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High fat diet effects on erythrophagocytosis and MCP-1 levels mice

A thesis submitted to the Graduate School of the University of Cincinnati in partial fulfillment of the requirements for the degree of

Master of Science

in the Department of Nutritional Sciences of the College of Allied Health

by

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ABSTRACT

Background: Atherosclerosis, characterized by the accumulation of lipid-laden plaques in vessel walls, is a known precursor to cardiovascular disease (CVD). Oxidation of cholesterol particles leads to endothelial damage and subsequent inflammation. Immune cells, such as monocytes, respond to chemokines and differentiate into macrophages, engulfing oxidized lipids, and forming foam cells within the vessel wall. Red blood cells (RBC) are known to bind chemokines through the Duffy antigen receptor for chemokines (DARC). RBCs may play a role in recruiting immune cells to lesions and contribute to the rupture of atherosclerotic plaques. Although exposure to a high fat diet (HFD) has been shown to alter RBC membrane properties, how this physiological change may affect RBC chemokine binding and macrophage interaction is less known.

Objective: To determine the effects of a high fat diet on murine RBC monocyte chemoattractant protein 1 (MCP-1) content and *ex vivo* murine macrophage erythrophagocytosis levels.

Methods: 6 week old C57BL/6 male mice were fed either chow diet (CD) as control (n=8) or a HFD (n=8) for 12 weeks. Body mass was measured upon arrival and at 18 weeks of age. Blood was collected via cardiac puncture and each sample divided into two treatments, one with heparin and one without for assessment of MCP-1 in total plasma and RBC bound. MCP-1 concentrations were measured by an ELISA for assessment of MCP-1 in total plasma and RBC bound. Erythrophagocytosis was determined *ex vivo* by exposing fluorescently labeled packed RBCs (pRBCs) to peritoneal macrophages harvested from C57BL/6 male mice fed a CD and measuring macrophage fluorescence.

Results: The HFD mice gained more weight over the 12 weeks than the CD mice (p < 0.01). Total plasma levels of MCP-1 increased in HFD mice compared to CD mice although the difference was not significant (p = 0.149). However, RBC bound MCP-1 levels increased significantly in HFD compared to CD (p < 0.05). Macrophage erythrophagocytosis was also 43% higher among the HFD mice as compared to the CD mice (p < 0.05).

Conclusion: The present study demonstrates that a high fat diet leads to increased likelihood of RBC clearance by macrophages, which could potentially contribute to inflammation. This change may be mediated by the altered RBC bound MCP-1 levels, although further work is necessary to determine cause and effect.

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Introduction

It is established that a high fat diet, more specifically one high in trans and saturated fatty acids, is associated with increased risk for atherosclerosis and cardiovascular disease (Bray et al., 2002; Calder et al., 2009; Nettleton et al., 2007; Salter, 2011). Atherosclerosis involves the accumulation of fat, cholesterol, and other debris along the arterial wall that leads to plaque formation. Plaque build-up hardens the arteries and decreases the vessel area limiting oxygenrich blood flow, stressing the cardiovascular system. The immune system responds to this condition by mounting an initial inflammatory response, with the goal of repairing the cellular damage. There is no true resolution given the perpetual nature of this condition, resulting in a chronic inflammatory state.

Immune and hormonal responses are recognized as having roles in the prevention of chronic inflammatory disease states. The role nutrition plays in inflammation continues to expand as more components of foods are found to possess pro or anti-inflammatory properties. For example, it has been shown that regular ingestion of certain foods can increase systemic inflammation, which can alter properties of adipocytes, immune cells, RBCs and even brain cells (Calder et al., 2009; Herder et al., 2009; Weisburg et al., 2003). These alterations have been linked to a number of diseases including stroke, heart disease, Alzheimer's disease, as well as certain cancers (Calder et al., 2009).

Literature Review

Immune system

Basic overview

The body's immune system functions to protect the body from foreign particles, such as microbes, that may invade and cause damage to cells and tissues. Immune system response to

infection or injury is crucial in deterring illness. An imbalance of immune response may lead to the development of some chronic diseases and as well as further spread of infection that may result from microbial invasion (Calder et al., 2009; Hansson et al., 2002; Henson & Hume, 2006).

When microbes invade and damage tissue, histamine is released by the damaged cells and blood flow to the surrounding area increases in order to deliver clotting factors and phagocytes. This is known as an acute inflammatory response. The result of this increased blood flow and immune cell movement into the tissue causes the surrounding bacteria, viruses, or apoptotic cells to be ingested by the phagocytes. This in turn limits tissue damage and enables clotting factors to signal platelet aggregation and seal the wound to prevent further invasion of bacteria (Hansson et al., 2002; Henson & Hume, 2006). The immune system is also able to recognize previous exposure to pathogens by increasing proliferation of the cells released to fight the initial exposure, which increases the level of circulating antibodies specific to previously encountered pathogen. Certain factors can result in a hypo functioning immune system while others may lead to a hyper functioning immune system. Balance of immune system regulation is key in the prevention of chronic inflammatory diseases and progression of infection.

