FINAL REPORT

1. SUMMARY

Obesity is a pathologic condition, in which the intra-abdominal white adipose tissue is characterized by hypoxia, intensive adipocyte cell death, expression of various danger signals, local inflammation and intensive macrophage infiltration. The recruited macrophages produce large amounts of inflammatory mediators, which trigger metabolic syndrome characterized by insulin resistance, low-grade inflammation and dysregulated metabolism.

It is well known, that the deficient clearance of dying cells (so called efferocytosis) leads to the accumulation of apoptotic cells, which favors to the development of chronic inflammatory diseases. Our hypothesis was that the disturbed apopto-phagocytosis program - caused by mutations affecting phagocytosis-related genes like tissue transglutaminase (TG2) - contributes to the development of low-grade systemic inflammation and insulin resistance related to diet-induced obesity.

Our data indicate that loss of TG2 indeed enhances obesity-related pathologies such as adipose tissue inflammation, adipocyte death, hepatic steatosis and insulin resistance in mice exposed to either high sucrose (HSD) or high fat (HFD) containing diet, and all this is a result of the loss of TG2 from bone marrow-deived (BMD) cells. In parallel, our results unexpectedly indicate, that phagocytosis-deficient TG2 null macrophages clear dying adipocytes more efficiently via lysosomal exocytosis, but produce more pro-inflammatory cytokines than the wild type ones.

Overall, our results confirmed and revealed the role of TG2 not only in the clearance of dying adipocytes but also in the development of certain obesity-triggered abnormalities like inflammation, insulin resistance and hepatic steatosis. However, further studies are needed to clarify the exact mechanism by which TG2 plays a role in these processes.

2. ALTERATION(S) FROM THE CONTRACT

The experiments (with MerTK and A2AR deficient mice) planned for the second and third years of the project were postponed, because (1) the reproduction of the MerTK and ADORA2 null mice was not sufficient to perform the feeding experiments (2) the results of the first 1.5 year pointed in a different direction, and we continued our work along this path.

3. DETAILED REPORT

To study the effect of improper efferocytosis on the development of obesity-related abnormalities we carried out 6 rounds of feeding experiment. During these studies we fed wild type and TG2 deficient mice with normal (ND), high-sucrose/low-fat (HSD) or high-fat (HFD) diet for 16 weeks. The body weight and food intake of the animals were registered weekly. Insulin resistance test and glucose tolerance test were performed on week 15 and

16. At the end of the feeding phase, the animals were sacrificed and various frozen and paraformaldehyde-fixed samples were collected and analysed by various methods.

To determine, whether loss of TG2 in BMD cells or in other cell types is responsible for the above changes, wild type or TG2 null mice were irradiated, and their bone marrow was replaced by a bone marrow originated from either wild type or TG2 null mice in each combination.

Loss of TG2 does not affect weight gain during diet induced obesity, but enhances the pro-inflammatory cytokine production of the gonadal adipose tissue

Wild type mice fed either on HFD or on HSD developed marked obesity compared with animals kept on a standard control diet. Loss of TG2 did not affect the weight gain of the animals kept on the three different types of diet. The food intake of mice was roughly the same in the different groups.

Adiponectin expression of adipocytes was significantly decreased, while resistin expressions significantly increased with weight gain in the adipocytes of gonadal white adipose tissue adipocytes of mice kept on HSD or HFD, as compared to mice kept on control diet. But the loss of TG2 did not affect the obesity-related alterations in the expression levels of these adipokines. The obesity-induced expressions of the pro-inflammatory cytokine TNF-alpha, the chemoattractant MCP-1 and leptin, the level of which is related to the energetic status of the adipocyte lipid stores, were significantly enhanced in the adipocytes of TG2 null animals kept on HFD.

Loss of TG2 in non-BMD cells leads to enhanced circulating plasma insulin levels and insulin resistance

In line with the enhanced pro-inflammatory cytokine and resistin production, we detected significantly enhanced fasting plasma insulin levels and increased insulin resistance in TG2 null mice as compared to their wild type counterparts kept on HFD. As compared to the wild type counterparts, loss of TG2 in the non-BMD cells resulted in significantly increased fasting circulating plasma insulin levels and increased insulin resistance.

Loss of TG2 from BMD cells is responsible for the obesity-related adipocyte cell death, inflammation and hepatic steatosis in TG2 null mice

Following 16 weeks of HSD or HFD the gWAT increased significantly in both wild type and TG2 null mice as compared to mice kept on control diet. However, in case of TG2 null mice kept on HFD the gWAT was significantly less than in wild type mice very likely due to the enhanced adipocyte apoptosis in TG2 null gWAT. To confirm this hypothesis we performed TUNEL assay and fluorescent macrophage labelling on paraffin-embedded gWAT slides. This confocal microscopy analysis revealed higher level of adipocyte death in the gWAT of mice kept on HFD in the absence of TG2. Indeed, in the gWAT of TG2 null mice kept on either HSD

or HFD significantly more dying cells were detected than in their wild type counterparts by identifying dead adipocytes. In parallel, we determined the expression of various pro- and antiapoptotic genes in the adipocytes of gWAT. We found that in the gWAT adipocytes the expression of the pro-apoptotic Bid and Bim was induced during diet-induced obesity. In addition, the anti-apoptotic Bcl-2 was also induced during diet-induced obesity, but the levels of Bcl-2 mRNA were significantly dropped in the TG2 null mice kept on HFD as compared to their wild type counterparts.

