Inflammation triggers high mobility group box 1 (HMGB1) secretion in adipose tissue, a potential link to obesity

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A B S T R A C T

Background: Low grade inflammation is one of the major metabolic disorders in case of obesity due to variable secretion of adipose derived cytokines called adipokines. Recently the nuclear protein HMGB1 was identified as an inflammatory alarmin in obesity associated diseases. However HMGB1 role in adipose tissue inflammation is not yet studied.

Objectives: The aim of this study was to prove the expression of HMGB1 in human adipose tissue and to assess the levels of expression between normo-weight and obese individuals. Furthermore we determined which type of cells within adipose tissue is involved in HMGB1 production under inflammatory signal.

Methods: Western-blot was performed on protein lysates from human normo-weight and obese adipose tissue to study the differential HMGB1 expression. Human normo-weight adipose tissue, adipose-derived stromal cells (ASCs) and adipocytes were cultured and stimulated with LPS to induce inflammation. HMGB1, IL-6 and MCP-1 secretion and gene expression were quantified by ELISA and Q-PCR respectively, as well as cell death by LDH assay. HMGB1 translocation during inflammation was tracked down by immunofluorescence in ASCs.

Results: HMGB1 was expressed 2-fold more in adipose tissue from obese compared to normo-weight individuals. LPS led to an up-regulation in HMGB1 secretion and gene expression in ASCs, while no change was noticed in adipocytes. Moreover, this HMGB1 release was not attributable to any cell death. In LPS-stimulated ASCs, HMGB1 translocation from nucleus to cytoplasm was detectable at 12 h and the nuclear HMGB1 was completely drained out after 24 h of treatment.

Conclusion: The expression level studies between adipose tissue from normo-weight and obese individuals together with in vitro results strongly suggest that adipose tissue secretes HMGB1 in response to inflammatory signals which characterized obesity.
pro-inflammatory cytokines and is characterized as an ‘alarmin’. Further, HMGB1 promotes the recruitment of inflammatory cells to damaged tissues [19,20], as in the case for atherosclerotic plaques [21,22].

In mouse model, Hmgb1−/− pups die within 24 h due to hypoglycemia and have no fat [23]. Moreover atherosclerotic hamsters fed with high fat diet exhibit higher level of circulating HMGB1 [24]. Mouse 3T3 fibroblasts and macrophages have been shown to express and release HMGB1 under signals such as oxidative stress (UVB) and inflammation (LPS) [25–27], and they are activated by HMGB1 [15,27]. As 3T3 fibroblasts are capable of differentiating into adipocytes and as macrophages infiltrate adipose tissue in case of obesity, these studies suggest a potential link between HMGB1 and obesity, although this link is not yet well understood. Moreover, a positive correlation has also been done between HMGB1 gene expression in human adipose tissue and one of the common polymorphism of the fat mass and obesity-associated (FTO) gene strongly linked to obesity [28]. Based on these data, we aimed to determine if HMGB1 is secreted by adipose tissue under low grade inflammation and which cell types within adipose tissue could be implicated in HMGB1 production.

The first aim of this work was to prove the expression of HMGB1 protein in human adipose tissue and to compare the expression level between normo-weight and obese individuals. Then from primary culture of human adipose tissue, adipocytes and adipose-derived stromal cells (ASCs) from normo-weight individuals, we compared the expression and secretion level of HMGB1 in normal and inflammatory conditions. Finally, immunostaining experiments were conducted on ASCs to study the cytoplasmic translocation of HMGB1 under inflammation.

2. Materials and methods

2.1. Reagents

Lipopolysaccharides (LPS from E. coli 0111:84 strain, batch #LPE-32-02) and TRIreagent were purchased from Sigma, France. Collagenase (NB4, 0.12 PZU/mg) was from SERVA, Germany. Sterilip 100 μm was purchased from Millipore, France. All reagents for cell culture were purchased from PAN Biotech (France) except insulin (Humuline Rapide, Lilly, France) and reagents for adipocyte differentiation (Sigma, France). Ringer Lactate was obtained from B. Braun, France.

