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Maternal low protein diet leads to placental angiogenic compensation via dysregulated M1/M2 macrophages and TNFα expression in Sprague-Dawley rats

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ABSTRACT

A maternal low-protein (LP) diet in Sprague-Dawley rats results in low birth weight, rapid adipose tissue catch-up growth, adult obesity, and insulin resistance. The placenta functions to fulfill the fetus' nutrient demands. Adequate angiogenic factor concentrations help to ensure normal growth and vasculature development of the placenta and, in turn, optimum maternal-to-fetal nutrient delivery. Maternal malnutrition creates a proinflammatory environment that leads to inhibition of placental tissue growth. Therefore, we hypothesized that a maternal LP diet will lead to abnormal angiogenesis via dysregulation of immune cells resulting in increased secretion of proinflammatory cytokines and reduced angiogenic factor expression. Sprague-Dawley dams were fed 8% LP or 20% normal protein diets for 3 weeks prior to breeding and throughout pregnancy. Placenta from dams fed a LP diet weighed less; had increased M2 macrophages producing TNFα, decreased M1 macrophages and iNKT cells; greater angiogenic factor (FGF2, VEGFR-1, IGF2) expression and protein content, and greater CD31/PECAM (platelet endothelial cell adhesion molecule) expression. Prenatal protein restriction may induce the placenta to upregulate compensatory mechanisms of angiogenesis in order to meet the nutrient demands of the fetus.
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1. Introduction

We have shown that a maternal low protein (LP) diet reduces the birth weight of male offspring which leads to rapid adipose tissue growth, adult-onset weight gain, and an increased risk of insulin resistance (Claycombe et al., 2015b; Claycombe et al., 2013a). Normal development of the fetus resulting in normal birthweight depends on adequate placental function (Anderson et al., 2005; Ouyang et al., 2013a; Murphy et al., 2006). Throughout gestation, the placental size gradually increases and is correlated with nutrient transfer capacity, which is especially important in the third trimester as this is the developmental period of the most rapid fetal growth. Placental dysfunction due to an increase in inflammation and/or immune dysfunction could lead to placental cell injury (Ouyang et al., 2013b). However, it is not yet known whether a maternal LP diet affects placental immune function and inflammatory responses and whether this is connected with reduced birth weight.

Pregnancy is considered an inflammatory state due to the activation of maternal leukocytes and increased acute phase reactants (*i.e.* C-reactive protein, complement factors, serum amyloid A) and proinflammatory cytokines (Brown et al., 2014; Challier et al., 2008; Hauguel-De Mouzon and Guerre-Millo, 2006). Macrophages are one of the major immune cell types that regulate the inflammatory response during pregnancy (Nagamatsu and Schust, 2010). Placental macrophages originate from both fetal (Hofbauer cells from fetal chorionic villi) as well as from maternal sources (decidual macrophages of the maternal decidua basalis) (Pinhal-Enfield et al., 2012).

Macrophages differentiate from monocytes that infiltrate the placental tissue from circulation. These cells activate into two major subphenotypes based on their local environment (Brown et al., 2014; Nagamatsu and Schust, 2010). M1 type (proinflammatory)

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macrophages are polarized by toll-like receptor 4 agonists such as lipopolysaccharide, IFNγ, TNFα, and GM-CSF. M1 macrophages secrete proinflammatory cytokines such as IL-12, IL-23, TNFα, and reactive oxygen species. Conversely, macrophages that are stimulated with IL-4, IL-10, IL-13, IL-33, TGFβ and/or M-CSF will form M2 type (anti-inflammatory) macrophages with an increased expression of mannose and scavenger receptors and produce anti-inflammatory cytokines such as IL-10 and TGFβ (Brown et al., 2014). M2 macrophages have a wide range of functions, so they have been further subdivided into M2a (IL-4 and IL-13 induced), M2b (exposed to immune complexes and toll-like receptor agonists), and M2c (IL-10 and glucocorticoid hormone induced) (Brown et al., 2014; Mantovani et al., 2004). M2a cells exhibit the alternatively active phenotype typically attributed to M2 cells. M2b cells have a Type II phenotype as they promote T-cell helper 2 responses and are able to produce both pro- and anti-inflammatory cytokines such as, TNFα, IL-1, IL-6, IL-10^{high}, and IL-12^{low}. M2c cells produce extracellular matrix components, IL-10, and TGF-β (Brown et al., 2014; Mantovani et al., 2004).

In addition to macrophages being key players in inflammatory processes, they are also important in promoting angiogenesis by producing pro-angiogenic cytokines such as TNFα and growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor 2 (FGF2) (Jetten et al., 2014). These pro-angiogenic properties are not shared by all subsets of macrophages as these angiogenic factors are more highly expressed by M2 rather than M1 macrophages. The ability of the M2 macrophages to express these pro-angiogenic factors provides them the ability to mediate each phase of the angiogenic cascade, including endothelial cell proliferation and migration and formation of vascular sprouts (Jetten et al., 2014). Taken together, we hypothesize that a maternal LP diet leads to reduced placental tissue growth due to a dysregulation of growth factors and M1 and M2 macrophage populations, and an increase in proinflammatory cytokines that promote angiogenic factors and compensatory angiogenesis to try to supply the fetuses with adequate nutrition.

