Immunohistochemical Detection of FOXP3 Shows Stimulatory Effect of Metformin on Thymus Regulatory T-Cells in Type 2 Diabetic Mice

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Abstract

Metformin is an adjuvant drug used in the treatment of obesity and diabetes, two conditions associated with stress and chronic inflammation that affects thymus structure and function. Recent evidence suggests a complex role of metformin in thymic homeostasis. The study was designed to develop an animal model of obesity and type 2 diabetes, and treat it with metformin to evaluate its effects on the thymus. In addition, its general effects on body weight and blood glucose level were also investigated. Over a period of 6 weeks, 30 albino male mice (4-5 weeks) were fed either regular chow (control group, N=10) or high fat diet (obese group). The obese group was then subjected to low dose streptozotocin induction of diabetes and divided into two subgroups, one of which was treated with metformin (N=10) while the other was not (N=10). Body weight, random blood sugar, relative thymus weight were recorded. Thymic tissue sections were stained with H & E to study general histology and with single immunohistochemical stain to detect regulatory T-cell using FOXP3 marker. Thymic corticomedullary ratio and regulator cell frequency were calculated. Metformin was successful in reducing body weight and blood glucose levels in treated animals. The thymus had higher relative weight and less cortical cellularity but more frequently counted regulatory cells. Untreated animals showed signs of thymic involution but increased corticomedullary ration in response to reduced regulator cell counts. Metformin has both direct and indirect effects in correcting metabolic abnormalities associated with obesity and diabetes. These effects are anti-inflammatory and may be responsible for stimulation of thymic immunosuppressive cells. Contrariwise, obesity and diabetes have detrimental effects on thymus structure and homeostasis.

1 Introduction

The global prevalence of obesity has increased more than twofold over the past 30 years. Diet-induced obesity is considered a chronic inflammatory condition. The thymus plays a central role in modifying the chronic inflammatory processes associated with obesity. Li and colleagues were successful in modifying type 2 diabetes by thymus implants, suggesting a role for autoimmunity in the pathophysiology of the disease.

Natural thymic Regulatory T-cells (Tregs), formerly known as suppressor T-cells, play an important role in immune homeostasis. Metformin is one of the first-line treatments for type 2 diabetes and the associated overweight.

The aim of the current study was to develop an animal model of obesity and types 2 diabetes, to examine the effects of these conditions on the thymus gland, and to discover whether metformin treatment has any effect on thymus structure and its production of regulatory T-cells.

2 Materials and Methods

2.1 Animals and study design

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Thirty adult male albino mice (Mus musculus) aged 4-5 weeks were used in the study. Animal ethical clearance was provided by the Research Ethics Committee at Al-Mustansiriyah University. The study lasted 6 weeks. The animals were divided into three groups, ten mice each:

- **Group I**: were fed regular rodent chow (Teklad®, 4% Fat Mouse/Rat Diet, Harlan Laboratories, Seoul-Korea) that provided 3 kcal/g of energy. These animals were the control healthy group.
- **Group II & Group III**: were fed high fat and high fructose diet throughout the study time. The diet provided 5.3 kcal/g of energy. This high fat diet (HFD) was prepared by mixing the powdered regular pellets with glucose, lard, butter and other ingredients and prepared as instructed before. At the end of the third week of the study, all animals received an injection to induce hyperglycemia similar to type 2 DM. Mice were considered to be diabetic when their random blood sugar (RBS) levels were ≥200 mg/dl. During the last 2 weeks of the study, Group II were continued on HFD without any treatment (designated OB+DM= obese diabetic). Group III animals (designated OB+DM+MET= obese diabetic on metformin) were treated with metformin hydrochloride (Merk Santé, France) by gastric gavage at twice daily dose. Therefore, data are presented as measures of mean ± standard deviation of each group. The statistical difference between the correlated groups was estimated by using analysis of variance (ANOVA) followed by the Student t-test.

### 2.2 Study parameters

The body weight of each animal was recorded on the 3rd and 5th day of each week and at the time of sacrifice. Random blood glucose level was recorded at the end of the third week of the study as a baseline measurement and twice-weekly thereafter using ACCU-CHEK® Compact Plus glucometer (Switzerland) with 1.5 µl sample size strips and obtaining blood from tail nips. At the end of the study, animals were euthanized under general anesthesia in accordance with laboratory animal protocols. The thymus gland was dissected and weighed before being fixed in 10% formaldehyde fixative.