Immune system components: adaptive versus innate

The immune system responds to injury or infection in two different ways, either by the innate or humoral/adaptive system. The adaptive system is cell-mediated, engaging both T and B-lymphocytes in antigen-specific responses that develop a memory from previous exposures to a particular pathogen. The innate system on the other hand, is rapid and non-specific, acting as the main mediator of response to injury or infection (Hansson et al., 2002; Henson & Hume,

2006). Inflammation associated with obesity and CVD primarily involve excessive (and often inappropriate) responses of the innate immune system (de Heredia et al., 2012). Similar to foreign invaders, as components of the diet accumulate in the body, phagocytes are recruited to eliminate them to maintain homeostasis. Excess dietary components such as fat, cholesterol, carbohydrates and even minerals such as calcium can threaten physiological functions as they buildup in the vessel walls and force storage in cells to a maximum. As phagocytic cells engulf and eliminate potentially harmful molecules, they release chemicals that can signal either the reduction of the inflammatory response or its continuation. As it is difficult to directly assess the immune cell activation within tissues, "biomarkers" of inflammation are often used to provide surrogate indicators of the body's overall "inflammatory state". Studies can focus on a specific biomarker of inflammation involved in these systems, measuring several different pro/anti-inflammatory agents.

Widely studied biomarkers of inflammation include protein molecules involved in cell signaling known as cytokines that are secreted by immune cells such as macrophages, lymphocytes, and leukocytes. Cytokines are small proteins synthesized and secreted by immune cells, involved in either the up regulation or down regulation of transcription factors that play a role in the inflammatory response (Calder et al., 2009). Interleukin-6 (IL-6), interleukin 1-beta (IL-1 β), tumor necrosis factor-alpha (TNF- α), and interferon-gamma (IFN- γ) are cytokines that have been identified as having a role in the inflammation process associated with obesity and CVD (Calder et al., 2009; Lionetti et al., 2009; Stapleton et al., 2008). Chemotactic cytokines or chemokines signal immune cells, recruiting them to an area in need of defense or "clean-up". MCP-1, a chemokine secreted by various cells including monocytes, endothelial cells, mast cells, and fibroblasts, will be discussed further (Schnabel et al., 2010).

Monocytes & macrophages

Monocytes are white blood cells produced in bone marrow that function as critical cells of the innate immune system (Ingersoll et al., 2011). Monocytes can be found circulating in the blood for immediate immune response or stored in the spleen for backup, when additional response to injury is necessary. Chemoattractants released by cells in need, activate monocytes, which then leave circulation by adhering to proteins on the surface of endothelial cells lining tissues like the blood vessel wall and transmigrating through the subendothelium (Libby, 2012). Once activated, monocytes have the ability to signal for additional monocyte recruitment and to differentiate into macrophages (Davies & Lloyd, 1989). Anchored monocytes undergo permanent differentiation in tissues resulting in macrophages that can more effectively phagocytose with enhanced ability to produce, release, and respond to several types of cytokines and chemokines (Zernecke & Weber 2010).

Immune Response

In response to excessive energy consumption, cellular stresses result in acute activation of the immune system, which can be beneficial in the short term to maintain homeostasis and limit potentially harmful effects on the body. However, continual excessive food/energy intake results in energy imbalances within the body leading to weight gain and possibly obesity. Chronic over feeding and obesity can result in over activation of the immune system resulting in prolonged & chronic deleterious responses throughout the body.

Activation of monocytes occurs through cytokine signaling as well as by presence of foreign and potentially harmful materials including microbes, apoptotic/necrotic cells, and even

low-density lipoproteins (LDLs) (Ley et al., 2011). Chemotactic response is gradient dependent, as the level of chemokines increases the rate of chemotaxis increases. Chemokines can be considered more potent as they act at a pg/mol concentration to be biologically active, compared to cytokines that act at μ g/mol. IFN- γ is a cytokine involved in both innate and adaptive immune response increasing macrophage activity and antigen presentation respectively (McGillicuddy et al., 2009). Tumor necrosis factor- alpha (TNF- α) is a cytokine produced primarily by macrophages known to induce inflammation and apoptosis (Sato et al., 1998). Abnormalities involving pro-inflammatory levels of these cytokines and their receptors can lead to activation of additional inflammatory mediators negatively affecting insulin, signaling, cell differentiation, lipid storage, and other metabolic pathways.

Monocytes are produced in bone marrow and are mainly stored in the spleen until they are needed. Some monocytes can be found circulating in the blood stream, ready to alert the immune system for recruitment if in the presence of a pathogen or area of injury by releasing chemokines (Swirski et al., 2009). Immune cells or monocytes in this case release chemokines, which target specific cells or an array of cells depending on their structure. Interleukin-8 (IL-8) and MCP-1 are notable mediators of inflammation. IL-8 is responsible mainly for neutrophil recruitment to a site requiring phagocytosis activity while MCP-1 is responsible for monocyte recruitment (Darbonne et al., 1991; Myhrtsad et al., 2011). As previously stated, several different cell types possess chemokine receptors and the need for chemokine signaling varies.