2. Methods

2.1. Study design and animals

Obese-prone Sprague-Dawley female rats (Charles River, Wilmington, MA) were fed an AIN93-based diet containing either 8% (low protein, LP) or 20% protein (normal protein, NP) starting 2 weeks prior to breeding and throughout pregnancy (Claycombe et al., 2015a; Claycombe et al., 2013b; Claycombe et al., 2015b). Male breeders were fed a normal chow diet. Placentas were harvested at day 19 or day 20 of gestation, which is late in the 3rd trimester when the greatest fetal growth occurs (Baumrucker and Stover, 1987). Dam euthanasia was by CO_2 inhalation according to animal use and care protocol approved by the USDA ARS Animal Care and Use Committee guidelines. Harvested placental tissues were either immediately frozen in liquid nitrogen or single-cell suspensions were prepared.

2.2. Tissue collection and flow cytometry

Single cell suspensions were prepared from placenta or spleen by mashing through a 100 μm sieve and a plunger from a 3 ml syringe in complete media (RPMI, 10% fetal bovine serum, 1% L-glutamine, 10 mM HEPES, 1x penicillin/streptomycin, 0.1% beta-mercaptoethanol). These single cell suspensions of placental cells, (Hauguel-De Mouzon and Guerre-Millo, 2006) from the stromal vascular fraction (SVF) or splenocytes were treated with one or two successive RBC lysis (Sigma-Aldrich, St. Louis, MO) treatments and filtering through a 100 μm sieve. Subcutaneous adipose tissue was minced and digested using collagenase type I (Sigma Aldrich, St. Louis, MO) at 37 °C, shaking for 1 h. Adipose tissue cells were filtered using 100 μm nylon cell strainers (Fisher, Pittsburgh, PA). The subcutaneous adipose SVF cells were separated from floating primary adipocytes by centrifugation (500 × *g*, 5 min). SVF cell pellet was treated with RBC lysis buffer for 5 min at room temperature, quenched with DMEM + 10% FBS and centrifuged. Splenocytes, subcutaneous SVFs or placental SVFs were stained with monoclonal antibodies for surface markers and intracellular markers for flow cytometry as stated in the supplemental methods.

2.3. Real-time qPCR

Total RNA was extracted using the RNeasy Lipid Tissue Mini kit and Qiacube (Qiagen, Valencia, CA) from whole, flash-frozen placental tissue or from single cell suspensions (as described above) of placental SVF cells, splenocytes and subcutaneous adipose SVF cells or splenocytes. Collected mRNA was converted to cDNA using the Quantitect Reverse Transcriptase kit (Qiagen, Valencia, CA) and then used to measure expression of genes in Supplemental Table S1 by qPCR (ABI Prism 7500 PCR System, Applied Biosystems, Foster City, CA). FastStart Universal Probe Master mix assay reagents were purchased from Roche (Indianapolis, IN). Primers were purchased from Integrated DNA Technology (IDT, Coralville, IA). The endogenous control (18S rRNA) was purchased from Applied Biosystems.

2.4. Western immunoblotting

Protein (25 μg) from isolated placenta tissue was suspended in 2.5 μl of loading sample buffer and 1 M DTT and run by electrophoresis on a 10% Bis-Tris gel (Life Technologies). Protein was transferred to a PVDF membrane, washed, and hybridized with primary FGF-basic antibody or primary VEGF antibody and primary αTubulin antibody as a loading reference (Abcam, Cambridge, MA) at 4 °C overnight. The membrane was washed with 1X PBS containing 0.1% Tween and hybridized with secondary antibody for 1 h in 0.1% Tween and 0.01% SDS buffer. Images were developed using an Odyssey Infrared Imager (Li-Cor Biosciences Inc., Lincoln, NE).

2.5. Immunohistochemistry

Placenta tissue was collected and immediately frozen in liquid nitrogen prior to cryosectioning at 10 μm thickness. Sections were air dried and post-fixed in 3.7% paraformaldehyde in phosphate buffered saline (PBS) for 10 min at room temperature (RT). For standard histological staining, slides were stained with Hematoxylin Solution Gill #2 (Sigma-Aldrich, St. Louis, MO) for 2 min, run through tap water and 0.05% Eosin Y in acidified alcohol solution (Sigma) for 1 min each, passed through an alcohol dehydration series prior to a 2 × 2-min exposure to xylene and then mounted with Permount™ solution (Vector Labs, Burlingame, CA). For immunolabeling, sections were blocked with 3% donkey serum, 2% bovine serum albumin, and 0.1% TritonX-100 in PBS overnight at 4° C in a moist chamber. Sections were labeled with CD31 antibody directly conjugated to phycoerythrin (Novusbio Inc., Littleton, CO; 10 μg/ml) and 4′,6-diamidino-2-phenylindole (DAPI, Sigma; 2 μg/ml) diluted in block solution for one hour at RT then washed 3 × 10 min with PBS and mounted with Vectashield mounting medium (Vector Labs). Stained sections were visualized using brightfield or fluorescent microscopy as appropriate on an Olympus BX51WI fluorescent scope and images processed using Adobe Photoshop (Version CS6 Extended, Adobe Systems, Incorporated) with scale bar magnifications listed in the figure legends.