2.2. Origin of human adipose tissue samples and harvesting protocol

Subcutaneous (abdominal, buttocks, hips and thighs) adipose tissue samples were obtained from normal weight (BMI < 25, mean body mass index = 22.65 ± 1.42 kg/m²) and obese women (BMI ≥ 30, mean body mass index = 35.6 ± 3.2 kg/m²) undergoing liposuction, performed under general anaesthesia for aesthetic reasons (aged from 25 to 60 years, mean 39 years). Apart from oral contraception, the subjects were not receiving any treatment with prescribed medication at the time of liposuction. Fat tissue was harvested mechanically with a vacuum pump after infiltration of a tumescence solution (40 mL lidocaine 2% + adrenalin 1 mg/L for 1 L Ringer Lactate) for local anaesthesia. A total of 22 samples were obtained and the study was approved by the Reunion Island ethics committee for the protection of persons undergoing biomedical research. Samples were handled within an hour after the end of the surgery.

2.3. Supplemented media

For all experiments, medium 199 was supplemented with 2 g/L glucose, 5 mg/ml amphotericin B, 0.2 mg/ml streptomycin and 200 U/mL penicillin. For adipose tissue and adipocytes, 1% fetal bovine serum (FBS) was added with 8 μg/mL biotin, 4 μg/mL pantethenate and 66 nM insulin. For ASCs, 20% FBS was added on the day of culture, then 10% the day after and for the rest of the culture period (media was changed each 2 days).

2.4. Adipose tissue preparation and primary culture

After liposuction, adipose tissue was rinsed thrice with Ringer Lactate. For primary culture, 200 μL of tissue was distributed in 24-well tissue culture plates with 300 μL of supplemented medium. Tissue was then maintained at 37 °C in 5% CO₂ for a period of 24 h prior to the experiments.

2.5. Adipocytes isolation and primary culture

Tissue samples obtained by liposuction were digested for 1 h at 37 °C in Ringer Lactate containing 0.5 mg/mL collagenase. The floating cells (adipocytes) were rinsed in Ringer-Lactate and plated (200 μL, ~30,000 cells) in 24-well tissue culture plates with 300 μL of supplemented medium. Cells were then maintained at 37 °C in 5% CO₂ for a period of 24 h prior to the experiments.

2.6. Stromal cells extraction and ASCs primary culture

After washing as described above, tissue was digested for 30 min at 37 °C in Ringer Lactate containing 0.5 mg/mL collagenase. The floating cells (adipocytes) were rinsed in Ringer-Lactate and plated (200 μL, ~30,000 cells) in 24-well tissue culture plates with 300 μL of supplemented medium. Cells were then maintained at 37 °C in 5% CO₂ for a period of 24 h prior to the experiments. By convention, the adherent and proliferated stromal vascular cells are called adipose-derived stromal cells (ASCs).

2.7. ASCs differentiation into adipocytes

The day after 80–90% confluence, a differentiation cocktail was added to the supplemented media [30]: 3% SVF, 200 nM insulin, 1 μM dexamethasone, 0.1 mM IBMX, 5 μM rosiglitazone, 16 μg/ml biotin and 7.4 μg/mL pantethenate. After 4 days, the differentiated adipocytes were maintained with 3% SVF, 200 nM insulin, 16 μg/ml biotin, 7.4 μg/mL pantethenate and medium was changed for every 3 days.

2.8. Adipose tissue protein extraction and western blot

Adipose tissue was lysed with protein lysis buffer (50 mM Tris–HCl, pH 7.6, 500 mM NaCl, 0.2 mM EDTA) using Dounce homogenizer (10 strokes) under ice-cold condition. The lysates were centrifuged at 15,000g for 30 min at 4 °C, the fat cake was removed and the aqueous protein phase was transferred to a new tube. Protein concentration was determined by Bradford method.

20 μg of proteins were separated by 12.5% SDS–PAGE and transferred to nitrocellulose membrane (Hybond C-EXTRA, Amersham bioscience, France). Membranes were blocked with 5% non-fat dried milk for 1 h at room temperature in Tris-buffered saline with 0.1% Tween-20 and then sequentially probed with mouse anti-human HMGB1 antibody (1:1000 dilution, clone 2F6, Sigma, France).
or control mouse anti-human β-actin antibody (1:1000 dilution, clone AC15, Sigma, France) and then with peroxidase labeled anti-mouse IgG (1:2000 dilution, Vector Laboratories Inc., USA).

Band detection using homemade chemiluminescent substrate (Luminol A8511 and p-Coumaric acid C9008, Sigma, France) was developed on a high performance autoradiography film (Amer sham Hyperfilm™, France) and digitized using EPSON perfection 2480 photo. Band intensities were analyzed using imageJ software (NIH, USA). HMGB1 signal intensity from samples was averaged, normalized to signal from β-actin and used to calculate the relative signal intensity and the statistical analysis.

