Unique Population of Immune Cells in Adipose Tissue after Gastric Bypass Surgery in Mice

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KEYWORDS. Roux-en-Y gastric bypass (RYGB), white adipose tissue (WAT), metabolism, macrophage, inflammation, apoptosis, immune cell

BRIEF. This study was an attempt to establish a link between roux-en-Y gastric bypass (RYGB), a procedure that reroutes one's intestinal track so as to induce weight loss through decreased food consumption, in mice and improved metabolism. Although the study was not powered to detect a statistical significance in inflammation, a novel immune cell was discovered.

ABSTRACT. The objective of this study was to establish a link between RYGB and improved metabolism. The key question is how RYGB improves metabolism even before weight loss. We know that weight loss leads to a decrease in white adipose tissue (WAT) in mice. Our hypothesis was that changes in WAT macrophage content reduce WAT inflammation and that apoptosis of WAT macrophages may be the mechanism by which they are reduced. The first goal of the study was to confirm whether WAT macrophage levels had decreased in mice that underwent the RYGB procedure. Next, we had to demonstrate whether inflammation had indeed been reduced as well. Lastly, we had to show if this in turn happened because of apoptosis. We used a 2-part method to do this – the first part was gene expression, which showed whether the amount of transcripts for genes of interest had changed. This would show whether macrophages, inflammation, and cell death had increased. While we were unable to show that RYGB impacted WAT macrophage or inflammation levels, out finding of a new population of unidentified granular cells is exciting. The fact that they only appear in tissue of mice that underwent RYGB presents an opportunity for concrete future directions in this study.

INTRODUCTION.

The world has seen a disconcerting increase in the prevalence of obesity in the last three decades, indiscriminate of ethnicity, race, and socioeconomic status [1, 2]. Obesity is closely positively correlated to chronic subclinical inflammation, which has been implicated in many obesity-related diseases [2, 3]. Key to the inflammation hypothesis is the role adipose tissue macrophages (ATM) play in affecting the inflammatory response to over-nutrition and overeating [1, 4]. The expansion of white adipose tissue (WAT) is associated with the progressive infiltration of macrophages in human and mouse models of obesity [5-7]. In this context, ATMs have been shown to heavily influence WAT function and chronic insulin resistance (IR) in obesity [5-8]. Apoptosis and efferocytosis are key mechanisms to this study as well, because an increase in the occurrence of the two reflects a decrease in inflammation and macrophage levels, indicating improved metabolism.

Current solutions for obesity and its associated metabolic effects (i.e. Type 2 Diabetes) are limited. Lifestyle changes and drug therapy are generally preferred over surgical solutions but are not nearly as effective and have been shown to be relatively unsuccessful. Bariatric surgery, though not a perfect solution, remains the most effective therapy in terms of inducing and maintaining weight loss. Over the past 5 years, scientific inquiry has shown that diabetic patients who receive RYGB start to show normal glucose levels and no longer need diabetes medications [1, 4]. Specifically, RYGB is one of the most commonly used bariatric surgical procedures in the United States [4]. RYGB is a procedure that reroutes the intestinal track so as to induce reduced food consumption. As a result, the patient consumes less and feels "full" faster, effectively inducing weight loss [7, 4]. An intriguing aspect of RYGB is its near-immediate influence on glucose tolerance and insulin sensitivity, as soon as 24 hours post-surgery, far before any weight loss is seen [4]. The reason for this sudden return to a normal glycemic state is unknown. Currently, there are no other surgical solutions for diabetes [1, 4]. RYGB comes closest to solving the problem, but still requires testing and studying; this project is one of those studies. The objective of this study was to establish a link between RYGB in mice and the anticipated improved metabolism in those mice for reasons other than sudden weight loss. This project is innovative in that the specific gene networks analyzed in this study have never been studied before, and that a unique, granular immune cell was discovered. Although its function or significance is unknown, it presents an exciting potential foray into the various effects of the RYGB procedure and future studies.

MATERIALS AND METHODS.

Experimental Design.

WAT tissue was taken from RYGB, Sham, and Sham Pair-Fed mice. The pair-fed group was used as a way to look at weight loss separate from surgery; in other words we wanted to see whether changes in metabolism were functions of direct weight loss or other factors that RYGB alone could induce. Four images each were taken from 3 mice per group to analyze the results.

Animals.