Inflammation

The inflammatory response is one of the first lines of defense of the immune system that functions to quickly achieve homeostasis of damaged tissues. The presence of invading particles

signals the release of chemical mediators that initiate immune cell recruitment to the affected area. Immune cells then release hormones or other proteins that aid in the destruction and subsequent clearance of the pathogens and begin repair processes for the damaged tissue. A normal inflammatory response, due to histamine release, presents clinically as redness, swelling, and pain due to increased blood flow and fluid to the affected area. This 'inflammation' serves a purpose to bring blood as well as immune cells (in the blood), nutrients, oxygen, and to remove waste. Importantly, negative feedback loops govern inflammation regulation in order to ensure it has resolved in a timely manner, after the threat is no longer present. Disruption of the regulatory processes, excessive tissue damage, and continual immune response can lead to the progression of chronic inflammation, perpetuating the disease state. Thus, it is important that this process is regulated. Negative feedback mechanisms involved in inflammation regulation include pro-inflammatory signaling cascade inhibition, anti-inflammatory cytokine secretion, and a decrease in inflammatory mediator receptors (Calder et al., 2009).

Regulation

Nuclear factor kappa beta (NF- κ B) is a redox sensitive transcription factor that increases transcription of inflammatory proteins (Tak & Firestein, 2001). Found in the cytoplasm in its inactive form, NF- κ B can become activated by exposure to pathogens and physiological stress (Chandel et al., 2000). Reactive oxygen species (ROS), tumor necrosis factor alpha (TNF- α), interleukin 1-beta (IL-1b) and lipopolysaccharides (LPS) are all known inducers of NF- κ B (Chandel et al., 2000; Cruz et al., 2007; Kim et al., 2012). NF- κ B increases expression of proinflammatory genes upon activation and has been found to be chronically active in several

inflammatory diseases including inflammatory bowel disease, asthma, psoriasis, rheumatoid arthritis, and atherosclerosis (Monaco et al., 2004; Tak & Firestein, 2001).

Role of inflammation in disease

The acute immune response results in the activation of a cascade of factors that increase the inflammatory response. However, with continual stimulation, the ability of immune responses to self-regulate begins to fail due to long-term activation of these "short term" mediators, resulting in sustained systemic inflammation. Continuously high levels of proinflammatory stimuli further activate immune cells to release pro-inflammatory cytokines in a feed forward mechanism to initiate phagocyte response and activate the endothelium. Transmembrane proteins are produced, allowing transmigration of immune cells into tissues via the blood vessel wall. A positive feedback loop is initiated as phagocytes arrive and continue to release pro-inflammatory cytokines in response to the present inflammatory stimuli (Lionetti et al., 2009). Endothelial cells chronically exposed to pro-inflammatory signals allow the accumulation of lipid-laden phagocytes (foam cells) within the blood vessel wall, which begins the formation of atherosclerotic plaques (Ingersoll et al., 2011).

Role of nutrition in inflammation

The condition of chronic overnutrition (obesity) is an inflammatory state. Chronic overnutrition leads to increased storage of fat in visceral adipose tissue, a fat depot that releases a multitude of cytokine and chemokine signals, thereby creating an ongoing state of inflammation (Lionetti et al., 2009). Notable resulting pathologies include: accumulation of ectopic fat, insulin resistance, endothelial dysfunction, and eventually diabetes (Bray et al., 2002; Calder et al.,

2009; Stapleton et al., 2008). Obesity develops from an energy imbalance caused by either excess energy intake, inadequate energy output or a combination of the two. Obesity is known to instigate an inflammatory response where secretion of excessive amounts of cytokines from adipose tissue induce a pro-inflammatory environment (Stapleton et al., 2008). Numerous signaling molecules contribute to this chronic low-grade inflammatory state associated with obesity (Calder et al., 2009). Energy imbalance leads to increased levels of circulating glucose and lipids that are eventually absorbed by the pancreas, liver, and adipose tissue in an attempt to maintain homeostasis.

A diet that limits pro-inflammatory food intake and promotes anti-inflammatory food intake can be beneficial, deterring diseases stemming from chronic inflammation (Nordmann et al., 2011). Specific nutritional components can play key roles in inflammation as foods rich in fatty acids and antioxidants are shown to influence systemic inflammation (Park et al., 2009; Galland, 2010). In terms of dietary fat and inflammation, *in vitro* studies show that cellular response depends on the type of fatty acid present, as omega-3 fats exhibit more anti-inflammatory effects, while other fatty acids (such as saturated and omega-6) exhibit more pro-inflammatory effects. Furthermore, lipid cholesterol influences membrane fluidity, having a negative impact on enzyme activity when at high levels.

