Hall, 1999). The stratum corneum functions as a permeability barrier to decrease water loss and block entry of allergens and microbes (Hall, 1999). As the keratinocytes move upwards they flatten due to the production of keratin filaments. They also increase their production of keratin and lamellar granules or lipid-filled vesicles. At the level of the stratum granulosum, several developments contribute to the formation of the epidermal water barrier. The keratinocytes' keratohyalin granules release filaggrin, a protein that binds the keratin filaments into tough bundles. Envelope proteins produced by the cells create a strong protein sac around the keratin bundles. The lamellar granules release a lipid mixture that spreads out over the cell surface and waterproofs it. Above the layer of the water barrier, the keratinocytes die as they are cut off from their nutrient supply. The tough waterproof sac that is left behind along with the tight junctions between the keratinocytes together with the intercellular lamellar material form the epidermal water barrier that is crucial for retaining body water. (Saladin, 2012)

Primary defects in the stratum corneum may be due to decreased production of filaggrin, or filament aggregating protein (Cork et al., 2006; Elias, 2010; Proksch et al., 2006). This view is supported by studies that find filaggrin gene mutations to be positively correlated with skin barrier dysfunction (Palmer et al., 2006). The filaggrin gene is located in the epidermal differentiation complex located on chromosome 1q21 and mutations of the gene predispose to the development of atopic dermatitis (Howell et al, 2007; ibid; Weidinger et al., 2008). A large number of individuals with atopic dermatitis have a filaggrin gene mutation with a prevalence of up to 50% (Elias, 2010; Jungersted, 2010.) Filaggrin gene mutation appears to be the strongest known risk factor for AD (Rodriguez et al., 2009). Low levels of filaggrin are especially prevalent in eczematous skin with visible lesions (Cork et al., 2006; Proksch et al., 2006). Interestingly, a study of infants with and without filaggrin gene mutations found that infants with the gene mutation had impaired epidermal barrier function even in the absence of eczematous skin lesions compared to those without the genetic mutations (Flohr, et al., 2010). It seems that skin barrier dysfunction precedes the development of clinically evident eczema when associated with the filaggrin gene mutation, but it is not proof that epidermal barrier defects are the initiating factor in the development of atopic dermatitis.

An important clinical measurement used in the description of AD is transepidermal barrier water loss (TEWL). TEWL measurements differ significantly between AD and healthy skin (Agner, 1991; Holm et al., 2006) with higher TEWL values for AD. These studies show that even in the absence of skin lesions, barrier function is impaired in AD. Jungersted et al. (2010) reported highest TEWL values for those with filaggrin mutations opposed to AD without the mutations.

Natural moisturizing factor is generated from the breakdown of filaggrin. High levels of natural moisturizing factor are becoming increasingly recognized as a crucial component in the formation of a healthy skin barrier. As a humectant, natural moisturizing factor attracts water to itself causing the corneocytes to swell and form a resilient barrier. Urea is a substance found in natural moisturizing factor that may be responsible for its humectant properties due to its sponge-like characteristics. Urea may in fact be used as part of atopic dermatitis treatment based on this premise (Cork & Danby, 2009). In eczematous skin, natural moisturizing factor levels are decreased and corneocytes shrink as a result (Cork et al., 2009) Shrunken corneocytes release cytokines, pro-inflammatory markers that cause itching. In addition, the shrunken corneocytes develop cracks between themselves which allows for the penetration of irritants and allergens and a subsequent inflammatory response. (Cork & Danby, 2009)

Thickness of the stratum corneum is maintained by the balance between proteases and protease inhibitors. Proteases allow for desquamation and turnover of the stratum corneum by breaking down corneodesmosomes, the link between corneocytes. Protease action is balanced by protease inhibitors such as LEKT1 to ensure a constant thickness. If the stratum corneum becomes too thin it allows for the penetration of allergens and irritants. Protease activity is enhanced by increased pH, which explains the effect of irritants that raise the pH of the skin (Cork & Danby, 2009).

Another contributing factor may be due to impairment in tight junctions. Tight junctions proteins are situated below the stratum corneum in the stratum granulosum and function as an additional skin barrier by regulating the selective permeability of the paracellular pathway (DeBenedetto et al., 2010; Kubo et al., 2012).

Research published in the Journal of Allergy and Clinical Immunology in December 2010 suggests that reduction in

claudin-1, a tight junction protein, results in increased permeability of the barrier. The research findings also imply that variants in the tight junction gene, Claudin-1 is associated specifically with atopic dermatitis.

The lipid content of the stratum corneum appears to play a role in atopic dermatitis. It is known that altered ceramide levels are present in AD (Proksch et al., 2008; Jungersted et al., 2008). Authors Danby and Cork liken the lipid lamellae to mortar around a corneocyte brick wall. The barrier lipids help keep the water content high in the corneocytes. Defective lipid lamellae present in eczematous skin increases water loss and shrinkage of corneocytes (Cork & Danby, 2009). However, it has not been established whether altered lipid content is associated with filaggrin mutations. Research is lacking in this area, but a more recent study by Jungersted et al. (2010) detected no clear relationship. The authors discerned that ceramide 1 and 4 are decreased in AD, while ceramide 7 is increased, a confounding finding. More research is necessary to determine the role of filaggrin gene mutations in relation to characteristic skin findings of AD.

It seems to be a difficult proposition to blame the filaggrin gene mutations for the sole underlying pathology in AD if these mutations are not present in all individuals with AD and are not associated with important AD characteristics, although additional research will hopefully provide further elucidation. Perhaps skin findings in AD such as the altered ceramide profile are not inherent to the pathogenesis of the disorder.

It is important to distinguish the difference between a characteristic finding and underlying pathogenesis in regard to the skin disorder. It is widely known that barrier dysfunction is present in AD. To this end researchers use isolated measures of barrier function and dysfunction for classification of disease severity (Danby et al., 2011; Mochizuki et al., 2008). The question is whether to take an out-in or in-out approach to understanding the responsible pathology.

The out-in or outside-inside hypothesis relies on the epidermal barrier dysfunction as the cause for the disease. (Cork & Danby, 2009; Elias & Wakefield, 2011; Elias et al., 2008; Palmer et al., 2006). It implies that skin barrier damage precedes immune dysregulation. Elias and others have been proposing this hypothesis since 1999 (Elias et al., 1999; Taieb, 1999), although it has only been gaining wider recognition in recent years. Authors Cork and Danby propose that skin barrier breakdown is the first event in the development of AD. The breakdown of the epidermal barrier is a result of interaction of environmental agents with several genetic mutations (Cork & Danby, 2009).

The most recent developments in the field provide supporting evidence for the epidermal barrier hypothesis. A study in mice discovered that a certain missing protein may be responsible for the development of AD. The COUP-TF interacting protein 2 (Ctip2) is important for maintaining the skin barrier and normal lipid metabolism. When functioning, it appears to suppress the skin inflammatory response. Ctip2 deficiency, studied by removing the protein from the epidermal layer of mice skin, caused atopic dermatitis lesions as well as a systemic inflammatory response. The absence of Ctip2 appears to increase expression of the thymic stromal lymphopoietin (TSLP) gene. The TSLP gene has been linked to asthma and a food allergy related disorder and is elevated in mice with atopic dermatitis. (Wang et al., 2012) These findings build on the premise of the epidermal barrier hypothesis where breakdown of the barrier is a trigger for AD and for wreaking havoc with the systemic immune system.

It is possible that the epidermal barrier dysfunction hypothesis can explain the pathogenesis of the AD triad, which consists of asthma, allergic rhinitis, and atopic dermatitis. Genetic research by Palmer et al., (2006) demonstrates evidence for a genetic defect that is common to atopic dermatitis and associated asthma. Two independent mutations of the filaggrin gene, 228del4 and R501X, appear to be the major variants in people of European origin, according to their study. These mutations were found to be a major risk factor for AD and due to their presence in people who subsequently develop asthma, a molecular mechanism can be provided for the asthma subtype that is associated with AD. Although these mutations are the most common and result in complete loss of function, there are likely mutations associated with reduced filaggrin expression, as well as different profiles for other populations.

The in-out or inside-outside hypothesis assumes that the barrier dysfunction is driven by immunologic abnormalities. Barrier dysfunction is due to the inflammatory response to irritants and allergen. Characteristics of the impaired immune response are both systemic and cutaneous and include excessive T-helper type 2 (Th2) cell signaling leading to increased interleukin-4 production, which promotes IgE production (Boguniewicz & Leung, 2011; Nicol, 2010). Although a debatable finding, studies have found increased serum IgE levels in up to 80% of patients with AD (Leung et al., 2004; Flohr et al., 2004). A proteomic study by Howell et al. found that an important protein, S100/A11, is downregulated in AD. This protein has an immunomodulatory effect on the filaggrin gene (Howell et al., 2008). Authors Boguniewicz and Leung interpret these findings to mean that there is immune dysregulation that affects the integrity of the skin barrier as well as the body's innate immune response (Boguniewicz & Leung, 2011).

More recent literature (Wolf & Wolf, 2012; Boguniewicz & Leung, 2011) remains inconclusive. The authors continue to support the existing immune theory although they show evidence to support the defective barrier theory.

Genetic studies provide support for both skin barrier defects and immunologic abnormalities in AD. Authors Boguniewicz and Leung suggest that immune and skin barrier genetic variations may work together to increase risk for AD (Boguniewicz & Leung, 2011). There may be a unique type of AD that is associated specifically with the filaggrin gene mutation. This phenotype of AD predisposes to early onset AD that persists into adulthood (Barker, 2007). Adults with AD are more

likely to have the filaggrin gene mutation although not all individuals with AD have the filaggrin gene mutation (O'Regan et al., 2008).

It may be too early to tell, but implications for treatment are enormous. If the skin barrier theory holds true, it implies that first line therapy should focus on restoration of the skin barrier. As of December 2012, American Academy of Dermatology (AAD) revised their recommendations for the public based on this theory. Their clinical guidelines for AD are in the development process and are not yet available. Other dermatology educational websites have followed the lead of the AAD including National Institutes of Health (NIH). National Jewish Health is also a proponent of barrier restoration therapy due to underlying genetic filaggrin mutation theory.

The leaky skin barrier is the driving force for inflammation and dryness that characterize atopic dermatitis. Allergen sensitization occurs with increased penetration of environmental antigens to the body. Sensitization results in subsequent immune system hyperactivity associated with atopic diseases such as asthma and allergic rhinitis (Elias, 2010). Research demonstrates that the atopic march occurs early; over 50% of children will have allergies and/or asthma by their third birthday (Kapoor et al., 2008). An additional effect of the damaged skin barrier is the increased incidence of secondary infections. Skin infections are common in individuals with atopic dermatitis and may be due to the disturbed antimicrobial barrier (Elias, 2010).

There may be aggravating factors that affect relapses and severity of atopic dermatitis. The avoidance of environmental stimulants is important in management (Buys, 2007; Hanifin et al., 2003). Factors such as allergens, irritants, temperature fluctuations, low humidity, and stress may be triggers in certain individuals (Habif, 2005).

Allergens include contact, food, and inhalant allergens. The role of food and environmental allergens in eliciting or maintaining eczematous skin lesions is debatable (Leung et al., 2004). Food and inhalant allergens are associated with infantile atopic dermatitis. A study of risk factors showed that sensitizations to certain allergens were more common in children with AD compared to healthy children. The most common sensitizing allergens in the study were house dust mites and grass pollen. Dogs, cats, Parietaria, milk, and eggs allergens were more prevalent in those with AD (Peroni et al., 2008). The 2003 "Guidelines of care for atopic dermatitis" technical report determined the evidence on the effectiveness of allergen avoidance to be inconclusive. The 2006 Clinical Review of atopic and non-atopic eczema published in the British Medical Journal maintains that food allergens are responsible for relapse in a small number of individuals and this is usually obvious to the patient or caregiver. Some research suggests that reducing house dust mites improves severe AD, but a similar more recent study has not produced the same effect (Brown & Reynolds, 2006).