2.9. HMGB1, IL-6 and MCP-1 ELISA

Following treatment, media samples were collected and assayed for HMGB1 (IBL International, Germany), IL-6 and MCP-1 (eBioscience, France), according to the manufacturer’s instructions. ELISA sensitivity: 0.2 ng/mL for HMGB1, 2 pg/mL for IL-6, 6 pg/mL for MCP-1.

2.10. Cytotoxicity assay

The release of lactate dehydrogenase (LDH) induced by plasma membrane disruption in culture medium was measured using LDH cytotoxicity assay kit from Science cell (Cliniscience, France) according to manufacturer’s instructions. LDH release was measured through an enzymatic reaction and read at 490 nm. As a positive control for cell death, tissue and cells were treated with 1% Triton X-100 (Sigma, France).

2.11. RNA extraction, reverse transcription and real-time quantitative PCR

Following treatment, total RNA was isolated using TRIreagent according to the manufacturer’s instructions. 2 µg of total RNA was reverse-transcribed using random hexamers and Reverse Transcriptase™ (Invitrogen, France). Quantitative PCR was performed on 1 µL of cDNA using ABI PRISM 7500 thermal cycler (Applied Biosystems, France) with qPCR supermix for ABI PRISM (Invitrogen, France). The 18S ribosomal RNA gene was used as a reference. Quantification of target mRNA was calculated using the comparative Ct method (ΔΔCt) and was normalized to the reference gene expression. Analysis was performed on 6 samples per condition. Primers sequences are listed below:

<table>
<thead>
<tr>
<th></th>
<th>IL-6</th>
<th>MCP-1</th>
<th>HMGB1</th>
<th>18S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>TCA CCT CAG AAC GAA TTG ACA</td>
<td>ATC ACC AGC AGC AAG TGT C</td>
<td>ACC CAG ATG CTT CAG TCA AC</td>
<td>GCC CGC TAG AGG TGA AAT TCT</td>
</tr>
<tr>
<td>Reverse</td>
<td>TCA C</td>
<td>TTG CTC</td>
<td>TCA C</td>
<td>TCT TGG CAA ATG TTC</td>
</tr>
</tbody>
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2.12. Immunofluorescence

ASCs were cultured in monolayer on glass coverslips. Following treatment, the culture medium was removed completely and washed thrice with ice-cold PBS to be fixed with ice-cold absolute ethanol for 5 min. Cells were immediately stained for immunofluorescence or air dried and stored at −20 °C until staining was performed. Immunofluorescence staining was carried out according to a standard protocol.

The fixed cells were incubated primarily with mouse anti-human HMGB1 antibody (1:1000 dilution, clone 2F6, Sigma, France) overnight at 4 °C. After PBS-Tween (0.05%) washing, cells were incubated with secondary antibody (1:2000 dilution, goat anti-mouse Alexa flour 488 IgG, Molecular Probes, Invitrogen, France) for 2 h in dark. Nuclei were stained with DAPI (D9542, Sigma, France) at a final concentration of 0.1 ng/mL. Coverslips were mounted in glass slides using Vectashield (Vector Labs, Cliniscience) and fluorescence was observed using a Nikon Eclips 80i microscope (Nikon, France). Images were obtained using the Nikon Digital camera system (Nikon, DXM1200C) and the imaging software NIS-Element BR version 3.1 (Nikon).

2.13. Statistics

All values were measured as mean ± S.D. Statistical analysis was performed using Graph pad PRISM 5 software (Windows). Differences between normal and obese adipose tissue were tested using non-parametric T-test (Mann–Whitney test). Differences between control and treated samples were tested for significance by one-way ANOVA and Dunnett post-test. P < 0.05 (**); P < 0.01 (***); P < 0.001 (****).

3. Results

3.1. High HMGB1 expression in adipose tissue from obese individuals

To determine the basal expression of HMGB1 protein in adipose tissue from normo-weight and obese individuals, western blot was performed. Regardless of the BMI, a specific band corresponding to HMGB1 protein was revealed from adipose tissue protein lysates of all the samples tested with the expected molecular weight (Fig. 1A). No oligomer or complex was revealed from the blot (data not shown). This ensures that HMGB1 protein was expressed in adipose tissue from both normal weight and obese persons. However, the HMGB1 bands from obese samples showed higher intensity in comparison with normal samples. This was confirmed after β-actin normalization of 20 samples analyzed: the average intensity for obese samples was significantly 2-fold higher than the average intensity for lean samples (Fig. 1B). Thus, HMGB1 protein was expressed more in adipose tissue from obese in comparison with normal weight individuals.