Free radicals created from the inflammatory process can alter DNA, causing damage to cells and potentially death. When the body cannot detoxify the free radicals at a sufficient rate to balance the production, oxidative stress occurs, disturbing signaling mechanisms of cells. Antioxidants prevent the transfer of electrons from a molecule to an oxidizing agent also known as oxidation by sequestering the free radicals. Antioxidants found in foods have the ability to neutralize free radicals, thereby preventing damage to other cells and halting the signaling of

inflammatory responses (Calder et al., 2009). Many antioxidants are found in a variety of fruits, vegetables, and whole grains, including vitamins A, C, and E as well as minerals such as zinc and selenium (Calder et al., 2009; Chandra, 1997).

Obesity, inflammation, and cellular activation

Adipocytes begin to increase in size as they accumulate more lipid droplets (Neels & Olefsky, 2006). Their physiology changes, as evident by insulin resistance and altered levels of metabolites and adipokines (cytokines released by adipocytes) (Hursting & Dunlap, 2012). Recruitment of macrophages to the adipose tissue amplifies the inflammatory response as these cells release additional chemokines and cytokines contributing to cellular dysfunction and as well as oxidative stress. Insulin resistance results from these cytokines as well as free fatty acids leading to impaired fasting glucose and impaired glucose tolerance.

Human studies focusing on markers of inflammation have shown altered levels and function of immune cells (Alissa et al., 2006). Individuals with obesity and type 2 diabetes have been found to express higher circulating levels oxidative stress biomarkers (Boden, 2011). Overfeeding (positive energy balance) involved high fat content has also been associated with increased levels of oxidative stress and inflammatory markers (Samocha-Bonet et al., 2012). For example, in patients with metabolic syndrome, several markers of oxidative stress including H_2O_2 , lipid oxidase, and superoxide dismutase were elevated in postprandial serum after a 12 week intervention on high saturated fatty acid diet (Cruz-Teno et al., 2012).

High fat diet effects on inflammation and cellular activation

The role of fatty acids in inflammation varies by the type of fatty acids, which depends on their chemical structure. Saturated fatty acids contain hydrocarbon chains of single bonds, are solids at room temperature, and are found in whole-milk products, butter, several cheeses, and certain types of meats such as beef, pork, and veal. Unsaturated fatty acids contain multiple bonds, creating a kink in their structure causing them to be liquids at room temperature. Monounsaturated fatty acids (MUFAs) contain one double bond and can be found in olive oil and various nuts. Polyunsaturated fatty acids (PUFAs) contain more than one double or triple bond and are found in walnuts, soybeans, fish, and safflower oil. Fatty acids are metabolized endogenously, producing a variety of bioactive compounds including eicosanoids such as prostaglandins, leukotrienes, and thromboxanes (Hu et al., 2001).

Intake and subsequent metabolism of long chain omega-3 fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), leads to the synthesis of antiinflammatory compounds, reduces vasoconstriction and increases anticoagulation (Calder et al., 2009; Park et al., 2009). These beneficial effects provide a healthier vasculature thus decreasing the risk of CVD (Caspar-Bauguil et al., 2010; Galland, 2010). Omega-6 fatty acids produce proinflammatory compounds, which participate in inflammation-related diseases (Hu et al., 2001). Polyunsaturated fatty acid (PUFA) intake recommendation is often expressed as an omega-6/omega-3 ratio, as omega-6 and omega-3 fatty acids compete for the same enzymes in the metabolic pathway that converts them into their bioactive form (Caspar-Bauguil et al., 2010).

HFD directly impacts immune cell activation as shown by increased expression of the MCP-1 receptor, CCR2, in obese mice when compared to lean mice (Weisberg et al., 2006). This study also compared adipose tissue macrophage (ATM) and adipocyte size of obese mice

with and without CCR2 to determine whether ATM content was dependent upon the receptor. Mice that were CCR2 deficient indicated lower ATM content in obese mice. CCR2 deficient mice also showed a greater expression of several genes involved in inflammation including TNF- α , indicating that changes in adipose gene expression under obese conditions are CCR2 dependent. It has been shown in animal studies that mice deficient in both MCP-1 and lipoprotein lipase receptor (LDLr) and fed a western diet had significantly less atherosclerotic lesions when compared to mice deficient in LDLr (Rull et al., 2010). This study also showed that MCP-1 may contribute to cytokine gene expression in the aorta, as MCP-1 deficient mice showed limited aortic cytokine gene expression.