Other aggravating factors may be irritants. Soaps and detergents can irritate the skin and worsen symptoms (Brown & Reynolds, 2006). They insult the skin barrier by raising the pH in the stratum corneum. A higher pH inhibits lipid synthesis and increases protease activity, both of which increase the breakdown of the epidermal barrier in AD (Cork & Danby, 2009). Shampoos, bubble baths, shower gels, and dishwashing liquids can be potential triggers according to the UK National Institute for Health and Clinical Excellence (NICE) guidelines (Carr et al., 2007). Patients are recommended to use mild unscented non-soap cleansers (Siegfried, 2009). Individuals with atopic dermatitis may be sensitive to detergents and fabric softeners. Recommendations are to use fragrance and dye free detergents and/or to double rinse (Siegfried, 2009). The 2003 Technical Report's analysis of the peer reviewed literature found only one investigation that studied the effects of avoidance of enzyme enriched detergents on symptom relief and showed no difference between placebo and control (Hanifin et al., 2003). Irritants vary for each individual, therefore expert opinion recommends patients to identify and avoid known personal triggers (Carr et al., 2007). In concordance with the skin barrier theory, irritants may further destroy the epidermal skin barrier leading to the worsening of symptoms (Bieber, 2008; Jungersted et al., 2010;) It is important to note that although a study by Jungersted et al. showed that irritants serve as triggers in AD, their results were not statistically significant. In addition, their sample size was small with only 49 participants, but their findings can indicate avenues for further research.

Pruritus induces scratching and perpetuates the itch-scratch-itch cycle by further damaging the skin barrier. Scratching also exposes the skin to secondary infection (Brown & Reynolds, 2006). Secondary infection in AD is common due to disturbance in the skin barrier (Elias, 2010). The majority of patients are colonized with Staphylococcus aureus infection. It is debatable whether there is a connection between S. Aureus infection and AD exacerbations (Buys, 2007). All of the above mentioned aggravating factors interact with the skin barrier and therefore can be used to demonstrate some support for the defective epidermal barrier theory in AD.

It is suggested that neuroimmunoregulation may be implicated in the effects of psychological stress on AD exacerbation (Brown & Reynolds, 2006). Several studies have investigated the effects of psychological treatment, including stress reduction techniques, on reducing symptoms (Hanifin, et al., 2003; Stabb et al., 2002; Stabb et al., 2006). Most found significant benefits for group therapy (Hanifin et al., 2003.). Neuorimmunoregulation directs support for the immune system theory, but there are insufficient studies to prove that reduced stress leads to the reduction of AD symptoms. A study that focused on the correlation between psychological stress levels and epidermal barrier dysfunction as measured by transepidermal water loss (TEWL) showed no significant correlation (Kepska et al., 2012). However a major study limitation was the small sample size. Although this study employed excellent design and had a superior idea to measure effects of psychological interventions on skin structure and function, if this was the best study on the topic there is clearly a need for more research in this area.

A cure is not possible for atopic dermatitis. It is a chronic skin condition with a fluctuating course that requires a multi-faceted treatment approach. Management focuses on preventing exacerbations and treating flare ups. A stepped approach is recommended by the National Institute for Health and Clinical Excellence (NICE) guidelines. The classification of AD used by the NICE guidelines is based on quality of life assessment including impact on everyday activities, sleep, and psychosocial wellbeing ranging from none to mild, moderate, and severe. The NICE recommendations are based on tailoring treatment according to severity and continual use of emollients even when skin is clear (Carr et al., 2007).

Emollients are considered the mainstay of treatment for prevention and maintenance (Brown & Reynolds 2006) to combat the almost universal feature of xerosis (Buys, 2007). Emollients soften and soothe dry, irritated skin. Emollients have been shown to reduce the requirement for topical corticosteroids by up to 50% (Lucky et al., 1997; Brown & Reynolds, 2006). Patients are recommended to apply emollients with or without moisturizer to the skin once or twice daily after showering or bathing when the skin is not fully dry, preferably within the first three minutes (Siegfried, 2009). Ointments are more efficacious than creams, but patients may find them too greasy. Lotions have a higher liquid content and are therefore not as superior as creams and ointments. Creams may be preferred for daytime use, while ointments may be better for nighttime (Buys, 2007). Emollient ingredients that include glycerol and urea combined in a complex emollient compound are useful for reducing the oil content while producing similar results to a greasier ointment product. Glycerol and urea are humectants that rehydrate the skin barrier, but are likely to be more cosmetically acceptable because they are less greasy (Cork & Danby, 2006). A placebo-controlled, double blind, randomized study of a glycerol-based emollient found it to have positive influence on AD skin with enhanced stratum corneum hydration (Breternitz et al., 2007).

The patient's prescribed topical medications are applied before moisturizing (Siegfried, 2009). Moisturizers may prevent penetration of medications into the skin (Nicol, 2010). In addition to moisturizing, adherence to an appropriate skin care regimen is important. Short daily baths or showers followed by the application of moisturizer are beneficial, contrary to previous beliefs that bathing should be kept to minimum. Warm water is preferred because hot water is drying to the skin (Siegfried, 2009).

Topical corticosteroids have been considered the mainstay of treatment for exacerbations (Buys, 2007) and are the standard to which other treatments are compared (Hanifin, et al., 2003). Steroids are classified according to their potency, ranging from high potency (class one) to low (class seven). The NICE guidelines recommend tailoring corticosteroids according to severity of AD (Carr et al., 2007). Mild potency steroids are to be used for mild AD, moderate potency for moderate AD, and potent steroids for severe AD under specialist supervision if used for a length of time. Low potency steroids are preferred in infants due to their higher ratio of skin surface area to body mass index and increased potential for systemic absorption (Buys, 2007). The duration of therapy, frequency of application and quantity of application are uncertainties due to limited data. A large systematic review found that there was no added effectiveness to using steroids twice daily as opposed to once daily. Long term intermittent use of topical steroids appears to be safe and effective (Hanifin et al., 2003). Expert opinion recommends treating flare ups with the shortest course of steroids necessary to control the exacerbation for a maximum of four weeks. A study found that patients generally underestimate the quantity of steroids and emollients needed for long term therapy (Buys, 2007).

Local side effects limit long term use of topical steroids (Hanifin et al., 2003). Cutaneous complications include striae, telangiectasia, atrophy, and acne and are more common on the face, groin, and axillae. Systemic side effects include hypothalamic-pituitary-adrenal axis suppression, reduced linear growth in children, and bone density changes in adults (Buys, 2007). Evidence of significant systemic effect from proper use of topical steroids is inconclusive (Hanifin et al., 2003).

Second line agents are topical calcineurin inhibitors (TCI). Pimecrolimus (Elidel) and tacrolimus (protopic) are immunosuppressants that alter T cell function (Buys, 2007). A systematic review of TCI efficacy and safety in pediatric patients identified twenty randomized controlled trials (Chen et al., 2010). They found no significant difference between 0.01% and 0.03% tacrolimus. 1% pimecrolimus compared to corticosteroids showed possible superiority at six months, but not at 12 months. Tacrolimus was found to be superior to pimecrolimus. The authors concluded that TCI are safe and effective in pediatric patients for the treatment on AD, but they did not address their possible carcinogenicity. There is an FDA "black box" warning about the possible link to skin cancer and lymphoma, but at present it is still unclear whether long term topical calcineurin inhibitors are associated with malignancy (Williams & Shams, 2010).The most common adverse effects are local skin burning and irritation and users need to use proper sun protection. The FDA limits use to children over two years and recommends avoiding long term use (Buys, 2007.). In the stepped treatment approach, TCI are added for moderate atopic eczema (Carr et al., 2007).

Phototherapy is a second line treatment used in moderate to severe AD (Carr et al., 2007). Ultraviolet phototherapy options include UVB, narrow-band UVB, UVA, or psoralen plus UVA (Buys, 2007). UVB appears to be more effective than UVA which is found in conventional sunbeds. The potential increased risk of skin cancer must be explained to the patient and monitored long term (Brown & Reynolds, 2006).

Systemic therapy may be necessary in the treatment of severe atopic dermatitis (Carr et al., 2007; Brown & Reynolds, 2006). Immunomodulators are useful in refractory AD that does not respond to topical agents (Hanifin et al., 2003). Cyclosporine (Sandimmune) is a systemic immunosuppressant used for treatment of moderate to severe AD (Buys, 2007; Brown & Reynolds, 2006). Its efficacy is well established,

although careful monitoring is necessary. Side effects include immunosuppression, nephrotoxicity, and increased risk of cancer (Brown & Reynolds, 2006). Evidence shows that interferon gamma-1b (Actimmune) may be effective for severe AD (Buys, 2007; Hanifin et al., 2003).

Systemic corticosteroids are effective for gaining short termcontrol, but should be limited to sparing short term use in adults and rarely in children (Buys, 2007). Short term oral prednisone or intramuscular injections of triamcinolone acetonide are used for major exacerbations. Their use is limited due to rebound flare of symptoms and diminishing effects (Hanifin et al., 2003).

Evidence for mycophenolate mofetil (Cellcept), azathioprine (Imuran), and intravenous immune globulin (human; Baygam) is conflicting; no definitive conclusion has been reached (Buys, 2007). There is insufficient evidence to support the use of leukotriene inhibitors, methotrexate, desensitization injections, theophylline, or oral pimecrolimus (Buys, 2007; Hanifin et al., 2003).

Occlusive clothing is often used in management of AD, and recently silk clothing is being touted as being helpful in improving eczema. DermaSilk is made from woven silk and impregnated with an anti-bacterial agent. A systematic review of the literature reveals only two non-randomized trials and although they showed benefits for DermaSilk, the inadequacies of the trials make it too early to suggest firm clinical benefit (Williams & Shams, 2010).

Unproven treatment strategies include Chinese herbal therapies, homeopathy, massage therapy, dietary restrictions including exclusion of sugar, and salt baths (Buys, 2007). Patients need to be aware about the potential toxicities of Chinese herbal therapy. Unproven prevention techniques include delayed introduction of solid foods in infants and prolonged breastfeeding (Carr et al., 2007; Hanifin et al., 2003). Exclusive breastfeeding may postpone emergence of symptoms until the third year or later (Hanifin et al., 2003). The evidence is inconclusive regarding maternal dietary restriction in pregnancy (Brown & Reynolds, 2006). Probiotics during pregnancy may delay the onset of AD (Hanifin et al., 2003). The use of bath emollients is discussed on the uncertainties page of the British Medical Journal in November 2009 (BMJ, 2009). They are prescribed as part of complete emollient therapy, to avoid the use of bubble baths, and as an easy way to apply an emollient to a large body area. However, patients' attention may be diverted away from direct application of emollients and most of it is lost down the drain (Williams & Shams, 2010). Current evidence shows that bath emollients offer little or no benefit, but further research may focus on the development of bath bubbles that young children can enjoy without irritating their skin (BMJ, 2009).

Prognosis for AD is good; the majority of patients outgrow their condition by adulthood. Atopic dermatitis in children becomes less severe in the teenage years (Habif, 2005). Complications can arise due to the development of eczema herpeticum, or widespread herpes simplex virus. Immediate referral to a dermatologist is necessary if eczema herpeticum is suspected. Comorbidities may develop due to poor control, including sleep disturbance and limitation of psychosocial functioning (Carr et al., 2007). Partial sleep disturbance results in significant neurocognitive impairment (Brown & Reynolds, 2006). Emotional and behavior problems may be common in children with moderate to severe AD (Habif, 2005).