![Fig. 1. HMGB1 protein expression in adipose tissue from lean and obese individuals](image)
3.2. HMGB1 secretion is up-regulated without gene up-regulation in adipose tissue under inflammation

After LPS treatment, inflammation was confirmed by the huge increase in IL-6 and MCP-1 secretion from adipose tissue: around 200 fold for IL-6 and 100 fold for MCP-1 since 12 h, until 400 and 200 fold respectively for 48 h (Fig. 2). LDH measurement from media remained the same between control and LPS-treated tissue, regardless of the time point (Fig. 2).

Regarding HMGB1 secretion, we observe that human adipose tissue was able to release HMGB1 at a low basal level (less than 5 ng/mL, Fig. 2) and that there was no accumulation in culture media. More interestingly, we show that under LPS treatment this secretion was up-regulated from 24 h of incubation (3-fold increase) and kept increasing until 48 h (5-fold more) reaching 17 ng/mL.

At gene expression level, although IL-6 and MCP-1 mRNA were up-regulated, we cannot detect any significant change in HMGB1 mRNA level between control and LPS-treated adipose tissue (Fig. 3).

3.3. Adipocytes inflammation doesn’t regulate HMGB1 secretion and gene expression

Despite the up-regulation of IL-6 and MCP-1 at both secretion (Fig. 4) and mRNA (Fig. 5) level, there was no change in HMGB1 secretion and mRNA expression in adipocytes after LPS treatment, regardless of the incubation time. Nevertheless, a basal level of released HMGB1 was detected (less than 5 ng/mL), without any accumulation in the culture media. Moreover, adipocytes viability was not affected by LPS treatment since there is no increase in LDH release (Fig. 4).

3.4. HMGB1 secretion and gene expression up-regulation in ASCs under inflammation

LPS-treated ASCs showed inflammation, as there was a significant up-regulation in IL-6 and MCP-1 secretion since 12–48 h (Fig. 6). HMGB1 was also released in higher levels after LPS treatment (Fig. 6). This increase was significant since 12 h although the level was low (less than 5 ng/mL) and at 48 h the increase was about 10-fold to reach more than 10 ng/mL. Moreover, LPS treatment did not induce any cell death in ASCs, as confirmed by the LDH assay (Fig. 6).

At gene expression level, inflammation was characterized by IL-6 and MCP-1 up-regulation (Fig. 7). Regarding HMGB1 mRNA, a 20% increase was found at 12 h treatment and at 24 h expression levels were doubled compared to control (Fig. 7). After 48 h treatment, HMGB1 gene expression was no more up-regulated and returned to normal.

3.5. HMGB1 secretion is not modulated by LPS in in vitro-differentiated adipocytes

At day 16, adipose differentiation was confirmed by multilocular droplets accumulation inside cells, and identified as triglycerides with Oil Red O staining (Fig. 8). These fully differentiated and hypertrophied cells secreted more than 3-fold IL-6 after 24 h LPS treatment, whereas no significant change was noticed in HMGB1 secretion. No cell death was detected insofar as LDH dosage was not significantly higher after LPS treatment (and compared to the first day of culture, data not shown).

3.6. HMGB1 translocation in ASCs under inflammation

In control cells, nuclei were stained in blue with DAPI, surrounded and punctuated by green points corresponding to HMGB1 (Fig. 9). The nuclear DAPI staining completely matched with HMGB1 staining, in accordance with the function of HMGB1 as a nuclear protein. In control cells, there was no change in HMGB1 localization until 24 h of culture.

Under LPS treatment at 6 h, the HMGB1 intra-nuclear localization was slightly modified to be higher in the nuclear crown. At 12 h treatment, HMGB1 translocation was effective and clearly visible, resulting in a cytoplasmic localization, although a slight signal...
was detected in the nucleus. After 24 h treatment, HMGB1 was completely drained out from nucleus to the cytoplasm, resulting in green signal only in the cytoplasmic compartment.