Red blood cells

Structure & function

Red blood cells or erythrocytes (from the Greek derivation meaning red hollow cells) are responsible for transporting oxygen and carbon dioxide to tissues throughout the body (Guest et al., 1963). RBCs are anucleate with a biconcave disc shape that increases the surface area, allowing for more rapid gas exchange to occur (Guest et al., 1963. The lipid bilayer of the RBC membrane allows for permeability, containing both phospholipids and cholesterol (Tziakas et al., 2010). Phosphatidylserine (PS) is found on the inner layer of the RBC membrane but can be moved to the outer layer by enzymes known as floppases (Daleke, 2007; Schroit et al., 1985), and then recognized by phagocytes for RBC clearance (Fens et al., 2012). PS is frequently used as a marker of oxidative stress; given that it is stimulated by reactive oxygen species (ROS), when at high levels can cause cell damage, triggering phagocytosis. RBCs lack several organelles, including a nucleus, to accommodate for maximum stores of the iron-containing

protein hemoglobin, which provides RBCs with red color as well as adenosine triphosphate (ATP), lipids, and carbonic anhydrase (Tziakas et al., 2010). However, this means that RBCs do not produce or secrete proteins such as cytokines or chemokines.

Hemoglobin contains four polypeptide chains (2 alpha globins and 2 beta globins) that are each linked to one heme molecule. One molecule of oxygen is able to bind to the one iron atom in heme therefore one hemoglobin molecule can transport up to four oxygen molecules at a time. Hemoglobin can also bind nitric oxide (NO) that is produced by endothelial cells found lining blood vessels (Martins et al., 2010). The enzyme nitric oxide synthase (NOS) catalyzes the conversion of L-arginine to L-citrulline and NO (Martins et al., 2010). NO contributes to the regulation of vascular smooth muscle relaxation as well as platelet aggregation and adhesion inhibition (Martins et al., 2010). Factors that inhibit this pathway including high fat diets and reactive oxygen species may promote atherosclerotic effects (Martins et al., 2010).

Formation

Erythropoiesis is the production of new RBCs that requires folate, vitamin B₁₂, and iron in adequate amounts (Koury & Ponka, 2004). All blood cells originate from stem cells, which undergo several mitotic divisions leading to the various types of blood cells. Immature red blood cells known as reticulocytes are formed after late erythroblasts lose their nuclei. These reticulocytes are released from bone marrow into circulation where ribosomes within the reticulocytes degrade to form mature RBCs. Erythropoiesis takes four days to complete and the typical life span of mature human RBCs is between 100-120 days (around 50 days for mice) with 1% of RBC replacement occurring daily (Ishikawa-Sekigami et al., 2006; Khandelwal et al., 2007). The glycoprotein erythropoietin is released from the kidneys to stimulate erythropoiesis

in red bone marrow when blood oxygen levels become low and as well to balance red blood cell clearance.

Metabolism

As RBC components (proteins, lipids, and enzymes) begin to degrade, the cells' ability to transport oxygen decreases, altering their shape, becoming more fragile and eventually phagocytized by cells of the spleen, liver, and bone marrow (Khandelwal et al., 2007). As RBCs age, components of their plasma membrane and proteins begin to degrade, since the ability to replace them is absent due to a lack of nuclei. This degradation sends out signals so that phagocytes can engulf RBCs for appropriate removal from the body and recycling of components such as iron, which takes place in the spleen (Ishikawa-Sekigami et al., 2006). RBCs release free radicals upon senescence. In an abnormal state, high RBC turnover can result in high levels of free radicals that if not resolved at the similar rate, may alter normal physiology.

Relationship with/effect on inflammation

RBCs are involved in NO metabolism, expressing endothelial NOS, which plays a role in NO synthesis (Kleinbongard et al., 2006; Martins et al., 2010). The Martins et al. (2010) study found that olive oil (consisting mainly of monounsaturated fatty acids known as MUFAs) was associated with an up-regulation of NOS enzymes with amino acids (namely L-arginine, involved in NO synthesis) in RBCs, ultimately leading to cardioprotective effects. PS location (either on the inner or outer RBC membrane layer) can determine the cell function and status depending on floppase activity. Phagocytes, such as macrophages, are able to recognize PS when it is located on the outer surface of the RBC (Beppu et al., 1994). RBC phagocytosis by

macrophages results in the release of the RBC contents including iron and free cholesterol. This action can result in free radical formation, which can lead to damage to surrounding tissues and increased inflammation. Inflammatory response may be exacerbated when lipid-laden blood cells signal apoptosis of RBCs, releasing free cholesterol near atherosclerotic lesions thereby contributing to plaque buildup on the arterial walls and increasing tissue damage with the release of free radicals.

DARC

As previously mentioned, there are receptors for chemokines located on the RBC membrane. These are receptors recognize chemokines such as IL-8 and MCP-1. DARC has been identified as a glycoprotein found on the surface of RBCs that serves as a nonspecific receptor, with the ability to bind various chemokines shown by Neote et al. (1993, 1994) using radioactively labeled chemokine from different classes, measuring binds to RBC surface receptors. This study suggests that DARC works as a chemokine clearance receptor in blood and other surrounding tissues. Darbonne et al. (1991) explains that IL-8 cannot communicate with its targets (neutrophils) to induce their migration once it is bound to DARC on RBCs. Consequently, neutrophil and IL-8 interaction is limited by the RBC DARC binding site, inhibiting inflammatory response.