The above analysis of available treatment options for AD demonstrates the importance of emollient therapy in conjunction with occasional steroid application for exacerbations. Stronger therapies may have to be added for very severe cases, but complete emollient therapy should never be neglected. Additional therapies may be useful if caregivers and patients find them to be easy and helpful. Health care providers should stress the benefits of emollient therapy by recommending beneficial moisturizer products, writing down their names on paper, and demonstrating how much to apply. Educating patients requires some extra time, a precious commodity in a busy pediatric office, but it is likely to show results because many of the above mentioned treatments rely on lifestyle management changes and proper usage of creams. For example, it would be useless to recommend a greasy ointment based on its superior efficacy if it will not be tolerated by the patient. In this case, it is the job of the health care provider to find out if the child refuses to use thick creams. It would make sense to recommend a milder lotion even if not as efficacious because it will be used and therefore be effective. A discussion of irritants as possible triggers is relevant to every patient, although based on available evidence it does not seem to be worth the trouble to investigate every possible food allergen. More worthwhile is education regarding avoidance of harsh soaps and detergents that raise the pH and further damage the skin barrier.

Conclusion

Atopic dermatitis is a very common and distressing skin disorder that requires a multifaceted treatment approach. Therapeutic interventions in the field are continuously developing as current research deepens and changes our understanding of the underlying pathogenesis. Current research is emerging in favor of epidermal skin barrier defect theory although the evidence remains inconclusive and we may hold on to the immune system theory at least a little longer. Further research may ultimately blend existing theories. Additional peer reviewed studies facilitate guideline refinement in the pursuit of evidence based medicine. Evolving research theories are likely to be reflected by updated clinical guidelines in the near future.

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Cytoplasmic Actin: Structure and Function

Justin Konig

Abstract

Cytoplasmic actin plays a crucial role in cellular structure, cell motility, intracellular transportation, and the cell cycle. Two isoforms of cytoplasmic actin have been identified, β and γ. Although their amino acid sequence is nearly identical, these two isoforms are encoded by different genes located on different chromosomes. Recent research has found that, despite their similarities, the two isoforms of cytoplasmic actin have distinct functions. This paper will review the structural and functional differences between the two isoforms, concluding with a discussion of some mutations that have been linked to disease.

Introduction

Actin is one of the most abundant proteins, accounting for 10% of protein content in muscular cells, and 1-5% of protein content in all other cells. Muscular actin, together with myosin, is responsible for the contraction of muscles. Cytoplasmic actin functions as a part of the cytoskeleton, and plays a role in cellular structure and motility, cytokinesis, phagocytosis, and intracellular transportation (Lodish et. al. 2013). Although it may have been observed as early as 1887 by W.D. Halliburton, actin was not purified in significant quantities until 1942 when Brunó Ferenc Straub developed a new technique for extracting muscle proteins. Soon after Straub's discovery, researchers reported finding actin in non-muscle cells. These findings were confirmed by Sadashi Hatano in 1968 (Schleicher and Jockusch, 2008). Since then, technological innovations have allowed researchers to solve its amino acid sequence and 3D structure.

Two isoforms of cytoplasmic actin have been identified, β and γ (also referred to as cytoplasmic actin 1 and 2, respectively). Although their amino acid sequence is nearly identical, these two isoforms are encoded by different genes located on different chromosomes; the gene for β actin is located on chromosome 7p ("Actin, Beta"); the gene for γ actin is located on chromosome 17q ("Actin, Gamma 1"). Both β and γ actin are necessary for proper cellular function. Mice in which the gene for β actin was erased on both chromosomes (actin knockout mice) died during early development. In contrast, mice engineered not to express γ actin are viable; however, they are smaller than the wild type and they experience a shortened lifespan. In humans, deficiencies in γ actin particularly affect the sensory hair cells of the inner ear. Scientists have proposed two theories to explain the distinct functions of β and γ actin. First, a number of actin binding proteins bind specifically to one isoform. The second theory is that the difference in function is due to the localization of the different isoforms by various cellular mechanisms. There is research that supports both theories (Perrin and Ervasti, 2010).

Structure

Quaternary Structure

Actin is found in cells as globular monomers (G-actin) and as filamentous polymers (F-actin). These filaments are made up of two strands of F-actin subunits wound around each other in a helical formation. Each filament has a (+) end and a (-) end, also referred to as the barbed end (+) and the pointed end (-). The (+) end polymerizes ten times quicker than the (-) end, and the (-) end de-polymerizes a little quicker. These filaments, called microfilaments, are highly organized and form part of the cytoskeleton. The organization of actin filaments in the cytoplasm differs from cell to cell, and is regulated by a number of proteins (Lodish et. al. 2013).

Actin is often associated with other molecules, most notably ATP and a divalent cation (usually Mg^{2+} or Ca^{2+}). Binding to ATP and Mg2+ changes the conformation of actin and is crucial to its polymerization. Myosin is another protein that is often associated with actin. Muscular actin combines with myosin to form contractile units called sarcomeres. In the cytoplasm, myosin motor proteins transport vesicles by moving along the actin microfilaments. There are also a number of proteins collectively referred to as actin binding proteins (ABPs) that bind to actin and regulate its polymerization and organization (Lodish et. al., 2013; Dominguez and Holmes, 2011).

Tertiary Structure

Actin is a globular protein that is divided by a cleft into two lobes. The upper cleft is the main binding site for ATP and Mg^{2+} ions; the lower part, lined with hydrophobic residues, is the main binding site for actin binding proteins (ABPs) (Domiguez ans Holmez, 2011). The molecule is further divided into four subunits numbered 1-4 (the bottom two domain are 1 & 3; the top two are 2 & 4). Both the N-teminus and the C-terminus ends are located in domain 1 (Lodish et. al. 2013). Domain 2, and particularly the DNase 1 - binding loop located in domain 2, play a critical role in polymerization. There is a small difference in conformation between G-actin and F-actin subunits. In G-actin domains 1 and 3 are rotated approximately 20º whereas F-actin subunits are flatter (Dominguez and Holmes, 2011).

Secondary Structure

SOPMA software predicts the following secondary structure for cytoplasmic actin 1 (β): alpha helix 33.60%, extended strand 22.40%, beta turn 6.67% and random coil 37.33%. The secondary structure for cytoplasmic actin 2 (γ) is as follows: alpha helix 34.67%, extended strand, 24.00%, beta turn 7.47%, random coil 33.87%. (Jonnalagedda et.al. 2012)

Primary Structure

Cytoplasmic actin 1 is comprised of 375 amino acids). It has an estimated molecular weight of 41,736g, and it is slightly

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acidic, with a pI of 5.29. It is noteworthy that actin has very high concentrations of glycine (7.5%) and proline (5.1%). This characteristic contributes to actin's compact structure. The different isoforms of human actin have a nearly identical amino acid sequence. Cytoplasmic actin 2, also made up of 375 amino acids, differs from cytoplasmic actin 1 in only four residues (2, 3, 4 & 9). Its PI is estimated to be 5.31, and it has an estimated molecular weight of 41,792.8g. α skeletal actin contains 377 amino acids. It differs from the cytoplasmic actins in only 25 residues, and 15 of those are considered to be similar (The UniProt Consortium, 2013, Jonnalagedda et. al., 2012).

Actin is highly conserved across different species. The actin found in the fungus Saccharomyces cerevisiae (baker's yeast) closely resembles cytoplasmic actin found in humans. It is also made of 375 amino acids, and it differs in only 42 of the residues, 30 of which are considered to be similar. The UniProt Consortium). S. cerevisiae actin has a MW of 41,689g, and a pI of 5.44, similar to that of human actin. (The secondary structure of S. cerevisiae actin is also very similar to human actin: alpha helix 33.87%, extended strand 24.00%, beta turn 6.93% and random coil 35.20%.

Functional Differences Between β and γ Actin

The amino acid sequences of the two isoforms are nearly identical; they differ in only four residues (2, 3, 4 and 9) (Figure 1). Interestingly, although their amino acid sequences are nearly identical, there are significant differences between the 5′ and 3′ un-translated regions surrounding the genes; these differences are conserved among species (Erba et-al). The gene encoding β actin is located on chromosome 7p; the gene encoding γ actin is located on chromosome 17q.

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MDDDIAALVVDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEAQS
MEEEIAALVIDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEAQS
*:::*****:**************************************************
KRGILTLKYPIEHGIVTNWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPLNPKANREKMT
KRGILTLKYPIEHGIVTNWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPLNPKANREKMT
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QIMFETFNTPAMYVAIQAVLSLYASGRTTGIVMDSGDGVTHTVPIYEGYALPHAILRLDL
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Figure 1: A comparison of the amino acid sequences of β and γ cytoplasmic actin. The top line is β actin; the bottom line is γ actin. The residues that differ have been highlighted. (The (:) symbol beneath these residues indicates that they are conserved mutations) (GeneCards.org).

Experimental evidence suggests that the two isoforms of cytoplasmic actin have some overlapping functions, but are not interchangeable. In γ actin knockout mice it was found that cellular concentrations of actin were normal, indicating that the body compensates for deficiencies in γ actin by up-regulating the expression of β actin. However, despite the fact that the total amount of actin was normal, these mice were smaller than the wild type, and significant numbers died due to developmental delays. In contrast to γ actin knockout mice, β actin knockout mice are not viable, demonstrating β actin's greater role in critical cellular functions (Perrin and Ervasti, 2010).

Experiments have shown that the relative concentrations of actin differ between cell types. Generally, β actin is present in greater concentrations than γ actin. However, in the cilia of the inner ear and epithelial cells of the intestinal tract γ actin dominates (Zhu et. al, 2003; Rendtorff et. al., 2006). Even within cells different isoforms of actin seem to be localized to specific areas. The mechanism that controls the localization of the different isoforms has not been identified. It is likely that the concentration in different cell types is regulated by the 5′ and 3′ UTRs, which regulate gene expression. As previously mentioned, significant differences have been found in the UTRs of the two genes. Differences in localization within the cell may be the result of differences in interactions with actin binding proteins (Perrin and Ervasti, 2010).

The differences in function between the two isoforms of cytoplasmic actin may be due to differences in the rate of polymerization. Under calcium-bound conditions β actin polymerized and de-polymerized at a quicker rate than γ actin. β actin and γ actin readily copolymerize, and the relative rates of polymerization of different filaments vary according to the ratio of the two isoforms. Because they polymerize and de-polymerize at a slower rate, filaments containing a higher ratio of gamma actin are more stable. The reason for these differences is unclear. One theory supported by research is that certain actin binding proteins that regulate polymerization are isoform-specific (Perrin and Ervasti, 2010).

Pathology

Hearing Loss

The function of the inner ear relies on the specialized structure of the auditory hair cell, and the function of these cells is closely related to the structure of their cytoskeleton. Mutations in cytoplasmic actin which result in malformations of the cytoskeleton have been found to cause hearing loss. These mutations affect the shape of the actin monomer, the stability of actin filaments, and actin's ability to bind to other proteins. Subtle differences in the onset and severity of the hearing loss have been observed in patients carrying different mutations (Zhu et. al..2003; Morin et. al.,2009). In one family a genetic mutation that causes isoleucine to be incorporated in place of threonine at position 278 (T278I) was observed. This is a non-conserved mutation; threonie is hydrophilic, and isoleucine is larger and hydrophobic. This mutation affects the 9 and 11 helices, structures that play a role in polymerization (Figure 2) (Van Wijk et. al, 2003).