In line with these findings, in TG2 null mice kept on HFD both the liver weight and the triacylglycerol content of the livers were significantly higher than that of the wild type mice kept on HFD indicating translocation of lipid depots from the gWAT into the liver. Tissue sections of livers stained with haematoxylin eosin confirmed these findings.

The absence of TG2 in BMD cells, promoted adipocyte death in the gWAT of mice exposed to HFD, compared to mice transplanted with wild type bone marrow, possibly due to the enhanced TNF-α and leptin production in these mice. In addition, significantly larger gWAT weight, increased liver weight and TAG content could be detected in animals lacking TG2 in the bone marrow compartment. In line with these data, in the absence of BMD cells' TG2 a significantly higher expression of adipose tissue TNF-α, IL-6, MCP-1, leptin and Bid mRNA-s were detected in mice exposed to HFD. Similarly, gWAT macrophage IL-6, MCP-1, TNFα mRNA levels were also significantly higher in the absence of TG2.

LXR agonist treatment reverts the HFD-induced phenotype in mice lacking TG2 in BMD cells

To test whether the development of obesity-related inflammation and insulin resistance could be prevented by the usage of phagocytosis-enhancing compound we used LXR agonist GW3965 mixed with their high fat containing food during the whole feeding period

LXR agonist treatment prevented body and gWAT weight gain in HFD-exposed mice. This was related to an enhanced white adipocyte apoptosis assessed by the enhanced Bid and Bim expression of gWAT adipocytes. In contrast, the percentage of dying adipocytes observed on tissue sections decreased in the gWAT of LXR-treated mice, if TG2 was missing from the BMD cells, perhaps due to their more efficient clearance. Similarly, significantly increased LXR activation-related hepatic steatosis was detected only in wild type mice. LXR agonist treated TG2 null gWAT macrophages produced significantly less MCP-1 and resistin, than their wild type counterparts, while the gWAT adipocytes from the same mice significantly more adiponectin reflecting an improved insulin sensitivity. Indeed, though there was no difference in the fasting circulating plasma insulin levels in the LXR agonist treated mice, animals lacking TG2 in BMD cells, showed increased insulin sensitivity.

Loss of TG2 in macrophages results in a more efficient adipocyte efferocytosis

In the next phase of the project we studied the potential role of macrophage TG2 in the clearance of apoptotic adipocytes. We exposed serum deprived, apoptotic 3T3 adipocytes to

both wild type and TG2 null BMD macrophages and their clearance was followed by laser scanning cytometry. The loss of macrophage TG2 did not delay, but accelerated the efferocytosis of dead adipocytes. TG2 null macrophages take up adipocyte-derived lipid-containing vesicles, and trigger the initiation of the classical apoptosis program characterized by membrane blebbing much faster. After the whole cytosolic content of the adipocyte is cleared, TG2 null macrophages digest finally the degrading nucleus.

Metabolically activated TG2 null macrophages produce more pro-inflammatory cytokines than their wild type counterparts due to enhanced c-Src signaling

Previous studies have demonstrated that TG2 deficiency leads to increased integrin β3 (ITGβ3) expression, enhanced c-Src tyrosine kinase activity and consequently to intensified macrophage inflammatory responses. We found that both the gene expression of ITGβ3 and pSrc level is significantly increased in TG2 null, metabolically activated (MMe) macrophages in the absence of TG2. We also found increased ITGβ3 mRNA levels in the gWAT macrophages of TG2 deficient HSD and HFD mice.

The induction of ITGβ3 expression in MMe macrophages was completely c-Src-dependent, as PP2, a Src inhibitor, prevented it. Metabolic activation also induced significantly higher TNF-α, IL-1β, and IL-6 mRNA levels in TG2 null Mme macrophages than in the wild type ones. Finally, the PP2 Src inhibitor and the integrin receptor inhibitor RGD peptide inhibited the induction of proinflammatory cytokine formation both in wild type and in TG2 null MMe macrophages, and in the presence of these inhibitors no difference in their metabolic activation induced pro-inflammatory cytokine induction was found.

4. LIST OF SCIENTIFIC PUBLICATIONS

Scientific publication(s):

Sághy T, **Köröskényi K**, Hegedűs K, Antal M, Bankó C, Bacsó Z, Papp A, Stienstra R, Szondy Z. Loss of transglutaminase 2 sensitizes for diet-induced obesity-related inflammation and insulin resistance due to enhanced macrophage c-Src signaling. Cell Death Dis. 2019 Jun 5;10(6):439. doi: 10.1038/s41419-019-1677-z.

Scientific presentation(s), poster(s):

Szondy Z, **Köröskényi K**, Sághy T, Antal M, Hegedűs K, Loss of transglutaminase 2 sensitizes for the development of inflammation, loss of insulin sensitivity and hepatosteatosis in mice kept on high fat diet. Keystone Sympoisia " Organ Crosstalk in Obesity and NAFLD" January 21-25, 2018, Keystone, Colorado, USA

Köröskényi K, Sághy T, Antal M, Hegedűs K, Szondy Z, Loss of transglutaminase 2 sensitizes for the development of inflammation, loss of insulin sensitivity and hepatosteatosis in mice kept on high fat diet. FEBS3+ Conference, September 2-5, 2018. Siófok, Hungary

Köröskényi K, Sághy T, Antal M, Hegedűs K, Szondy Z, Loss of Transglutaminase 2 Sensitizes for Diet-Induced Obesity-Related Inflammation and Insulin Resistance due to an Enhanced Macrophage c-Src Signaling. Gordon Research Conference: Phagocyte Functions Through Life: Development, Defense and Disease, June 2-7, 2019; Waterville Valley, NH, USA