Fat intake and high fat diet effects on RBCs

RBCs appear to influence communication between chemokines and immune cells. High fat diets have an important role in the inflammatory response given that these diets may alter RBCs in a way that increases affinity for chemokine binding to its DARC. Further studies

should be conducted comparing chronic inflammatory disease states focusing on diet, biochemical changes of the RBC, as well as chemokine levels.

Lipid-laden membranes of RBCs can contribute to cholesterol build up in atherosclerotic plaques (Tziakas et al., 2010). This build up could lead to a change in RBC shape, affecting its function. As RBCs are disposed of during senescence, they release cholesterol that was being held by their membranes. This free cholesterol accumulation can lead to plaque vulnerability during atherosclerotic lesion progression and possibly contribute to rupture (Tziakas et al., 2010). Iron can also accumulate within this plaque due to RBC breakdown, which can catalyze free oxygen radical formation, leading to tissue damage, inflammatory response and plaque rupture. Several receptors located on the RBC membranes are known to promote the inflammatory process. Pro-inflammatory chemokines bind to these receptors triggering monocyte movement from the blood to nearby tissues where they will then act as macrophages, phagocytizing the toxic contents.

Influence of fat type on RBCs

The intake of trans and saturated (pro-atherogenic) fatty acids can lead to their accumulation within the RBC membrane. This excess of cholesterol can directly initiate morphologic changes as the RBC begins to breakdown due to fragility. This morphology can cause the RBCs to lyse and release of several pro-inflammatory and oxidative stress inducing components.

Low levels of certain trans-fatty acids in RBCs along with increased vitamin (those involved in RBC formation and oxygen transport) and omega-3 fatty acid intake has been associated with decreased risk of atherosclerosis (Park et al., 2009). Caspar-Bauguil et al. (2010)

compared RBC membrane fatty acid composition in patients with acute coronary syndrome before and after nutritional and lifestyle education. Following the healthy lifestyle adjustments, patients had a decreased omega-6/omega-3 ratio of erythrocyte phospholipids suggesting potential for anti-inflammatory and anti-atherosclerotic effects with greater intake of omega-3 fatty acids and limited intake of omega-6 fatty acids. Since RBCs possess no nucleus and therefore no DNA, genetic factors are not anticipated as direct contributors to any physiological change that the RBCs membranes may undergo. The authors suggested that RBC phospholipid fatty acid composition might be used to evaluate compliance of dietary recommendations to patients. This evaluation would be helpful to CVD, diabetic, obese, and other inflammatory disease patients to observe efficacy of a new recommendation or therapy per the individual.

RBC role in immune cell function

Implications

Red blood cells (RBCs) are present in vast quantities and potentially play an important role in immune cell function as more studies have specifically investigated the DARC role because of chemokine binding. Mediators of inflammation such as MCP-1 have been shown to increase in the state of overnutrition leading to a prolonged inflammatory response. MCP-1 signals monocyte/macrophage recruitment for clearance of potentially damaging cells such as lipid-laden RBCs, eventually leading to the formation of foam cells. Foam cells can buildup in the vasculature forming plaques, which have the potential to rupture, contributing to atherosclerosis. RBCs have been previously dismissed as having any significant role in the inflammation process largely due to the length of their lifespan and the fact that they are without a nucleus, harboring no DNA or genetic factors, reducing their ability to acutely respond to

inflammatory stimuli. However RBCs may execute a small, yet possibly substantial role in the inflammatory response to diet.

Research question: How does a high fat diet affect erythrophagocytosis and RBC MCP-1 levels in mice?

Hypothesis: The high fat diet induced obese state of mice will result in an increase in both erythrophagocytosis and RBC MCP-1 levels.

Methods

Animals and diets

C57BL/6 male mice obtained from Jackson Laboratory were housed 4 to a cage and kept under standard conditions (12 h light/dark cycles and temperature of 23 ± 2 °C in a controlled animal care facility at the University of Cincinnati. Mice were fed chow diet (CD) control (n=8) or 60% high fat diet (HFD), (n=8) group for 11 weeks, previously fed CD (Research Diets, D12450B, New Brunswick, NJ) or 60% HFD (Research Diets, D12492, New Brunswick, NJ) at Jackson Laboratory. Mice were received at 17 weeks of age allowed to acclimate for 1 week, to account for possible effects of transportation stress, while continuing their respective diets prior to sacrifice. Water and food were available *ad libitum*. Body mass was measured upon arrival and on the day of sacrifice. Eight mice were dedicated to MCP-1 data collection and the remaining eight dedicated to erythrophagocytosis data collection. Diet composition of the CD and HFD is given in Table 1.