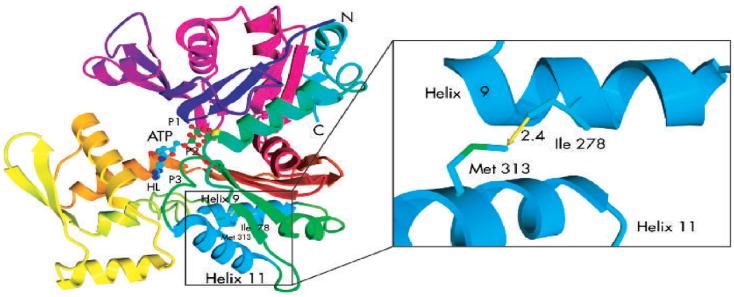
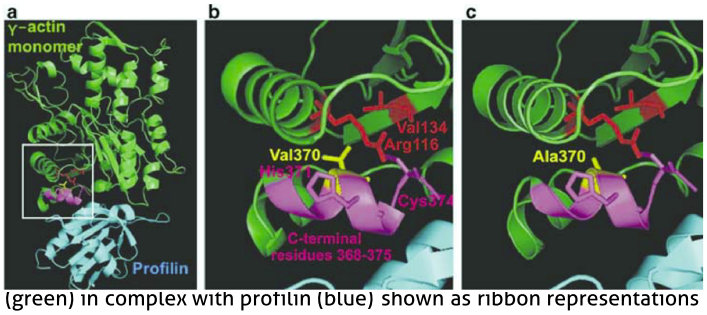


Figure 2: Structure of actin from D. discoideum. The protein is colored with a gradient from blue (N-terminus) to cyan (C-terminus), except for helices 9 and 11, which are shown in light blue. The three ATP binding loops (P1 to P3) and the hydrophobic loop involved in polymerization (HL) are marked. At position 278, the predicted side chain conformation of the isoleucine mutant is indicated, causing a strong bump with Met 313 (distance is 2.4 A°). The image was created with YASARA (www.yasara.org) (Van Wijk et. al., 2003).

Another mutation that has been studied is caused by the substitution of valine for alanine at position 370 (V370A). The valine at position 370 engages in hydrophobic interactions with neighboring side chains, stabilizing the C-terminal tail, a region that plays a critical role in the binding of some APBs. Alanine's side chain is too short to establish these interactions, destabilizing the C-terminal tail (Figure 3) (Rendtorff et.al., 2006).



(green) in complex with profilin (blue) shown as ribbon representations with side chains of residues discussed in the text. V370 is shown in yellow and the C-terminus of g-actin and residues herein that interact with V370 in magenta. Residues elsewhere in g-actin that interact with V370 are shown in red. (b) Enlarged view of the boxed region in (a). (c) As in (b), except that g-actin is the p.V370A mutant (Rendtorff et. al., 2006).

It is interesting to note that although γ actin is the prevalent isoform in the epithelial cells of the intestinal tract, these mutations have not been linked to any pathology of the intestines. This may be due to the fact that the cilia of the inner ear are subjected to high amount of mechanical trauma making them more susceptible to structural damage. Additionally, the cells of the intestinal tract are frequently renewed, making them less susceptible to such damage (Zhu et. al., 2003; Rendtorf et. al., 2006).

Muscular Disorders

Although muscular actin is the predominant isoform in muscle cells, mutations in cytoplasmic actin have also been linked to muscular disorders. One mutation in β actin that substitutes tryptophan for arginine at position 183 (R183W) has been linked to delayed onset dystonia. The mutated actin is more acidic than the wild type (PI 5.21 instead of 5.29), and slows depolymerization, making the actin filaments more rigid. This mutation has also been linked to hearing loss. Mutations in β actin have been linked to other muscular disorders as well, including, actin myopathy, nemaline myopathy, and intranuclear rod myopathy (Procaccio et. al., 2006).

Baraitser-Winter Syndrome

Mutations in both β and γ actin genes have been identified in patients with Baraitser-Winter Syndrome, a rare neurological disease. The two mutations that appeared most often caused a substitution of histidine in place of arginine at position 196 in β actin, and a substitution of serine for phenylalanine at position 155 in γ actin. The fact that mutations in both isoforms have been implicated suggests that the mutation affects a function that is common to both isoforms (Riviere et. al., 2013).

Conclusion

Cytoplasmic actin plays a crucial role in cellular structure, cell motility, intracellular transportation, and the cell cycle. As such, mutations in cytoplasmic actin can have wide-ranging deleterious effects on the organism. Because of their close similarity, researchers have had trouble identifying the functions that are unique to the different isoforms. However, it is clear at despite their overlapping functions the two isoforms are not interchangeable.

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The Effects of Emotional Experiences on Memory Processing

Naomi Berkowitz

Abstract

Neural regions, specifically the amygdala, hippocampus, and prefrontal cortex overlap in functions of emotion and memory, indicating a degree of interrelatedness between the two functions. Lesions in medial temporal lobe regions result in an impairment of memory processes specific to emotional stimuli. Additionally, amygdala activity is increased for all valence memory as opposed to neutral. Arousal levels of high and low valence memories affect the pathway for encoding in the brain, and determine the vividness and episodic detail with which a memory will be recorded. The amygdala-hippocampal network is involved in high arousal memory, while a prefrontal cortex-hippocampal network is involved in low arousal. Because of the different neural pathways, negative memory is better remembered, while positive memory is better known. Males and females display the same abilities in working memory, yet have differing neural pathways. Because males' memory networks are more associated with the prefrontal cortex, they have better cognitive control than females for emotional events. Some suggest that because of the implications of a prefrontal vs. amygdala memory encoding, emotional regulation at the onset may be key to preventing traumatic memories from ever developing. Further research should be done in defining the link between emotional and memory processing, to better understand and provide therapy for various neurological disorders.

Introduction

The field of affective neuroscience addresses the biological basis of emotions. Affective neuroscientists attempt to explain what an emotion is, and how emotional processes interact with other areas and functions of the brain. Neuroscientists over the years have associated emotions with a network of structures in the brain, specifically the limbic system. The limbic system includes parts of the orbital and medial prefrontal cortices, part of the thalamus, the amygdala, the hippocampus, and the cingulate cortex (Figure 1). Many of these same structures, aside from control of emotions, are responsible for various functions of memory. Specifically, the prefrontal cortex, hippocampus, and amygdala are known to play significant roles in memory processing, encoding, and storage. Because of the overlap of brain structures for these two different functions, there is strong evidence of a connection between emotion and memory. Many neuroscientists have asked and will continue to ask what the exact interrelatedness of these functions is.

Aside from understanding the relationship between various memory processes and different types of emotional experiences, many new questions emerge because of it. Are there differences in male and female memory processing because of the way emotions affect each of them? How does a disturbance in this relationship affect people? Does it explain any mental abnormalities? Furthermore, this connection, should it be biologically proven, leads the way to areas of human manipulation in encoding memory, based on their emotional experiences. Questions of memory repression and other similar issues can also be biologically tested.

Emotion

The Limbic System is specifically associated with emotion, yet a conclusive localization of specificity of brain structures for each emotion has not been clarified. Neuroscientists have discovered different activity for positive versus negative emotion, but not complete specificity for a particular positive or negative emotion. The most consistent research regarding specificity of emotional function is in regard to a lateralization of function. Increased activity in the left frontal and temporal lobes is associated with the Behavioral Activation System, emotions which cause a person to outwardly react, such as happiness or anger. Emotions such as fear and disgust, which inhibit a person's behaviors, are associated with increased activity in the right frontal and temporal lobes (Kalat 2013).

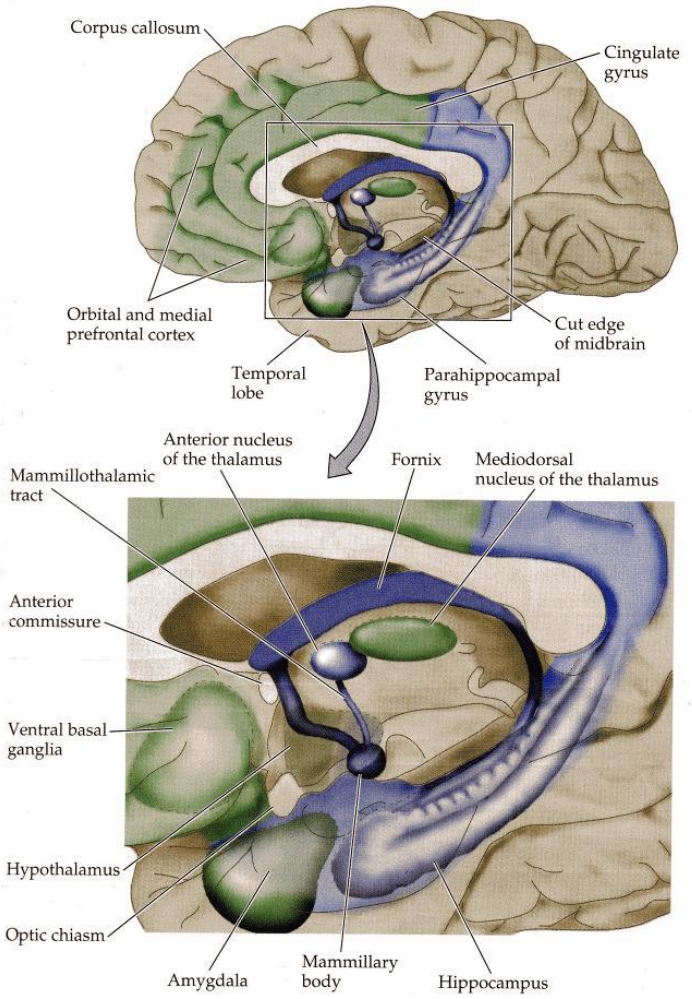

Figure 1: The Limbic System. Source: Purves et al, 2001.

Naomi graduated in 2013 with a B.S. degree in biology.

The major neural structures for emotional processing discussed in this paper include the amygdala, hippocampus, and prefrontal cortex (PFC). Most research has been conducted regarding the amygdala, because of its primary role in emotional functions.

When classifying an emotion, the experience is rated based on its valence and arousal. The valence of an emotion refers to how positive or negative an experience is. High valence is a pleasant feeling, and low valence is an unpleasant one. Arousal is the degree of intensity of emotion that an experience causes. High arousal results in feeling excited or anxious, while low arousal in feeling calm or subdued. An experience can be high valence, high arousal (winning the lottery), high valence, low arousal (getting a massage), low valence, high arousal (a screaming match), or low valence, low arousal (mourning). These factors are key in regard to measuring specific brain activity.

Memory

The two basic divisions for memory are short term memory and long term memory. Short term memory, now known as working memory, is a piece of information that our brain holds onto temporarily, as long as it's being rehearsed and focused on. There are two parts to processing long term memory. First, there is the original formation and encoding an image or event. Later there is the recall or retrieval of the memory, which is referred to as the subsequent memory.

Long term memory is memory that is stored by the brain, and can be subdivided into two major categories: explicit and implicit memory. Explicit, or declarative, memory refers to things that the brain consciously committed to memory. Things unconsciously absorbed by the brain are considered implicit memory. Implicit memory is known as procedural memory; it does not just include events or images, but refers to skills or procedures that are learned. Explicit memory can further be subdivided into episodic and semantic memory. Episodic memory is also known as autobiographical memory, because it refers to memories related to a specific occasion in a person's lifetime. Other pieces of known information, which are basically general knowledge that a person acquired, are semantic memories.

The major brain areas involved in memory functions include regions in the prefrontal cortex, hippocampus, amygdala, cerebellum and basal ganglia. Each structure plays a part in the overall processing of memory. Two major functions of the PFC are in working memory and autobiographical memory. The hippocampus is necessary for explicit memory, spatial memory, and episodic memory. The amygdala's function is in encoding and enhancing emotional memory. The cerebellum and basal ganglia play a major role in implicit memory. The processes of the PFC, hippocampus, and amygdala will all be discussed because of the emotional function of these structures as well.

Measuring Brain Activity

Studies conducted by affective neuroscientists generally have a behavioral phase and a neurological phase. Most experiments involve presenting various standard neutral or emotionally stimulating images or verbal cues. The cues can be classified based on valence and arousal for a more specific study. These images or words are then evaluated emotionally by the subjects, both by recording their behavioral responses and by viewing their brain function under an EEG, fMRI, or PET scan. Later on, these images are displayed again, and the subjects recall is evaluated, both by their behavioral responses and by neuroimaging.

