Brief Communication

MOLECULAR METABOLISM

Osteopontin is a key player for local adipose tissue macrophage proliferation in obesity

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ABSTRACT

Objective: Recent findings point towards an important role of local macrophage proliferation also in obesity-induced adipose tissue inflammation that underlies insulin resistance and type 2 diabetes. Osteopontin (OPN) is an inflammatory cytokine highly upregulated in adipose tissue (AT) of obese and has repeatedly been shown to be functionally involved in adipose-tissue inflammation and metabolic sequelae. In the present work, we aimed at unveiling both the role of OPN in human monocyte and macrophage proliferation as well as the impact of OPN deficiency on local macrophage proliferation in a mouse model for diet-induced obesity.

Methods: The impact of recombinant OPN on viability, apoptosis, and proliferation was analyzed in human peripheral blood monocytes and derived macrophages. Wild type (WT) and OPN knockout mice (SPP1KO) were compared with respect to *in vivo* adipose tissue macrophage and *in vitro* bone marrow-derived macrophage (BMDM) proliferation.

Results: OPN not only enhanced survival and decreased apoptosis of human monocytes but also induced proliferation similar to macrophage colony stimulating factor (M-CSF). Even in fully differentiated monocyte-derived macrophages, OPN induced a proliferative response. Moreover, proliferation of adipose tissue macrophages in obese mice was detectable in WT but virtually absent in SPP1KO. In BMDM, OPN also induced proliferation while OPN as well as M-CSF-induced proliferation was similar in WT and SPP1KO.

Conclusions: These data confirm that monocytes and macrophages not only are responsive to OPN and migrate to sites of inflammation but also they survive and proliferate more in the presence of OPN, a mechanism also strongly confirmed *in vivo*. Therefore, secreted OPN appears to be an essential player in AT inflammation, not only by driving monocyte chemotaxis and macrophage differentiation but also by facilitating local proliferation of macrophages.

Keywords OPN; Obesity; Inflammation; Adipose tissue; Adipose tissue macrophage

1. INTRODUCTION

Macrophage infiltration of the adipose tissue (AT) is a hallmark of the so-called obesity-associated low-grade inflammation that occurs in obesity and drives insulin resistance and development of type 2 diabetes. *In situ* proliferation of adipose tissue macrophages (ATMs) has been shown to take place at early stages of obesity and is associated with different cytokines [1,2]. This topic has become in vogue in recent years as an increasing number of studies described ATMs accumulation as the main driver of obesity-associated inflammation. Macrophages were shown to proliferate in atherosclerotic plaques [3], another inflammation-driven disorder. They also manifest increased proliferation in AT in response to cytokines such as monocyte chemoattractant protein-1 (MCP-1) and interleukin 4 (IL-4) [1,4]. However, prerequisite mediators for AT macrophage proliferation in obesity have not yet been found.

Osteopontin (OPN) is a secreted glycoprotein involved in a wide variety of physiological and pathological conditions, including inflammatory processes [5–7]. OPN was found to be expressed in different cell types such as activated macrophages and T-cells, epithelial cells, and osteoclasts [8,9]. It contributes to mineralization of bones and kidney, tumor development and metastasis, and atherosclerosis [10]. OPN is actively expressed and secreted in macrophages at sites of inflammation, playing an important role in cell-mediated immunity [11,12]. An intracellular variant has also been described in cytoplasm and nucleus, with biological functions different from the secreted form and involved in signaling transduction pathways and cytoskeletal rearrangements [13,14]. OPN is also described as a migratory cytokine for monocytes and macrophages [15] and has also been shown to act as a survival factor for monocytes [16], while neutralizing of OPN resulted in increased macrophage apoptosis in AT and liver of obese animals [17]. A link between OPN and inflammation, obesity, and insulin resistance

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became well described during recent years [18,19]; however a putative involvement of OPN in macrophage proliferation remains unexplored.
In the present work, we aimed at identifying the effects of OPN on monocyte and macrophage proliferation and their relevance in the context of obesity-driven AT inflammation. We focused at the beginning on proliferation and survival in human monocytes, discovering that OPN-treated cells outnumbered controls while diminishing apoptosis in the same experimental set up. Furthermore, OPN enhanced proliferation rates not only in human peripheral blood monocytes but also in *in vitro* differentiated macrophages. Notably, macrophages expressing proliferation marker Ki67 were virtually absent in genetically OPN-deficient (SP1KO) obese mice. Hence, local macrophage proliferation in obese AT is facilitated by OPN, thereby pointing to a novel mechanism that might trigger and maintain low-grade inflammation in obesity.

2. MATERIALS AND METHODS

2.1. Isolation and culture of human monocyte and macrophages

Monocytes were obtained from peripheral blood of healthy individuals by using a density gradient centrifugation (GE Healthcare, Little Chalfont, United Kingdom) and separated with a CD14-positive magnetic activated cell sorting — MACS (Miltenyi Biotech, Bergisch Gladbach, Germany) according to manufacturer's protocol. Human monocytes were differentiated to macrophages for 6 days in presence of 50 ng/ml M-CSF [20,21] (Peprotech, Rocky Hill, NJ, USA). The study was approved by the local ethics committee (EK 1241/2015).