	Diets	
Nutrient (kcal% diet)	10% CD	60% HFD
Protein	20	20
Carbohydrate	70	20
Fat	10	60
Corn Starch	1260	0
Maltodextrin 10	140	500
Sucrose	1400	275.2
Soybean Oil	225	225
Lard	180	2205
Cholesterol (mg/total kcal)	19	232.8
Research Diets, 2006		
CD (control diet) D12450B		
HFD (high fat diet) D12492		

Table 1. Diet Composition of CD and HFD

Cells

Blood from the mice was collected via cardiac puncture and immediately divided equally into two EDTA lined tubes (one for 50 U/mL heparin treatments and one without heparin treatment) for each mouse. Tubes were kept on ice and periodically inverted during two-hour incubation. Hematocrits were recorded and samples were then centrifuged at 1020 x g for 20 minutes at 4°C. Plasma was removed, placed in separate microcentrifuge tubes, and stored at -80°C until analyzed. Packed RBCs (pRBCs) were resuspended in supplemented media (described below) and then labeled using Calcein AM fluorescent dye at 1.0 µM. All pRBCs and plasma samples were kept on ice for same-day analysis.

Peritoneal macrophages were harvested from C57BL/6 male mice fed a standard chow diet. Macrophages were recruited to the peritoneal cavity using thioglycolate and harvested after four days. Cells were seeded in T75 flasks overnight using DMEM media supplemented with 20% fetal bovine serum (FBS) and kept under normal cell culture conditions, 5% CO₂ at 37°C.

Non-adherent cells were removed with washing and the remaining cells were gently dislodged, counted, and seeded evenly in 24 well plates.

Assays

MCP-1 concentrations in plasma were determined using an ELISA DuoSet kit (R&D Systems, Minneapolis, MN). For *ex vivo* erythrophagocytosis, Calcein AM labeled pRBC samples were pooled two per diet treatment (CD and HFD) and added to the seeded peritoneal macrophages seeded in 24-well plates and incubated for four hours at room temperature. Cells were then washed with PBS and RBC lysis buffer to remove any unbound RBCs. Triton X was used to lyse the macrophages and resulting fluorescence was measured.

Data analysis

Body weight, MCP-1, and erythrophagocytosis data was analyzed using the independent t-test. P values < 0.05 were considered significant. Statistical analyses were carried out using SPSS 21. RBC MCP-1 levels were determined by normalizing samples using the non-heparin treated MCP-1 levels; to determine (by difference) the MCP-1 content released from RBCs following treatment with heparin, a DARC competitor.

Results

Body weight

HFD mice (n=8) gained significantly more weight (p=0.006) when compared with the CD mice (n=8) over the 12 week diet duration (Table 2). The weight gain reported after 12 weeks is consistent with the ranges provided by Jackson Laboratory for the given diet types.

MCP-1

Each sample analyzed for MCP-1 concentration was treated with heparin and without heparin. The non-heparin treatment is indicative of basal plasma levels of MCP-1. The addition of heparin to whole blood upon collection releases MCP-1 bound to DARC on the RBCs, as heparin is known to compete with MCP-1 for DARC binding. Therefore, heparin treatment represents both MCP-1 free in the plasma as well as RBC bound MCP-1. Although total MCP-1 levels were increased by 41% in HFD mice compared to the CD mice, the difference was only a trend (p=0.149) and not statistically significant (Figure 1). However, the difference in MCP-1 levels between heparin treated and non-heparin treated plasma samples is due to RBC MCP-1 content. Following adjustment of the heparin treatment MCP-1 levels for the basal plasma MCP-1 levels (non-heparin treated levels), the difference between diet groups of the RBC MCP-1 content was also assessed and the RBC MCP-1 levels between the two groups was significantly different, as HFD RBC MCP-1 level was significantly higher (p=0.023) than the CD RBC MCP-1 levels.

Erythrophagocytosis

Peritoneal macrophage engulfment of HFD pRBCs (packed red blood cells) was 43% higher (p=0.027) than CD pRBC engulfment (Table 2).

	Body Weight (g)	MCP-1 (pg/mL)	Erythrophagocytosis (fold change)		
n	8	4	4		
CD mice	31.3 ± 1.2	46.05 ± 18.8	1.00 ± 0.03		
HFD mice	36.6 ± 3.9	64.75 ± 12.49	1.43 ± 0.22		
p value	0.006	0.149	0.027		
Data are mean ± standard error. Chow diet (CD); High fat diet (HFD). Body weight measured in grams, at time					
of sacrifice. Monocyte chemoattractant protein 1 (MCP-1) levels of heparin treated plasma expressed in					
pg/mL. Erythrophagocytosis by cultured peritoneal macrophages harvested from chow fed mice					

Table 2. Analysis after 12-week diet duration



Figure 1. Heparin-induced release of MCP-1 in plasma of mice fed CD versus HFD for 12 weeks (n=4).

Data are expressed as mean \pm SD. Chow diet (CD); High fat diet (HFD). Monocyte chemoattractant protein 1 (MCP-1) levels of heparin treated versus no treatment of plasma expressed in pg/mL. Heparin treatment of CD versus HFD: p = 0.149.