An EEG, or electroencephalogram, measures brain activity via electrodes by the electrical current being produced. The electrical activity of brain regions is measured as the subject is responding to a behavioral cue. For 3D imaging, PET scans and fMRI scans are conducted. PET scans involve the injection of a radioactive tracer, which can highlight brain regions based on the oxygen or glucose levels in those regions. An fMRI scan measures localized brain activity by measuring the levels of cerebral blood flow based on the oxygen levels of neural regions. Because of the advantages of projecting a 3D image and a lack of radioactive injections, fMRI scans are the most commonly used to determine brain function.

Aside from neuroimaging, researchers study brain activity in patients with lesions to various brain structures in comparison to the average human brain. For these studies, comparing behavioral results explains the effects of a removal of certain neural regions.

Methods

The research for this paper was collected via searches on the Touro College library database. Databases where articles were found include PubMed, ScienceDirect,, MD Consult, ProQuest, and EBSCO. The above listed databases include a vast library of journal articles related to medicine and the health sciences. The studies were collectively analyzed to draw consistent conclusions, regarding research in the area of emotion and memory.

Discussion

Neutral VS. Valence Memory

To establish a direct link between emotional arousal and memory processing, researchers studied patients with lesions in their medial temporal lobes (Ahs et al, 2010). The medial temporal lobe (MTL) contains the anterior part of the hippocampus and the amygdala. The hippocampus is involved in retrieval of all memory, and the amygdala is involved in the encoding of emotional memory, which enhances the memory performance for high arousal stimuli. The amygdala is also involved in enhancing emotional declarative memory. A study was conducted with control subjects and patients who had medial temporal lobe lobotomies, in either their left or right hemispheres. Various emotional images were displayed to the

subjects, and the recognition of these images was tested immediately afterward. The results revealed no difference between the controls and patients for neutral items, yet there was a significantly higher percentage of recall for all emotional items in the control subjects. This held true for all valence items, regardless of their arousal. Within the control group, there was a slight incremental increase in recall for valence items of higher arousal. There was no observed difference for arousal memories among patients with a lesion to the medial temporal lobe in one hemisphere or the other. The study did not reveal significant difference in recall for patients with right versus left MTL lobotomies. Other studies, however, do reveal poorer recall for those with left versus right MTL lesions (Buchanan et al, 2001). They studied how neutral versus emotional verbal cues affect memory processing. There is left lateralization for verbal processing of emotional information, which explains why those with a left MTL lobotomy have worse retention for emotional, verbal stimuli.(Buchanan et al. 2001)

In investigations of people with MTL lobotomies, the focus of the experiments was the amygdala. However, these studies were not conducted with patients who exclusively had lesions in the amygdala. Therefore, a more selective study was done, using subjects with damage to their amygdalae specifically (Adolphs et al, 2005). This study more accurately concluded revealed similar results as the above mentioned studies; enhanced processing for emotional memory occurs when the amygdala is involved in encoding. However, this study made a further, more specific observation, in terms of the amygdala's role in memory consolidation and retrieval. After studying patients with amygdala damage, these researchers compared the MRI scans of subjects with MTL damage. The volume of amygdala damage in the MRI directly correlated with the impairment of the general gist of emotional memories, while the volume of hippocampal damage directly correlated with the impairment of the contextual details of those memories.

Emotional Enhancement of Memory: Which Functions?

Neuroimaging allows for localization of brain activity under various environmental stimuli. It is known that increased amygdala activity leads to a better encoding of emotional memory. However, it is unclear whether this refers to the vividness of the memory or its episodic details. , Researchers used an fMRI study to determine what sort of emotional memory is better encoded by presenting subjects with images of various arousal and valence levels (Kensinger et al, 2011). They stated that there are different processes which reflect the vividness of a memory versus its episodic details. The fMRI scan revealed increased amygdala activity for memories with increased vividness, but not episodic detail. This held true for all types of emotional memory, regardless of the memory's valence or arousal level. However, higher activity in many regions of the prefrontal cortex was associated with an increase in vividness for high arousal memories. In contrast, increased activity in the occipital and inferior temporal lobes was related to increased vividness for low arousal memories. These different memory paths have significance in explaining behavioral tests of memory. High arousal memories are often reported to be more vividly recalled than low arousal memories. Because of the involvement of the prefrontal cortex, this greater vividness is due to other types of memory functions of the PFC, such as autobiographical memory.

Another study, specific to the role of the PFC in emotional processing, revealed increased activity in an fMRI scan in various PFC regions, depending on the sort of emotional experience (Dolcos et al, 2004). There was overall greater activity in the left ventrolateral and dorsolateral PFC for emotional versus neutral stimuli. The left dorsolateral PFC revealed greater activation for positive images, while the right ventrolateral region revealed greater activation for negative. This is consistent with the lateralization effect for emotional experiences.

When an emotional memory is being encoded, a high arousal memory is often more confidently vividly recalled, but there is actually no increase in encoding of details of the memory. Low arousal memories are just the opposite. They are not reported to be as vivid, but are more accurately recalled and contain contextual details, because of the sensory pathway involved in forming low arousal memory. In terms of increased episodic detail, the fMRI scan revealed no increased activity in the amygdala for better encoding of details (Kensinger et al, 2011). There was activity in the lateral prefrontal cortex, hippocampus, and anterior cingulate gyrus; these areas are all associated with binding contextual details, and are involved in neutral and emotional memory. There was increase in activity in the occipital and inferior temporal lobes for better recall of episodic detail in negative valence memory (more so than positive). This is why negative memory is encoded with more sensory detail than positive memory. The fMRI also revealed greater activity in the frontal and temporo-parietal regions for greater episodic recall of low arousal memory. This is consistent with the knowledge that those regions are associated with elaborative and semantic memory processing.

Another study was conducted to test a "trade off" effect for emotional enhancement of a memory versus the background details (Waring & Kensinger, 2011). Based on the valence and arousal of the stimuli, a memory is encoded via different pathways in the brain, which result in this trade off. Specifically, high arousal valence experiences are processed via an amygdala-hippocampal pathway, while low arousal valence experiences are processed via a PFC-hippocampal network (Kensinger & Corkin, 2003). Though studies proved a greater recall rate for valence, low arousal over neutral memory, the encoding pathway is quite similar. Subjects of an experiment were observed under an fMRI, while being presented with words that were either neutral, negative high arousal, or negative low arousal. After a short delay, these subjects were again observed under fMRI for their ability to recall these words. The scan for initial formation revealed increased activity in the left hippocampus, amygdala, and inferior parietal lobule for all the negative words versus the neutral words. (The inferior parietal lobule is believed to be involved in processing

verbal information that is related to the self, attention, and emotional content.) The left inferior and dorsolateral prefrontal cortices showed greatest activation for negative low-arousal words than for any other words, because of the left PFCs function in semantic memory. When the subjects were evaluated for subsequent memory, the fMRI scan showed increased activity in the left amygdala and hippocampus for the high-arousal words (Kensinger & Corkin, 2003). The recalled negative non-arousing and neutral words revealed increased activity in the left inferior PFC and left hippocampus.

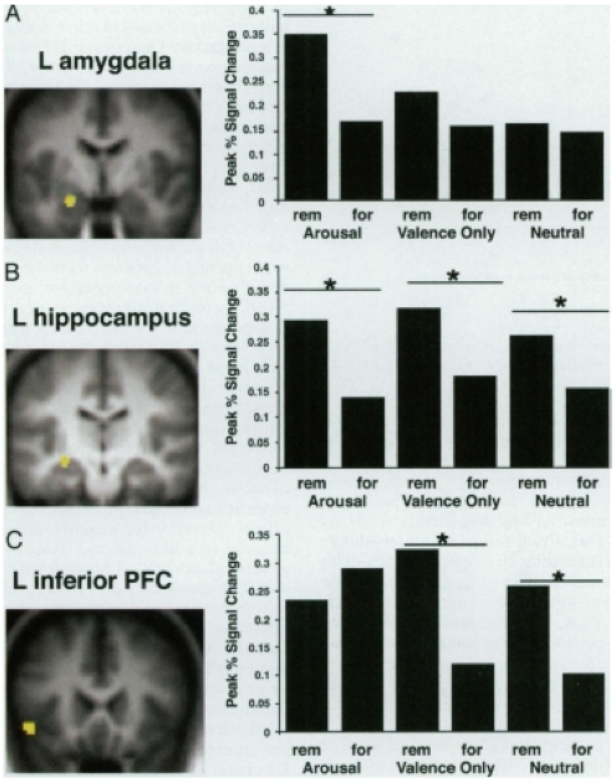

Figure 2: Graphs with their corresponding imaging scans, comparing activation in the amygdala, hippocampus, and prefrontal cortex for remembered (rem) and forgotten (for) neutral, valence, and arousal memories. Source: Kensinger & Corkin, 2003.

The results of many studies (Kensinger & Corkin, 2003, Kensinger et al, 2011, Dolcos et al, 2004) conclude that the amygdala is involved in an increase of the vividness of the memory because of encoding a particular set of the details. Contextual and episodic details, overall, are not better recalled because of the amygdala. These results are consistent with the above mentioned studies of amygdala and MTL lobotomies, which conclude that the amygdala is involved in emotional enhancement of memories, processing of the gist of the memory, but not encoding details (Adolphs et al, 2005). This memory recorded via the amygdala is often very subjective, as the person very vividly remembers only certain details of the experience.

An advantage of the amygdala-hippocampus pathway for high arousal items is that it occurs almost always and automatically (Kensinger & Corkin, 2003). In contrast, the PFC-hippocampus pathway for low arousal items requires attention and control. A small behavioral study to the same subjects of the fMRI scan was conducted, during which retrieval for neutral, low arousal, and high arousal was measured while the subjects were multitasking. The results revealed only a significantly greater recall percentage for the high arousal words over the neutral words while the subjects had to focus on other things.

High and Low Valence

The above studies show that arousal levels affect memory processing, but do not differentiate between positive and negative experiences. To gain this understanding, a study was conducted to examine the effects of valence on memory (Mickley & Kensinger, 2008). The study established two distinctions. First, that all emotional stimuli are remembered via the orbitofrontal cortex. However, after a longer delay of re-displaying the stimuli, negative information was "remembered" more than positive memory was. Positive information was remembered less, but had a higher rate of being "known". Under fMRI imaging, areas of the temporo-occipital lobe displayed high levels of activity for low valence emotional experiences. This is because negative memory is associated with higher sensory encoding, and is therefore more likely to be remembered. In contrast, positive memory shows high activity in the cingulate gyrus and parietal lobe. These areas are associated with both semantic and episodic memory retrieval and with poor encoding of specific details. This explains why positive memory is known rather than remembered after a long time delay. When a positive image is displayed, often a person gets distracted by personal positive memories that are triggered, because of association with that image. Therefore, the small details of the specific positive image are lost, because more memory is involved with a positive memory. Both emotionally positive and negative stimuli have pathways in the brain for better memory encoding, more so than neutral memory. Each category of emotional memory, however, has its own specific pathway related to the emotional experience.

Gender Differences

Because of the relationship between emotion and memory, researchers asked if there are any gender-related differences in these processes. Women are generally more emotional than men; biologically this means that women's orbitofrontal cortices are more activated than men when processing emotions induced by olfactory stimuli. Statistically, prevalence rates for emotional disorders are higher in women than men. Additionally, women are more prone to display physiological signs of emotion than men are. Men, however, display greater cognitive control over their negative emotions. In a controlled experiment, male and female subjects were presented with a behavioral test for working memory (Koch et al, 2007). The subjects were prompted to focus on one letter being displayed, and also verbally recall a letter that they had just seen. While they were involved in this task, a negative odor was periodically being sprayed into the room. Both males and females were observed to have impaired working memory while the negative

odor was presented. However, the females did not perform any worse on the tasks than the males. An fMRI scan was taken of the subjects while they were conducting this task with a neutral odor, and with a negative one. Males and females revealed activation in different brain areas for working memory, regardless of the odor. Males revealed greater activity than females in the lingual gyrus, while women displayed greater activity than men in a complex neural network, including areas of the prefrontal cortex, temporal lobe, and cingulate gyrus.