2.2. Viability and apoptosis assays

Survival of monocytes was determined by using Cell Titer-Glo Luminescent Cell Viability Assay (Promega, Wisconsin, USA). Briefly, 1×10^5 cells were seeded in a 96-well plate, incubated with either OPN (1 μg/ml) or 50 ng/ml M-CSF as viability control or left untreated for 48 h. A total of 50 μl buffers were added, according to manufacturer's instructions. The plate was then shaken for 2 min and luminescence was detected by a plate reader (EnSpire, Perkin Elmer). To test for apoptosis, freshly isolated monocytes were seeded in a concentration of 1×10^6/well into 24-well plates. Cells were incubated with either 1 μg/ml OPN, 50 ng/ml M-CSF or left untreated for 24—48 h. Apoptosis was determined by TUNEL (Terminal deoxynucleotidyl transferase deoxyuracil triphosphate nick end labeling) assay using the In Situ Cell Death Detection Kit, Fluorescein (Roche, Basel, Switzerland). Cell suspensions were analyzed in flow cytometry quantifying integrated Fluorescein-dUTPs.

2.3. Proliferation assay

Monocytes were seeded in 24-well cell plates with normal RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) for 24 h; the following day, they were stimulated with 1 μg/ml of recombinant full length OPN (PeproTech). After 24 and 48 h stimulation, cells were harvested and prepared for CSFE Cell trace proliferation kit (Thermo Fisher Scientific). Briefly, cells were centrifuged, washed twice with cold PBS, resuspended in PBS with a final concentration of CSFE cell trace of 1 μM, and incubated for 1 h at 37 °C. Afterwards, cells were washed twice with cold PBS and resuspended in warm DMEM (Thermo Fisher Scientific) for 10 min at RT in the dark and washed and resuspended again in cold PBS and proceeded to flow cytometric analysis. Flow cytometry was performed with BD FACSCanto™ II and BD FACSDiva™ software (Becton Dickinson New Jersey, USA).
For human macrophages, proliferation was followed up to 48 h with a live cell movie analyzer (Juli Br, NanoEnTeck Inc., Seoul, Korea), which took pictures of the indicated area every 5 min, generating a video and quantitative output. Control, untreated wells were compared and recorded at the same time with the OPN stimulated ones (1 μg/ml).

2.4. Diet induced obesity mouse study

Eight male WT (C57BL/6J) and eight male OPN-knockout mice (SPP1KO; B6.Cg-Spp1tm1Blh/J) were purchased by Charles River (Sulzfeld, Germany) and fed a high-fat diet (HFD, 60 kcal %, D12492; Research Diets, New Brunswick, NJ, USA) for 8 or 12 weeks. After the indicated time, mice were sacrificed and gonadal white adipose tissue (GWAT) was collected. The protocol was approved by the local ethics committee for animal studies.

2.5. Immunohistochemistry

Formalin-fixed GWAT was sectioned, de-paraffinized, and blocked for 60 min in blocking buffer (PBS, 5% normal goat serum, 0.3% Triton™ X-100). Blocking buffer was aspirated and sections were incubated overnight at 4 °C with 1:200 dilution of the monoclonal rat anti-mouse MAC-2 antibody (Cedarlane labs, Burlington, Canada) in PBS with 5% normal goat serum (Dako, Glostrup Municipality, Denmark). Slides were washed three times in PBS and incubated for 1 h at RT with 1:500 dilution of the monoclonal rabbit anti-mouse Ki67 antibody (Abcam, Cambridge, United Kingdom) in PBS with 5% normal goat serum (Dako). Slides were then washed three times in PBS and incubated for 1 h at RT in the darkness with 1:500 dilution of the Alexa Fluor 488 goat anti rat IgG for MAC-2 (Thermo scientific). The process was repeated with Alexa Fluor 594 goat anti rabbit IgG for Ki67 (Thermo scientific). Nuclei were counterstained with DAPI (1 μg/ml in ddH_2O) for 10 min, washed three times in PBS, and mounted (VECTASHIELD® Mounting Medium for fluorescence, Vector Laboratories, Burlingame, CA USA) for microscope analysis (EVOS® FLoid® Cell Imaging Station) [22].

2.6. Bone marrow derived macrophages isolation

In order to isolate bone marrow derived macrophages (BMDMs), 6 WT and 6 SPP1KO mice were sacrificed, disinfected; femurs were extracted and cleaned with bone cleansing solution (PBS, 1% BSA, 100 μg/ml streptomycin, 100 U/ml penicillin, 100 μg/ml Amphotericin B) and seeded in a 12 well plate at a concentration of 10×10^4 cells per well for 6 days with Mφ diff. medium (DMEM, 10% FBS, L929 conditioned medium, 100 μg/ml streptomycin, 100 U/ml penicillin, 2 mM L-glutamine, 50 μM β-mercaptoethanol). On day 6, medium was changed to normal medium with either 50 ng/ml murine M-CSF (Peprotech) or murine OPN 1 μg/ml (Sigma—Aldrich, St. Louis, Missouri, USA). The following day, cells were harvested and prepared for CSFE Cell trace proliferation kit as previously described (Thermo Fisher Scientific).

2.7. Statistics

Data are presented as mean values ± standard error of the mean (SEM), and significance was assessed by Student's t-test. Dunnett-T *post-hoc* testing was employed to compare 2 different treatments to the same control. A p-value <0.05 was considered statistically significant. All statistics were calculated using SPSS 22.0 software (Chicago, IL, USA).

3. RESULTS

3.1. Human primary monocytes proliferate in presence of OPN

Monocytes are recruited to inflamed tissues such as obese AT and differentiate into macrophages in response to several stimuli [23]. OPN was previously described to be an anti-apoptotic factor for human