4. Discussion

The role of HMGB1 in the initiation of certain diseases has been reported as well as its expression [31]. For instance, this is the case for the non-alcoholic fatty liver disease [32] or for atherosclerosis [16,24,33]. The common feature is low-grade inflammation, which participates from the onset to the maintenance and aggravation of these diseases. In the context of obesity, a recent work from Arrigo et al., has identified blood HMGB1 as a new biomarker for metabolic syndrome [34] in obese children, but nothing is known about HMGB1 expression and its role in human adipose tissue. Here we have investigated if HMGB1 could be possibly linked to adipose tissue inflammation and obesity.

On all patients analyzed in this study, we observed that there was a strong positive correlation between BMI and HMGB1 protein expression within adipose tissue: the expression was higher in adipose tissue from obese persons than normal weight individuals. Our results further supports the study of Lappalainen et al., in which they showed a positive correlation between HMGB1 gene expression in adipose tissue and one of the common polymorphism of the FTO gene strongly linked to obesity [28]. Our study further confirms that adipose tissue HMGB1 is dysregulated and strongly expressed in case of obesity. This increase in HMGB1 protein level could probably partly due to leukocytes infiltration, which happens in case of obesity [35,36], as these cells are known to be a source of HMGB1 [25,37]. We wanted next to determine if adipose cells themselves (stromal cells or adipocytes) have a role in
this expression. Then, for all in vitro experiments we have conducted, we used samples from normo-weight individuals, which contain no or really few infiltrated leukocytes (unpublished data).

It’s now well established that overweight and obesity exhibit a chronic and low-grade inflammation [38,39]. Thus, in order to know if the high level of HMGB1 expression in case of obesity is linked to low grade inflammation, adipose tissue from normal weight patients was cultured and treated with LPS to induce inflammation. We proved for the first time that human adipose tissue was able to release HMGB1 at a low basal level. More interestingly, we showed that under inflammation, HMGB1 secretion was upregulated. This increase in HMGB1 secretion following LPS treatment has never been published concerning adipose tissue, and this new result is totally in accordance with in vivo and in vitro studies in other models [25,40].

Since HMGB1 has been considered for a long time only as a cell death marker released passively by necrotic cells [41], we have also performed cytotoxicity assay by measuring lactate dehydrogenase (LDH) release. In our experiments, the level of LDH was low and the same in all supernatants (control versus LPS), ruling out the possibility that LPS-induced HMGB1 secretion was due to cell lysis. This secretion is thus specifically attributable to inflammation and could be associated to an active and regulated secretion, different from the passive mechanism that occurs during cell death. Nevertheless, the HMGB1 basal detectable level noticed in control adipose tissue could probably be attributable to basal cell death in culture, at least partly.

Our data were totally new and led us to investigate which type of cells within adipose tissue was responsible for this HMGB1 upregulation. Indeed, adipose tissue is composed by different kind of cells: after collagenase digestion, we can separate the floating adipocytes and the pelleted stromal vascular fraction. These two cell populations were then plated separately and inflammation was induced through LPS incubation.
From our results, we can conclude that the general HMGB1 production observed in adipose tissue comes exclusively from cells from the stromal vascular fraction, insofar as ASCs were able to respond to inflammation by up-regulating HMGB1 gene expression and secretion in a time-dependant manner. Neither freshly cultured adipocytes, nor in vitro-differentiated adipocytes showed a...
Moreover, no one has elucidated how this nuclear HMGB1 is secreted because HMGB1 does not have a leader sequence to follow the classical pathway of endoplasmic reticulum and Golgi apparatus [52]. For instance, in LPS-activated monocytes, it has been shown that once released from nucleus, HMGB1 is accumulated in lysosomal vesicles [53,54], whereas it is spread in all cytoplasm in mouse fibroblasts [53]. This lysosomes exocytosis is triggered by lysophosphatidyl choline, which is generated later under inflammation [54]. Nevertheless, it has been suggested that most of the early release (until 12 h) is from preformed HMGB1 (probably in nucleus) inactivated mouse RAW264.7 cells [43]. From our results, although no cytoplasmic vesicle was seen, we need additional experiments to well understand this mechanism in this new model.

5. Conclusions

In summary, we propose through our study that HMGB1 is more expressed in adipose tissue from obese persons and that this dysregulation is attributable to inflammation. We have also identified ASCs as the major source of HMGB1 in adipose tissue during inflammation. In the context of using HMGB1 as a therapeutic target to prevent and treat obesity associated diseases [55], further studies have to be investigated on human adipose cells to study what are the signal transduction pathways and the paracrine or autocrine effects due to this damage signal.

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