When the negative odor was presented, women revealed greater activation in the left superior temporal gyrus, right inferior frontal gyrus, and left insula. In comparison of the interaction between working memory and the negative olfaction, males displayed higher activity in the left inferior parietal lobe, right middle temporal gyrus, and left superior occipital lobe than females. Females displayed greater activation in the left amygdala and right orbitofrontal cortex (OFC).

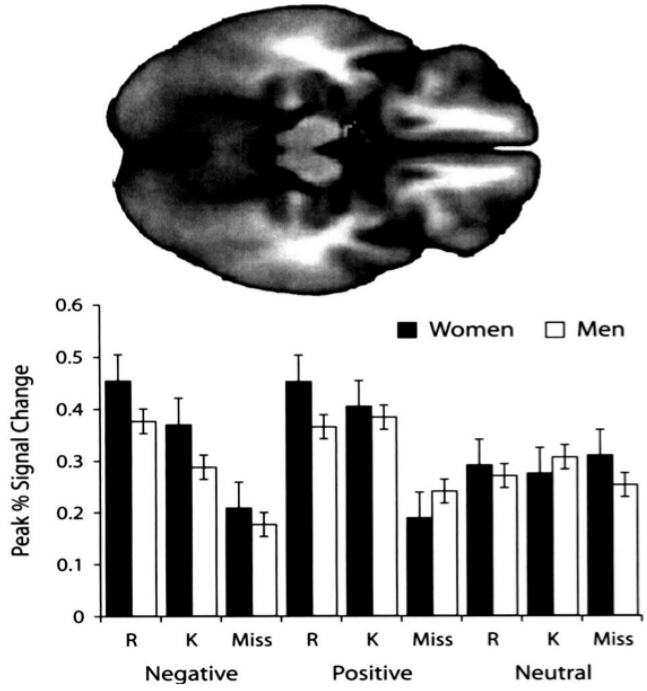

Figure 3: Graph comparing increased amygdala activation in males and females, for subsequent memories of varying valence experiences, that were remembered (R), known (K), or missed (Miss). Source: Koch et al, 2007.

The neural regions where females displayed greater activity when the negative olfaction was introduced are associated with processing negative emotions. In females, emotion and cognition are two parallel networks; the network for working memory in females was not affected when an emotional stimulus was introduced. However, in males, there was increased activity in areas which overlap with cognitive function. The cognitive and emotional processes in males are activated via the same brain pathway, as was observed in comparison of the fMRI results for an interaction between the memory task and the odor. Because of this shared pathway, men are better able to cognitively control their emotions.

Emotional Regulation: Affects on Memory Encoding and Retrieval

Research has shown that when the amygdala is activated because of an emotional experience, long term memory is better encoded (Dolcos et al, 2004). A study was conducted to test whether this applies when people regulate their emotions, as opposed to emotions that are experienced naturally (Erk et al, 2010). Subjects viewed neutral and negative images, and were asked for some of these images to allow themselves to naturally feel, and for some to regulate their emotions. One year later, some of the same images as well as new images where were displayed to the participants, and they were prompted to respond if they recalled the images. An fMRI scan of the subjects revealed that for all the negative images, there was brain activity in the amygdala, prefrontal cortex, occipito-temporal cortex, and brainstem. When an image was correctly recalled, there was brain activity in regions of the left prefrontal cortex, midbrain, parietal lobe, hippocampus, and amygdala. Regarding the question of emotions regulated or naturally experienced, the fMRI showed activity in the right amygdala when the naturally experienced emotional images were recalled. The behavioral results exhibited a similar percentage of correctly recalled memories for all negative images. However, for those that were regulated, there was no increased activity in the right amygdala. The regulated memory

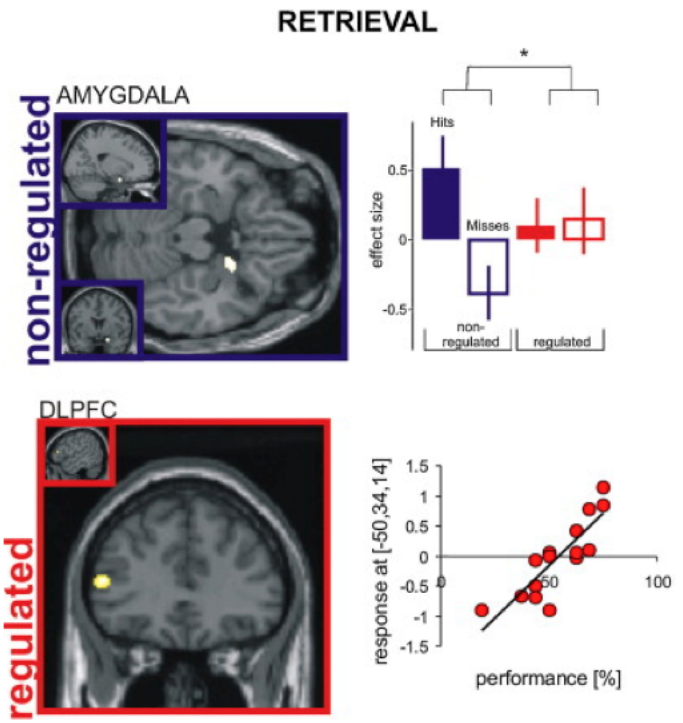

Figure 4: Scans comparing amygdala activity for non-regulated emotional stimuli and activity in the dorsolateral PFC for regulated emotional stimuli. Source: Erk et al, 2010.

showed increased activity in the right dorsolateral prefrontal cortex. This explains that all negative memory has a greater recall accuracy than neutral memory, but that the encoding pathways of the brain differ. The neural route of a memory is dependent whether or not it was experienced emotionally (Ochsner et al, 2002). When negative information is encoded as the emotion is processed, then the amygdala will be active when the memory is recalled (Erk et al, 2010). This resurfacing memory does not only bring forth the image, but also the emotions associated with it. If the negative information is originally encoded by emotional regulation, then when the memory is recalled via the prefrontal cortex, it will only be a cognitive experience (Ochsner et al, 2002). These results suggest that if the brain can regulate its recording of negative events, then these events will not, in the future, cause emotional stress or trauma.

Conclusion

This review concludes that emotional stimuli affect the pathways of memory encoding. Specifically, when the amygdala is activated at encoding, the memory that is stored will be vivid and may arouse emotion when retrieved. Many factors may contribute to a neural network with less amygdala involvement, and instead cause the prefrontal cortex to be more active in memory formation. There is greater cognitive control, in terms of episodic detail and emotional regulation, when memory is processed via the PFC. More research in this area should be conducted, because of the potential benefits that a clearer understand of encoding of emotional memories may have on patients with depression, post-traumatic stress disorder, and other mood and anxiety disorders.

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Microparticle Function

Leorra Kohen

Abstract

Microparticles are emerging as an integral part of the vascular system. The microparticles are derived from stimulated or apoptotic endothelial cells, platelets, and leukocytes. They are involved in coagulation and regular cell function. Excessive numbers of microparticles contribute to artherosclerosis, chronic renal failure, and metabolic syndrome. Microparticle levels may be reduced by medications, relieving symptoms for awhile. Studies are being done to predict disease by counting microparticles. Further research must be done to understand and make use of microparticles.

Introduction

Endothelial microparticles (EMPs) are small (> 1 μm in diameter), "non-nucleated phospholipid vesicles shed from the endothelial cell membrane in states of endothelial activation or apoptosis and express on their cell surface various antigens specific to the state of their parental endothelial cell" (Weber et al 2011). They were thought to have been markers for endothelial dysfunction. Recently, studies have shown that increased circulating microparticles (MP), which now include platelet microparticles (PMP) and leukocyte microparticles (LMP), also have a significant effect in contributing to endothelial dysfunction in the human body. They both mark and augment pre-existing conditions. MP's contribute to: apoptosis, thrombosis and arterial stiffness. Patients with kidney disease, obesity, and pulmonary hypertension are more susceptible to higher levels of microparticles than their healthy counterparts. The increased numbers may be caused by triggers, released as a byproduct of the diseases. High MP levels may lead to cardiovascular disease, one of the leading causes of death in the United States. This article will explore what affects and reactions microparticles may cause (Morel et al 2006). In addition, it has been found that MP's can be used as a biological tool. While microparticles have many functions based on origin, this paper will be focusing on the MP's that affect endothelial dysfunction, mainly: EMP, PMP, and LMP.

Methods

Searches were done primarily from PubMed. Once an article was deemed helpful, the PubMed ID was put into Touro's library ID search which gave access to the article through EBSCO, Proquest Medical Library, and Medline. Articles were also taken from Google Scholar. The first articles were found using the following methods. Keywords: microparticles, endothelial microparticles, platelet microparticles, microparticles and coagulation, microparticles and pulmonary disease, microparticles and atherosclerosis, microparticles and renal failure, mircroparticles and obesity, . Articles were filtered within the past 10 years, peer-reveiwed, clinical trial, and meta-analysis. Sources found in these articles were also used as references when further clarification was needed.

Definition

Microparticles are small membrane-bound vesicles that are released from the cell during cell stimulation or cell death. Electron microscopy techniques allowed Wolf to identify microparticles released from platelets, a particle that had been speculated upon but had never been seen. He further discovered that they were released from the cell after being triggered by chemicals such as cytokines, thrombin, and endotoxins (Budaj et al 2012). Chrinios et al (2005) believe that MP's are triggered by cell activation due to the expression of tissue factor, the main trigger for thrombin generation.

MP's can be found in multiple places, including blood, urine, ascetic fluids, and synovial fluids (Bundaj et al 2012).Contained on the EMP cell surface are many proteins including vascular endothelial cadherin, platelet endothelial cell adhesion molecule-1, endothelial NO synthase (also found on PMP), and vascular endothelial growth factor receptor. The last protein is particularly important in tagging EMP's in the blood (Dignat-George and Boulangar 2011).

Different types of Microparticles

The three groups of MP's are classified by their origins. EMP's are derived from the membrane of the vascular endothelial cell. For this reason, microparticles are mostly studied along with their effect on vascular and cardiological diseases. EMP's are involved in endothelial cell function. Chrinios et al (2005) have found that EMP's "express many receptors of the parent endothelial cell" and can help thrombin generation through the tissue factor pathway. Tissue factor is the main cellular initiator of blood coagulation. During EMP production, phosphatidylserine is released, an important molecule that enhances the procoagulant activities of tissue factor. In addition to coagulation and thrombosis, EMP's contribute to other factors of endothelial dysfunction as well. This includes changes in the nitric oxide (NO) synthase expression and local prostacyclin synthesis (Amabile et al 2008). These two molecules will be discussed in further detail below.

PMP's and LMP's are now emerging in new studies of MP's. The studies have shown that they are involved in coagulation, but they are still being researched. PMP's are frequently counted by the P-selectin count, a recognized marker of platelet activation in aggregation between platelets, monocytes, and neutrophils.The coagulation process will generally favor the seeding of LMP followed by PMP and EMP aggregation (Chirinos et al 2005).

Release and Inhibition

Under normal conditions, vessel homeostasis is controlled by

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Under normal conditions, vessel homeostasis is controlled by the intact endothelial cell monolayer which has anti-inflammatory, anti-atherogenic, and anti-thrombotic properties. It is maintained by low continuous cell regeneration. The endothelial activation remains local, low-grade, and reversible. Circulating MP's are barely detectable due to their low count. However, in case of injury cells may respond with apoptosis and MP generation along with other factors leading to a pro-coagulant state (Chironi et al 2009).

One hypothesis for the release of MP's may be that they are released as a way of preservation. The cell will attempt to evade phagocytosis by releasing the phosphatidylserine and caspase 3, both signals that alert the phagocyte. By shedding these factors, phagocytes will not take notice of the cell (Boulanger et al 2006). Another theory suggests that MP's are released in response to certain signals, such as tumor necrosis factor-α; a cytokine. A study has shown that EMP release istriggered by tumor necrosis factor-α and subsequently increased the release of ICAM-1, a T-cell. This allowed a paracrine loop, boosting the endothelial response to inflammation (Dignat-George and Boulanger 2011).