At the end of the study, images were obtained by Digital camera (Akkila, USA) via single intraperitoneal injection of streptozotocin (Sigma-Aldrich, USA) via single intraperitoneal injection. Heat activity was blocked with methanol containing 0.3% peroxide. After deparaffinization and rehydration, endogenous peroxidase activity was blocked with methanol containing 0.3% peroxide. Heat-induced antigen retrieval was performed in a microwave oven for 15 min. Then sections were covered with serum-free protein block (Dako), followed by the primary antibody. After incubation with anti-FOXP3 (clone 236A/E7; Abcam, UK), anti-mouse polymer was used as second step. Finally, peroxidase activity was developed using a liquid diaminobenzidine (DAB+) chromogen system (Dako). Slide background was counterstained with haematoxylin.

### 2.3 Digital image processing

Digital images of the histological sections were obtained using Micros microscope (Austria) with built in Micros Camera using the provided NMS Software. Images were scaled and examined using Image J software. In H&E sections, corticomedullary ratio was calculated as area percentage at X100 magnification. According to cellular density, images were converted to binary colors; one for each of the medulla and cortex, and the surface area percentage of each color was demarcated and calculated. Ten sections were examined for each animal.

In immunohistochemical sections, the cellular density of FOXP3-positive Tregs was calculated as percent of total nuclear points in the field using Image J automatic nuclear count plugin. At least four of the medullae and surrounding corticomedullary junction were examined in each section at X400 magnification.

### 2.4 Statistical analysis

By using analysis of variance (SPSS Software, version 20.0 2012), the results were evaluated statistically, and whenever there was a difference between the correlated groups, student t-test was applied to estimate the degree of significance by comparing the mean of data and standard deviation of each group. Therefore, data are presented as measures of mean ± standard deviation, at 95% confidence interval.

### 3 Results

#### 3.1 Anatomical and biochemical results

HFD feeding resulted in a rapid increase in animal weight that was obvious grossly within 3 weeks (Fig 1).

**Fig 1**: Difference in gross appearance of body weight between a mouse on regular chow (right) and another on HFD (left)

HFD fed animals gained a range of 4-5 grams/ week, compared to 2-2.5 grams/week for regular chow fed animals (Fig 2).
The weight gain continued at the same rate in control animals and obese diabetic animals but decreased at a rate of 2g/week in metformin treated group. This resulted in a statistically significant difference among the three groups.

Random blood sugar levels were normal in all groups (<200 mg/dl) but increased significantly in obese mice after streptozotocin administration. The levels decreased significantly to normal levels in metformin treated group but remained high in untreated group (Fig 3).

The relative thymus weight was significantly reduced in the OB+DM group (Fig 4). The corticomedullary ratio represented as surface area percentage was comparable in control and metformin treated animals. The cortical area, however, has significantly increased in OB+DM animals on the expense of significantly reduced medullary area (Fig 5).

Examination of H&E sections (Fig 6) showed normal histology of the thymus in control animals with clearly defined lobules separated by interlobular septa, dark staining cellular cortex and lighter staining less cellular medulla. Similar features were seen in OB+DM+MET animals but interlobular septa were less demarcated and peri-thymic fat was more developed.

In OB+DM animals, cortical thickness and cellularity was increased and interlobular fat accumulation was evident. On higher magnification, pyknotic cells were most pronounced in OB+DM animals while macrophages and Hassall’s corpuscles were more obvious in OB+DM+MET group.

Immunohistochemical detection of FOXP3 positive regulatory T-cells showed a significant change in frequency and distribution (Fig 7). The cells were mainly found at the corticomedullary junctions in control animals. In OB+DM they were sparsely distributed closer to the center of the medulla. OB+DM+MET treated animals revealed more intense signal and greater numbers and were scattered from the deep cortex to the medulla through the corticomedullary junction.

OB+DM animals had the least frequency of Tregs while OB+DM+MET animals had significantly higher frequency (Fig 8).

4 Discussions

High fat/high fructose diet was successful in producing obesity but not diabetes. Low dose streptozotocin was needed to cause mild to moderate hyperglycemic state. Obesity on its own is a chronic process that takes a long time to cause pancreatic beta cell exhaustion, insulin resistance and hyperglycemia. Low dose streptozotocin is expected to cause damage to 40%-60% of pancreatic beta cells resulting in hyperglycemia due to
insulin insufficiency within 2-5 days of injection. Obesity is known to be associated with high levels of the fat-secreted hormone, leptin. Metformin had positive effects on both body weight and blood sugar levels in the treated group possibly by interaction with leptin and other satiety factors. Metformin is known to reduce food intake by increasing the plasma activity of the anorexigenic glucagon-like peptide 1 (GLP-1). GLP-1 is an incretin released from the intestinal enteroendocrine L-cells to promote satiety and inhibit food intake. However, GLP-1 is rapidly degraded (within 1-2 minutes) by the enzyme dipeptidyl peptidase IV (DPPIV). Leptin also increases the release of GLP-1 in both humans and rodents, but also has an inhibitory effect on DPPIV. These interactions reduced the food intake in OB+DMMET group resulting in weight loss.