Figure 2. Comparison of mouse RBC bound MCP-1 levels of CD versus HFD groups (n=4). Data are presented as mean \pm SD. Chow diet (CD); High fat diet (HFD). Monocyte chemoattractant protein 1 (MCP-1) levels of heparin treated plasma expressed in pg/mL. HFD versus CD: *p = 0.023.

Discussion

Altered chemokine binding affecting recruitment

Total plasma MCP-1 (without heparin treatment) was unchanged; however, the level of RBC MCP-1 was significantly increased in the HFD compared to the CD. Increased levels of RBC MCP-1 were observed with high fat feeding indicating that there was more MCP-1 present in circulation that was picked up by RBCs. The increased total MCP-1 levels and presumed subsequent binding to RBC DARC in a high fat fed state may be the result of increased systemic or adipose tissue inflammation. HFD RBCs in this state are therefore likely to be more pro-inflammatory and could contribute to atherosclerosis. These preliminary results verify the increasing atherogenic potential of these HFD RBCs by the fact that HFD RBCs contained significantly high amounts of MCP-1 and that erythrophagocytosis by macrophages (immune cells) increased by 43%. HFD RBCs thus appear to be altered in a way that makes them more susceptible to be cleared from the system. Increased engulfment of HFD RBCs could lead to

increased lipid accumulation in macrophages and subsequent foam cell formation, which has been shown to negatively impact vascular properties (Ingersoll et al., 2001; Ley et al., 2011; Tabas, 2005). Foam cell accumulation in the periphery is thought to be a key player in the development of atherosclerotic plaques. Once these plaques have formed, their stability becomes crucial, as plaque cell rupture may contribute to a hemorrhage. Phagocytes are signaled from nearby areas as well as unanticipated areas in an attempt to swiftly and efficiently remove the contents that have potential initiate endothelial dysfunction. Therefore, plaque formation and subsequent stability status may have more adverse effects than previously thought in terms of plaque hemorrhages.

It should be noted that DARC is a nonspecific receptor, resulting in competitive binding between several other chemokines in addition to MCP-1 (Schnabel et al., 2010). MCP-1 may be a key, yet not exclusive marker of RBC inflammation during a high fat fed state thus, other chemokines may also contribute. However, when only considering MCP-1, as MCP-1 levels increase, the level of monocyte attraction will likely increase. MCP-1 bound to the DARC on RBCs attracts monocytes to RBCs specifically, wherever they may be. Increased chemokine binding to RBCs in a high fat fed state can enhance recruitment of phagocytic cells to a point of excess, increasing RBC uptake, and clearance, exacerbating the inflammatory response. A large response of macrophages may hinder their ability to initiate their own clearance resulting in accumulation, increased differentiation to foam cells, and plaque formation.

Under high fat feeding conditions, excess chemokines in circulation result in increased RBC MCP-1 content, which are then exposed to immune cells in the blood filtering organs (splenic monocytes/macrophages), potentially stimulating an inflammatory response from them. RBC can become loaded with chemokines, altering splenic macrophages by exposure to a high

inflammatory state stimulating continual phagocytosis, leading to chronic inflammation with the potential for atherosclerosis. Clotting has also been shown to release MCP-1 from DARC (Schnabel et al., 2010). In a disease state with clotting in the vasculature, additional or enhanced macrophage recruitment would be expected. Plaques in capillaries filled with RBCs may become destabilized as lesions accumulate the RBCs. Clots may form with potential to rupture, releasing more MCP-1 and attracting more pro-inflammatory immune cells into the vessel wall.

Morphology, oxidative stress, fragility of RBCs

Macrophages engulf RBCs as they become damaged because they are potentially harmful if they should lyse in circulation. As mentioned previously, erythrophagocytosis is a normal process to prevent any future harm to the body. As RBCs are the most abundant cell in the body, factors affecting RBC metabolism, such as fat intake (based on either frequency or duration of exposure) may contribute significantly to atherosclerosis due to the sheer number of RBCs in the body. As the most abundant cell in the circulation by far, RBCs have the potential to cause damage and dysregulation throughout the vasculature. Increased phagocytosis due to uncontrolled immune response under disease conditions can lead to chronic immune cell activation and inflammation. This study utilized a 12-week HFD exposure to determine the effects on erythrophagocytosis. Increased engulfment of HFD RBCs by peritoneal macrophages suggests that some change involving the RBC in response to exposure to the HFD and the amount of dietary fat introduced triggers this uptake. The altered lipid and cholesterol levels between high fat and low fat diets have been shown to increase oxidative stress, which is associated with engulfment (Martins et al., 2010). Altered morphology and increased fragility

of the RBCs induced by a high fat diet may be the cause of the increased erythrophagocytosis seen in this study.

Conclusion

A high fat diet leads to increased RBC MCP-1 levels and increased RBC uptake by macrophages. These conditions may promote a pro-inflammatory state in the vasculature and in turn, a more atherogenic state that may lead to cardiovascular disease.

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