Microparticles are produced in response to changes within the body as well. MPs derived from platelets, endothelial cells, and erythrocytes are produced in response to metabolic perturbation. The monocyte derived MP is produced as a subsequent step in response to more severe conditions, such as diabetes and cardiovascular disease (Helal et al (2012). Oxidative stress has been shown to be a significant factor in MP release. Reactive oxygen species have been shown to reduce the availability of NO, which is a mitochondrial stabilizer, the release of cytochrome c, and caspase activation. These contribute to endothelial dysfunction, leading to the release of EMP's (Morel et al 2003).

Studies have shown that vascular endothelial-cadherin, an adhesion molecule found at junctions between endothelial cells, regulates many cell processes including cell proliferation, apoptosis, and modulation of vascular endothelial growth factor receptor functions (Petzelbauer et al 2000). A study attempted to stimulate in vitro MP release. It was reported that apoptotic and activated cells respond differently depending on the stimulus. Cells were stimulated while deprived of endothelial growth factor. Findings indicate that renal endothelial cells release certain EMP's in larger amounts from apoptotic cells than cells activated by tumor necrosis factor. In contrast, other strains of EMP are significantly higher when activated by tumor necrosis factor than apoptosis. This study also shows that MP's are phenotypically different and may lead to further studies of different types of EMP's in response to different injuries (Jimenezet al 2003).

Conversely, the MP's also have an inhibition cycle in place. Firstly, endogenous NO dampens the release of EMP on stimulation with C-reactive protein by a mechanism involving tetrahydrobiopterin. Studies have also shown the importance of the Rho kinase pathway (figure 1). While its importance is understood in EMP release, the exact mechanism has not yet been reported.

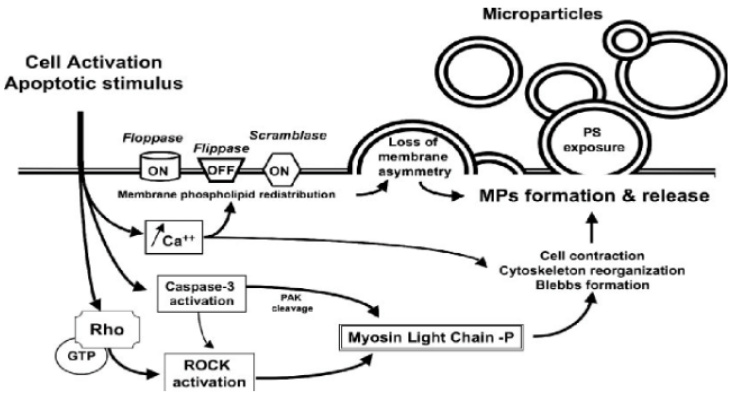

Figure 1: EMP release pathway. Release is stimulated by an increase in Calcium or the Rho kinase pathway (still under study). This causes an imbalance in the membrane or cytoskeleton restructure leading to MP release. Source: Boulangar et al., 2006

The Rho family is an important regulator in cell adhesion and cytoskeleton. The Rho GTPases attach themselves onto the cell membrane and cause subsequent activation of the Rho-kinase (Tramontano et al 2004). This activation reduces the stability of the endothelial NO synthase pathway (Davignon and Ganz 2004). An in vitro study showed that statins anti-inflammatory property is a result of Rho-kinase pathway inhibition. It can be inferred from here that MP's are released in response to inflammation and may be suppressed by the statins (Tramontano et al 2004).

Formation

A cell membrane at rest will prominently display inward translocation (flip). During this time, phosphatidylserine and phosphatidylethanolamine are stored in the inner leaflet of the cell membrane. At stimulation, the molecules will reverse to outward translocation (flop). At hemostasis, aminophspholipids are stationed on the inner leaflet and neutral phospholipids stationed on the outer leaflet. To control the asymmetric distribution, the flop enzyme is only activated by high levels of calcium. During flop, there is a swift movement of the aminophospholippase to the outer leaflet. This causes an extreme imbalance between the inner and outer leaflets (Baron et al 2012). To resolve the imbalance, the cell will begin membrane budding. Membrane budding causes the formation of membrane blebs (figure 1). The cell membrane must undergo changes in membrane lipids, cytoskeleton reorganization, and increase in intracellular calcium (Diehl et al 2011)

The buds cannot retain the symmetric membrane found on the parent cell. They are asymmetric and carry their annexin v (an anticoagulant protein) on the outward surface of the membrane (Kornak and Schuppan 2012). The buds are microparticles containing phosphatidylserine , a molecule that causes coagulation. This feature exposes more procoagulant molecules to the bloodstream proving that MP's are necessary

to achieve the required optimal environment for thrombin generation (Morel et al 2006). However, the presence of phosphatidylserine does not always mean they were released by MP's. MP release may be regulated by the level of intracellular calcium, significantly affecting other studies that used phosphatidylserine as a measurement (Boulangar et al 2006). Phosphatidylserine binds to the annexin-V. It can be inferred from here that this action causes less annexin V to circulate, allowing the thrombus to form (Dignat-George and Boulangar 2011).

Apoptotic cells differ from activated cells in protein and lipid composition. As opposed to cell stimulation, apoptotic cells release DNA fragments to microparticles with the help of an ezyme. The function of the genetic material has not yet been determined. However, certain studies have shown that mRNA transfer from the EMP to other endothelial cells promotes angiogenesis after protein kinase B and NO synthase expression. Blebbing occurs rapidly after the cell enters the apoptotic process. The vesicle formation depends on actin molecules within the cell, regulated by Rho kinase I activation (figure 1). The Rho kinase is needed to relocate the DNA fragments to the MP. Cell activated MP's can be identified by the proteins they contain. The MP's protein will retain traces of the original cell and the stimulus that triggered the release (Boulangar et al 2006). MP's were found to communicate through the use of antigens on the membrane surface. They are able to transfer the antigen to the other cells, thus able to transform the biological function of the cell. (Budaj et al 2012).

Coagulation Pathway

Thrombus Growth

According to a study done by Muller, PMP's are the main source of coagulation, with EMP's and LMP's supplementing. MP's are also known to have cytoplasmic effectors such as selectins, arachidonic acid, and thromboxine which are able to promote prothrombotic responses (Muller et al 2003). It has been shown that PMP's are 100 times more procoagulant than activated platelets, showing the importance of the PMP in a blood clot. PMP's contain arachidonic acid, a vasodilator, increasing aortic tone (Pfister 2004). MP's are then able to transfer their procoagulant potential to the target cell. For example, studies have shown PMP's can bind to and deliver the fibrinogen to the thrombus, promoting coagulation. Interactions between the MP's and monocytes promote mRNA expression of tissue factor, which is a major coagulation effector. EMP's and LMP's are released after activation by bacterial lipopolysaccharides, aggregated low-density lipoproteins, and reactive-oxygen species. Studies have shown that intracellular calcium at the site of vesicle formation is a critical step for MP release (Boulanger et al 2006).

P-selectin is an integral aspect of a thrombus. The P-selectin molecule is an adhesion molecule that is necessary for tissue factor accumulation and incorporation of the leukocytes into the thrombus. The molecule is generally expressed at platelet and endothelial cell surfaces. The buildup of cell-derived tissue factor correlates to the large number of MP's present before the leukocyte-thrombus interaction. In a study involving lab rats with hemophilia A, it was found that soluble P-selectin promoted the shedding of leukocyte-derived MP's carrying tissue factor. This product helps with hemostasis. Studies have shown that MP levels increase as people age. A study done with P-selectin glycoprotein ligand-1in mice has indicated that the MP level is dependent on the contribution of P-selectin, showing the importance of the P-selectin glycoprotein ligand pathway. If the P-selectin was decreased, as in this case, the MP's were decreased as well (Hrachovinova et al 2003).

Stability of the Thrombus

The thrombus may be stabilized by fibrin formation, induced by P-selectin. However, P-selectin glycoprotein ligand interactions are generally noted by their unstable rolling and other stabilizations are needed. Cytoadhesions, a β integrin on the LMP, contribute to stabilization of the thrombus. EMP's exhibit unusually large von Willebrand glycoproteins that promote platelet aggregation. The thrombus is regulated by phagocytes in order to prevent inflammatory responses. Little is known about MP clearance within the thrombus. However, studies on mice have shown that oxidized LDL can either blunt or saturate phagocytosis of apoptotic cells within the thrombus. The oxidized LDL interferes with recognition of MP phosphatidylcholine moieties by macrophage scavenger receptors. Mice infused with lysophosphatidylcholine showed impairment of apoptotic clearance. This can cause a chain reaction in which the macrophage undergoes apoptosis, causing more MP shedding. If the thrombus is disrupted, the arsenal of MP's and procoagulant molecules can be released (Morel et al 2006).

During the course of thrombus buildup, PMP's may modulate angiogenesis, causing the thrombus to be vulnerable. While productive in ischemic muscle, it can prove fatal and noxious in the thrombus (Virmani et al 2005). The PMP may cause angiogenesis whereas the EMP may enhance oxidative stress leading to cell destruction. The LMP could hinder endothelial NO synthesis, leading to endothelial apoptosis.

Tissue factor was believed to have been the major initiator for a thrombus. A study involving reciprocal bone-marrow transplants between mice showed that while vessel-wall tissue factor initiates the thrombus, blood-borne tissue factor, spread by microparticles is responsible for the propagation. They reasoned that vessel-wall tissue factor is quickly covered by the plaque, and is unable to reach circulating clotting factor. PMP's and LMP's are able to circulate, alerting and gathering the needed factors (Chou et al 2004).

Artheroscelrosis

Artherosclerosis is the disease of the blood vessels by deposits of fatty plaques on vessel walls. A low level of high density lippoprotien (HDL) in the blood is a predictive factor in coronary artery disease. It is believed that the HDL is able to carry excess cholesterol and transport it to the liver. In addition,

HDL's promote endothelial cell growth and suppress apoptosis. When HDL levels are low, the endothelial cell is no longer protected and apoptosis occurs, releasing pro-coagulant microparticles (Nofer et al 2001).

Early and constant endothelial dysfunction is a promoter of artheroscelrosis. The main contributor to the buildup of plaques is MP's carrying tissue factor. Doctors generally treat this problem with percutaneous coronary intervention, or more commonly known as the angioplasty. This procedure has shown to lead to procoagulant MP release. The now circulating MP can trigger more PMP release and MP shedding. Circulating MP's carry oxidized pohsholipids on their surface, which in turn express adhesion molecules. This triggers the release of chemokines by endothelial cells. Studies have shown that circulating PMP's reached a peak 8 hours after an angioplasty parallel to the decrease in platelet count (Tramontano et al 2004). An in vitro study has shown that EMP's can induce the release of matrix metalloproteinase -2 and -9. These enzymes degrade the matrix surrounding them, contributing to endothelial dysfunction (Taraboletti et al 2002).

It has been shown that LMP levels are higher in cardiovascular and artheroscleric patients as opposed to other diseases. However, the author notates the reason for this disparity in observations may be due to different methods used to conduct the experiment. For example, different methods of counting the MP's were used, with different centrifuge steps. Furthermore, the criteria for each patient pool differed significantly (Helal et al 2011). Further testing must be done before reaching a decision.

The MPs remain circulating in the myocardial vasostructure, increasing the chances for more plaques to develop. MPs can limit myocardial perfusion through many different pathways. The NO synthase pathway is involved in angiogenesis and neural development through cellular signaling. In addition, the NO is a vasodilator as opposed to its agonist, cytokines (tumor necrosis factor) which are vasoconstrictors (Cody et al 1992).The NO synthase is produced by endothelial cells. The MP's impair the regular endothelial function, disrupting NO synthase and promoting production of cytokines. This causes vasoconstriction in the blood vessel, limiting the blood flow. PMP's can control vascular tone, as shown in an experiment involving rabbits' aortic tone (Pfister 2004).