Fig 6: H&E stained sections of mouse thymus. Control animals (top panel) showed normal histology with clear lobules. OB+DM animals (middle panel) had increased cortical cellularity and thickness with interlobular fat accumulation (yellow arrows) and pronounced amount of pyknotic cells (red arrows). OB+DM+MET animals showed more regular general histology and less cortical cellularity but more obvious macrophages (red arrow heads) and Hassall’s corpuscles (yellow arrow). (C= cortex, M= medulla, F= fat). X40 (left panel), X400 (right panel)

HFD combined with partial pancreatic beta cell damage reduce insulin sensitivity by reducing the expression of insulin receptors. HFD also inhibits fatty acid oxidation in skeletal muscles and reduces the intracellular content and translocation of GLUT4 receptors to the cell membrane. These factors result in the hyperglycemia and hyperinsulinemia characteristic of type 2 DM. The direct action of metformin is involved in reducing glycogenolysis and gluconeogenesis by three distinct mechanisms. Metformin inhibits the activity of the enzyme...
glucose-6-phosphatase which is involved in release of hepatic glucose from glycogenolysis.

This gives a sparing effect on glucose-6-phosphate to be used for glycogen storage not glucose output\(^7\). Secondly, metformin reduces the hepatic uptake of substrates (especially lactate) that can be used in gluconeogenesis. It can also shift energy metabolism towards fatty acid oxidation rather than glucose utilization\(^8\). An indirect effect of metformin may be related to its interaction with the high levels of leptin seen in obese animals. Previous studies have shown that metformin treatment can enhance central and peripheral leptin receptor availability and increases its signal transduction in HFD-fed obese animals and in aged obese non-diabetic animals as well\(^9\).

The changes in relative thymus weight in the current study are in agreement with previous work on obesity\(^10\). While obesity accelerates thymic involution, possibly due to related high levels of cortisol, the increased body weight also exceeds the normal

\[\text{Fig 7: Detection of FOXP3 positive Tregs in mouse thymus. Control animals (Top panel) had medium cell signal mainly at the corticomedullary junction. OB+DM animals (middle panel) had the least frequency of cells mainly at the medulla. OB+DM+MET animals (bottom panel) had highest signal of Tregs scattered in the deeper cortex (C), medulla (M) and corticomedullary junction. (Haematoxylin counter stain). X100 (left panel), X400 (right panel)}\]

\[\text{Fig 8: Percentage of nuclear count of FOXP3 positive Tregs in mouse thymus (%/field). (Mean±Standard deviation, **= High statistical significance, P<0.01)}\]
growth of the thymus resulting in a drop of its relative weight. In the current work, OB+DM animals showed increased fat infiltration of the thymus which goes with features of thymic involution but at the same time showed signs of increased cortical cellularity which is indicative of proinflammatory activation. Thymic lymphocytes numbers increase due to antigenic stimulation associated with inflammation. The corticomedullary ratio, normally 2:1, is affected by that increase. Thymic Tregs may mediate the effects of obesity and metformin on such thymic changes.

Naturally occurring regulatory T cells (Tregs), are a special subset of immunosuppressive T cells that are positive for CD4, CD25 and FOXP3. They comprise 5-10% of the mature CD4+ cells. They play a crucial role in the maintenance of tolerance to self-antigens and prevention of autoimmune responses. Tregs have high lipid oxidation rates in vitro. With increased adiposity, Tregs are replaced by proinflammatory cells. This may explain the reduced frequency of Tregs in OB+DM animals and the subsequent increased lymphopoiesis in their thymic cortices (due to reduced immunosuppression). In metformin treated animals, the increased frequency of Tregs may be related to the drugs antiglycolytic properties. Treg cell generation is stimulated when glycolytic pathways are blocked. Similar results were obtained by other workers in the context of other inflammatory diseases.

Obesity is also associated with hyperliptinemia and leptin is considered to be proinflammatory. Metformin can also reduce leptin levels in obese and non-obese states, and this may have reduced the proinflammatory stimulation of the thymus.

5 Conclusion

In addition to the documented beneficial effects of metformin in the treatment of obesity and diabetes, the current work provides evidence that metformin enhances thymic architecture and immune response by stimulating proliferation of regulatory T cells through its direct metabolic actions and via interaction with other mediators of inflammation and obesity.

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7 Competing Interests

Authors have declared that no competing interests exist.

8 Author’s contributions

SSA carried out literature review and animal handling. AFH was responsible for histological staining and calculations. MMI reviewed the statistical data and finalized the manuscript. All authors read and approved the final manuscript.

9 References