All animal experiments were conducted according to the rules and regulations of the Vanderbilt IACUC. Eight-week-old C57BL6 mice were purchased from Jackson Labs and placed on a high-fat diet – 60% kcal from fat (HFD). After 8 weeks of HFD feeding, mice underwent either RYGB or a sham operation as described (1). After a 1-week recovery period, half of the mice receiving the sham operation were pair-fed along the RYGB mice for the subsequent 3 weeks when mice were euthanized by Isoflurane overdose and cervical dislocation. Body composition throughout the feeding period was measured by nuclear magnetic resonance (NMR) in the Vanderbilt MMPC on the Bruker Minispec. NMR uses a magnetic field to absorb and re-emit electromagnetic radiation and masses are calculated using corresponding frequencies. Total lean tissue mass and adipose tissue mass are reported in grams. The mice were perfused with 1X Phosphate Buffered Saline solution (1xPBS), and tissues of interest were either fixed in 10% formalin or snap-frozen in liquid nitrogen and stored at -80° C.

Realtime RT-PCR (Reverse-Transcriptase Polymerase Chain Reaction).

RNA was isolated using an RNeasy lipid tissue kit (Qiagen) from 50mg of epididymal WAT from the mice. cDNA was made from 400ng of the extracted RNA using an iScript cDNA synthesis kit (Bio-Rad). Realtime RT-PCR was performed on the Biorad IQ5 thermal cycler using IQ Supermix and FAM-conjugated primer probe specific for each gene analyzed (Applied Biosystems). Cross/cycle threshold (Ct) values obtained from realtime experiments were analyzed using the Pfaffl method to control for the efficiency of the PCR reaction and the gene expression data was normalized to the housekeeping gene 18S. Gene expression data is represented graphically using Graphpad Prism 4.0 statistics software.

Imaging.

Adipose tissue was fixed overnight in 10% formalin and then processed and embedded in paraffin wax for sectioning. Tissue blocks were cut on a Microm microtome into 7-micron thick sections and dried overnight on Superfrost microscope slides. The slides were then incubated at 60°C for 1 hour, cleared, rehydrated, and stained with toluidine blue O (TBO). Following TBO staining, the slides were covered with coverslips and left to dry overnight. Four images each were taken from 3 mice per group on an Olympus BX-51 microscope at 100x magnification. The numbers of adipocytes, crown-like structures (CLS), and brown-like cells were quantified using Histometrix 6.0 software and repre

sented graphically using Graphpad Prism 4.0 statistics software. Those counts show the effect of the RYGB procedure on macrophage levels, inflammation, and apoptosis in an alternate method to gene expression.

RESULTS.

Body Weight Curves.

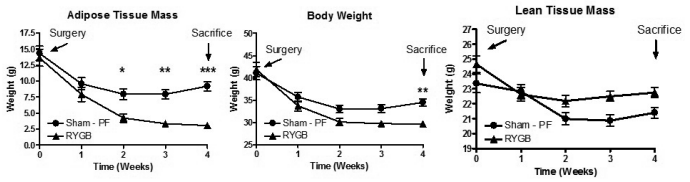

Figure 1. Body weight curves for Sham Pair-Fed and RYGB groups. Weight was measured within 0.1g accuracy and the average for each group is reported for the four weeks following surgery. Body composition was measured by NMR in the Vanderbilt MMPC on the Bruker Minispec. Total lean tissue mass and adipose tissue mass are reported in grams. * -P value = 0.05; ** -P value = 0.01; *** -P value = 0.001.

The body weight curves reflect the fluctuations in weight before the mice were sacrificed and the tissue was stored for processing for this study. The group of mice that underwent RYGB has lower values in all three categories, reflecting weight loss, and more importantly, fat loss.

Gene Expression.

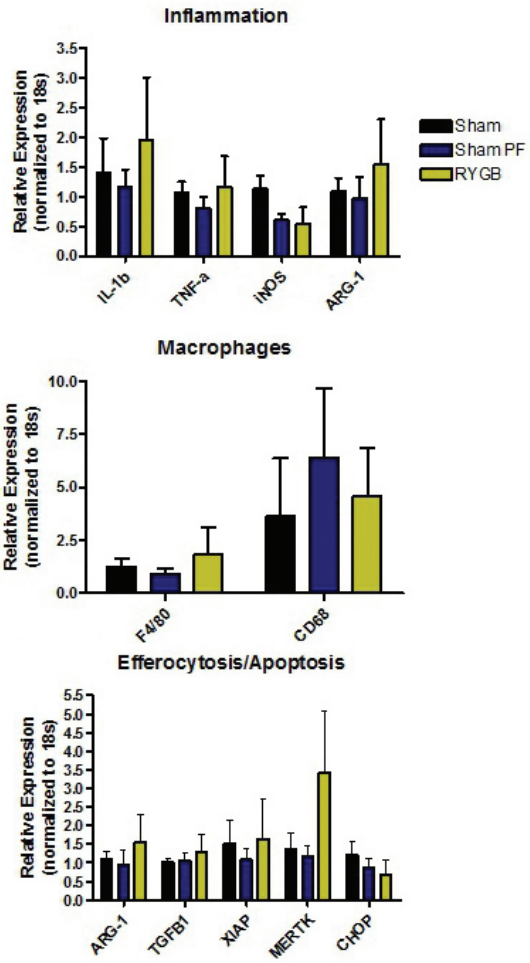

Figure 2. RNA was isolated, PCR was performed to produce cDNA, and realtime RT-PCR was used to determine the expression for several gene groups relative to the housekeeping gene 18S.