Pulmonary Hypertension

Pulmonary hypertension is associated with a pulmonary arterial pressure greater than 25 mm Hg. It is caused by severe obstruction in the pulmonary vessel wall, thrombosis, and vasoconstriction. It has been shown that the endothelial cell is changed during pulmonary hypertension. The cell is activated causing many cell factors to be released, among them MP's. Patients suffering from pulmonary hypertension will have a higher number of circulating MP's. The circulating MP's will release many coagulant factors, such as cytokines which induce thrombo-embolic conditions (Diehl et al 2011). The increased numbers may correlate to the structural damage in the vessel wall (Amabile et al 2008).In addition, PMP's may induce smooth muscle proliferation in the vessel wall and intima thickening, both contributing to pulmonary hypertension progression (Diehl et al 2011).

Scientists had believed that EMP levels have a direct effect on pulmonary arterial pressure as shown when measured by echocardiogram. However, it has since been proven that the echocardiogram severely limits the results: therefore it is suggested to use the catheter to better test for severity of arterial pressure. In a study involving 16 patients, scientists have shown that EMP's can be both a marker of dysfunction and a contributor to impairment of vascular function in pulmonary hypertension.

EMP's can interact with the endothelium and restrict NO synthesis. By doing so, EMP's act as a paracrine factor Inhibiting NO dependent vasodilation, leading to restriction in the blood flow when needed (Cody et al 1992). The disease will progress with more changes made to the arterial wall as a result. Furthermore, EMP release may have been caused by high-sensitivity C-reactive protein and LMP's showing that MP's have many different sources and reasons for release (Amabile et al 2008) Hypertriglyceridemia enhances the production of tumor-necrosis factor and C-reactive protein, molecules shown to affect EMP release (Ferrereira et al 2004).

It is interesting to note that annexin-V was not seen on the MP surface. The author theorized that the lack of apoptosis observed as well as data collection method are responsible for this lack (Amabile et al 2008. In addition, LMP's are known to be responsible for cell adhesion. However, the leukocyte counts were similar between the patients and the controls. The author theorized that functional status of leukocytes and not their absolute number was altered (Diehl et al 2011). For both experiments the patient pool was too small as well as too heterogeneous, more experimentation is needed.

Chronic Renal Failure

Cardiovascular disease is the main cause of death in patients with chronic renal failure. Endothelial dysfunction often occurs in the beginning stage of kidney failure. Blood pressure rises as glomerular filtration rate drops. However, Faure et al (2005) have found that there is no correlation between blood pressure, glomerular filtration and MP's. Scientists used an ultrasound to measure risk assessment. The study measured MP levels before and after dialysis. Results show that compared to controls, patients suffering from kidney disease had higher MP levels. Additionally, the study showed that uremic toxins had a positive correlation with the release of EMP's. Uremia affects the vascular structure in arterial stiffness and early stages of atheromatous disease (Dursan et al 2009).

Scientists injected frogs with uremic toxins from dialysis patients. Results showed that the frog's blood vessels were eight times more permeable after being injected by uremic toxins. An abstract was published in which (Adamson 1990) said permeability occurred with uremic toxins from healthy patients, but the authors were unable to reproduce the result. They believe that results would be considerably less in humans.

However, the situation can become a risk factor if the vessel wall becomes permeable to LDL's and albumin (Harper et al 2002). In addition, there was a 37% increase in EMP release in response to the release of indoxyl sulfate (a molecule in uraemic acid). Renal failure patients show excessive formation of MP, not inclusive to EMP. EMP and LMP levels remained constant before and after the dialysis session. However, PMP count was enhanced after the session. This may be as a result of mechanical stress on the platelets which stimulates MP shedding.

The uremic state of renal failure patients can cause endothelial dysfunction on its own. However, the MP's amplify the endothelial dysfunction already in place, such as the tissue factor and NO pathways along with vasorelaxation. Renal failure patients are dependent on the endothelium for a vasodilation response (NO synthase) and suffer decreased response to cytokines. Analysis has shown that patients without a history of vascular disease before suffering from renal failure have similar levels of MP's to those who had a past history.

P-cresol, a molecule known to induce endothelial dysfunction, which impairs endothelial barrier function and response to inflammatory cytokines, was induced in vitro to an endothelial cell culture. The p-cresol promoted the formation of MP's in the cells. It affected the endothelial cell skeleton on the Rho kinase pathway. Because the cytoskeleton is important to MP release, the author theorized that MP release was induced through this pathway, not as believed before by the apoptotic process. This is specific only for renal failure patients. Further evidence shows that endothelial activation is a response to an accumulation of many toxins, not only p-cresol and indoxyl sulfate. Evidence has shown uremia to be a risk factor for diabetes, however further research must be done (Faure et al 2005).

Obesity and Metabolic Syndrome

Obesity is an increasing problem among children and adults. It has been shown that obesity correlates to increased levels of clotting factors and increased coaguable state. Oxidative stress may affect the release of MPs. Obesity is a chronic state of oxidative stress and inflammation. Obesity is one of many factors involved in metabolic syndrome. Patients suffering from metabolic syndrome are commonly susceptible to endothelial dysfunction, triggering platelet aggregation with further risk in venous thrombus formation. In addition, patients are prone to resistance to insulin, low glucose tolerance, dyslipidemia, and hypertension (Nieuwdorp et al., 2005). Metabolic syndrome patients are also at increased risk for type 2 diabetes, cardiovascular disease, and mortality (Helal et al 2011).

In a study involving obese children, it was discovered that thrombin generation is increased. Obese children have a shorter lag time (exposure) and time to peak height (highest number of MPs), higher peak height, and higher thrombin potential. The study further revealed that obese children have a shorter MP release time, allowing the coagulation pathway to begin at a faster rate. The author hypothesized that the prothrombic state is founded in obesity. He furthers this hypothesis by stating that prothrombic state will accelerate to cardiovascular disease. Helal et al (2011) show that obesity causes a chronic state of oxidative stress and inflammation, leading to cardiovascular disease. In order to prevent cardiovascular disease, control of obesity should be done at an early stage. One theory suggests that the fat depots found in obese patients lead to MP overproduction. Furthering this line of thought, it was discovered that a high-fat meal induces the release of MP's. However, a fasting blood test, even in the metabolic syndrome patient did not correlate with the controls in other studies, showing that the high-fat meal affected the blood test more than metabolic syndrome in comparison between the patients and controls. However, the study only involved 18 subjects over a space of 2 days and more research must be done (Ferreria et al 2004).

Siklar et al (2011) found that insulin and glucose levels did not affect the MP levels in the patients. This may be as a result of the use of obese children as opposed to other findings in which they used overweight children and adults with type 2 diabetes. A study involving 88 metabolic syndrome patients not currently suffering from any other diseases or syndromes, tested for MP levels. It was found that Annexin-V, PMP, EMP, and LMP levels were significantly higher than controls. The hypothesis is that platelet, erythrocyte, and endothelial derived MP's were released in a general response and the monocyte derived MP's are released by more severe conditions, such as cardiovascular disease (Helal et al 2011). Arteaga et al (2006) conducted a study involving 33 patients with no previous history. He found that only the EMP levels were raised in the metabolic syndrome patient.

Biomarker

As previously stated, many diseases have higher levels of MP when compared to controls. In order to use microparticles as a tool, further studies must be done to accurately count the MP levels in the blood. As of now, methods are not completely developed (Kornek and Schuppan 2012). Current ways to count MP are: flow cytometry, fluorescent monoclonal antibodies, and two step differential centrifugation. Flow cytometry depends on membrane-specific antigens. The blood sample is incubated with an antibody for 30 minutes. At the end of the prescribed time, the sample is diluted and count beads are added to each sample according to internal standard (Faure et al 2006). A recent innovation adds different colors to the beads, allowing different phenotypes to be easily differentiated (Tramontano et al 2004). The sample is then counted by the flow cytometer. MP's are defined by their sum (>1μm) (Faure et al 2006).

Fluorescent monoclonal antibody count depends on antigen and antibody interactions. The specific antibody is labeled with a fluorescein isothiocyanate. The sample is then triggered by signals, and results are charted (Arteaga et al 2006). The final method is centrifugation. Most studies used a two-step centrifugation to get rid of cells that were previously treated. Time length and centrifuge speed differ based on authors preferences. The sample is then centrifuged a second

time at a faster speed and longer period of time in a microcentrifuge to isolate the MP pellet. The isolated MP pellet is then suspended in a buffered saline at one-tenth the original volume. The MP is then counted by flow cytometer (Ulal and Pitsetsky 2010). The different methods may be mixed and matched, as done by Helalet al(2011). The study used centrifugation, flurochrome technology, and flow cytometry to attempt to achieve as accurate a number as possible.

Pharmacology

Inflammation is a significant contributor to endothelial dysfunction and subsequently, artheroscelrosis. MP's promote endothelial dysfunction with the help of pro-inflammatory cellular adhesion molecules triggered by tumor necrosis factor. Tumor necrosis factor also activates the Rho kinase pathway. Statins are commonly used to treat artherosclerosis. They are known for their anti-inflammatory effects and LDL inhibition. Fluvastatin has been shown to reduce the synthesis of cellular adhesion molecules as well as inhibit Rho activation. While the exact mechanism is not yet understood, it is believed that Rho activation plays an important role in EMP release. In addition, Rho kinase is involved in cytoskeleton organization. By suppressing Rho kinase activation, actin cytoskeleton organization is also inhibited, leading to EMP inhibition as well (Tramontano et al., 2004). Another statin, Pravastatin has been shown to reduce the fibrinogen receptor on MPs in diabetes type 2 patients. Fibrinogen is an important aspect in thrombus growth. With repression of the receptor, thrombus growth can be lessened and cardiovascular events can be reduced (Baron et al 2012).

Conversely, in a study involving patients with chronic renal failure, statins did not show any decrease in MP levels when compared to controls. This may be a result of other factors in the patients, such as uremia levels (Faure et al 2006)

Oxidative stress has been shown to affect cardiovascular disease. Low levels of antioxidants (vitamin c) have been associated with inflammation and severity of disease. Vasospasms and neonintimal thickening after ballooning were improved after being treated with antioxidants. Morel et al (2003) conducted a placebo trial study with 61 myocardial infarction patients. Half of the group was placed on a vitamin C supplement for 5 days while others were given a placebo. Results were modest, a 10% reduction in overall MP count. However, the placebo group suffered an up to 44% increase in MP levels. It is believed that antioxidants prevent the ongoing process of MP shedding. This inhibition result was not observed in low-risk patients, perhaps due to the different nature of oxidative stress in the low-risk as opposed to high-risk patients. Additionally, vitamin C reduced cell apoptosis, with up to 70% decrease of EMP in diabetic patients. An early treatment of antioxidants can reduce redox reactions, leading to a decrease of cardiovascular disease (Morel et al 2003).

MP's can also be used as a treatment for bleeding disorders. In a trial run by Hrachovinova et al (2003), hemophilic mice were infused with P-selectins and immunoglobins. This procedure was done in vitro as well. This in turn initiated the release of tissue-factor containing MP's, causing coagulation to occur. Fibrin formation occurred at a faster rate after incubation with p-selectin and immunoglobins. This shows that hemophiliacs may have other treatments available to help in thrombus formation.

Conclusion

Microparticles have come a long way since discovered in 1969. They have been shown to be involved in many pathways of the human body, such as NO synthase and Rho kinase. Their main contribution lies in thrombus formation, helping the body recover from injury. However, in case of metabolic disturbance, the MP may become a pathogen to the patient. It will inhibit pathways needed and contribute to cardiovascular disease. Studies are currently being done to examine the exact pathway and correct the problem. As of now, certain medications have been discovered to help control MP levels in the blood. In addition, scientists are considering using MP levels as a biomarker. The MP levels can become a useful tool to predict cardiovascular and other diseases. MP is also emerging as a healing tool for hemophiliacs and other bleeding disorders.

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