The genes used were for 3 different categories: inflammation (IL-1β, TNF-α, INOS, ARG-1), macrophages (F4/80, CD68), and efferocytosis/apoptosis (ARG-1, TGFB1, XIAP, MERTK, and CHOP). These genes were chosen due to their common use in this area of inflammation and resolution. The hypothesis was that markers of inflammation and macrophages would be lower while genes involved in efferocytosis/apoptosis would be elevated for the RYGB group of mice. We did not see these expected differences in the RYGB mice. Given our sample size and the variability in gene expression, we were not able to make any statistical conclusions from this data.

Imaging.

The micrographs show there is a distinct population of dark, granular cells in the RYGB tissue that is not present in the other two groups' tissue samples. We did not investigate further into what exactly identity or implications of these cells or if they had any significance. However, they were a novel observation and possibly reflect RYGB-specific change to immune cell populations in WAT.

CONCLUSIONS & DISCUSSION.

This is the first study to evaluate macrophage, inflammation, and efferocytosis/apoptosis gene networks in mice following RYGB. It doesn't build the desired link between RYGB and improved metabolism in mice, as no significant differences were seen in the number of WAT macrophages among the RYGB, Sham, and Sham Pair-Fed groups. No significant differences were seen in inflammation in WAT in mice from the RYGB, Sham, and Sham Pair-Fed groups. Apoptosis and efferocytosis markers increased in the RYGB group as indicated by the quantification of gene expression. The link between the RYGB procedure and improved metabolism is dependent on a reduction of macrophage counts and inflammation due to an increase in apoptosis. Therefore, the hypothesis was that markers of the first two categories would be lower in RYGB tissue, while the third would be higher; whether this was the case cannot be discerned. It remains unclear whether apoptosis/efferocytosis were the reasons for the inconclusive changes in WAT macrophage quantity and inflammation. However, a distribution of unique, dark granular immune cells was found while imaging sections of tissue for cell counts. Their presence was not expected and neither their identity nor their function is established at this time. Further tests are required to identify these cells and establish their function. Although at times inconclusive, these results are significant because they are novel and help bridge the gap between surgery and therapeutics in terms of diabetes

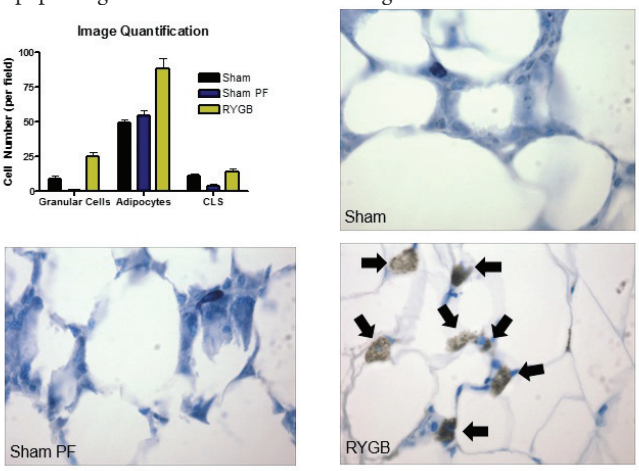

Figure 3. Toluidine Blue O staining of 7 μm sections of epididymal adipose tissue embedded in paraffin. Images taken at 400x magnification. The white structures, adipocytes, are 50-100 μm in length. The black arrows indicate the granular cells.

FUTURE DIRECTIONS.

There are a few key future directions for this study. First, due to the variety of markers of macrophages, inflammation, apoptosis, and efferocytosis, it would be beneficial to conduct a similar study with several more markers so as to account for error and contradiction in data analysis. Second, it is known that WAT macrophages decrease with weight loss; to further investigate the effects of the kind of weight loss induced by RYGB in mice, a longer study could be useful in confirming the eventual reduction of macrophages and inflammation in the same type of mice. Third and finally, additional staining and analysis of the unprecedented granular cells seen while imaging can be done to determine the nature, function, and potential medical significance of these unique immune cells. These analyses could include but would not be limited to the following: staining through ORO or some other lipid stain on the paraffin sections, F4/80 or CD68 stain on paraffin sections, staining for collagen or some other matrix protein on paraffin sections, possible new RYGB studies with sacrifices at earlier and/or later times, and atherosclerotic cells lesions and the effects of RYGB on them.

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