Placenta 34 (2013) 231–239

Contents lists available at SciVerse ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Immunoregulatory cytokines in mouse placental extracts inhibit in vitro osteoclast differentiation of murine macrophages

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ARTICLE INFO

Article history:
Accepted 30 December 2012

Keywords:
Osteoclastogenesis
Placental extracts
Cytokines
NFAT

ABSTRACT

Introduction: Previous studies showed that placental extracts (PE) alleviates arthritic symptoms in animal models of arthritis.
Methods: To evaluate whether murine PEs obtained at embryonic days 7.5 (PE7) and 17.5 (PE18) regulate RANKL-induced osteoclast differentiation, RAW 264.7 cells were cultured with RANKL and MCSF in presence or not of PEs. Tartrate-resistant acid phosphatase (TRAP) was stained and multinucleated TRAP positive cells were visualized under a light microscope. *Cathepsin K* and metalloprotease expression was assessed by RT-PCR and gelatin zymography respectively. NFATc1 expression was determined by immunoblot. To analyze NFAT-dependent transcription, macrophages were transfected with a luciferase reporter plasmid. Cytokines were determined in PEs by ELISA and immunoblot. Transforming growth factor (TGF)- beta and Interleukin (IL)-10 receptor were inhibited in cell cultures with specific antibodies.
Results: PE7 and PE18 inhibited RANKL-induced multinucleated TRAP positive cells, *Cathepsin K* expression and metalloprotease activity, as well as NFATc1 expression and activity, thereby inhibiting osteoclast differentiation of RAW cells. Inflammatory/Regulatory cytokine ratio was higher in PE7 than in PE18. Blocking TGF-beta abolished the effect of both, PE7 and PE18, on multinucleated TRAP positive cells and metalloprotease expression, whereas blocking IL-10 receptor reverted the effect of PE18 but not of PE7.
Discussion: Inhibition of osteoclast differentiation by PEs was not unexpected, since cytokines detected in extracts were previously found to regulate osteoclast differentiation.
Conclusions: PEs inhibited osteoclast differentiation of macrophages in vitro. Downregulation of NFATc1 might be involved in this effect. Regulatory/Th2 cytokines play a role in the effect of PEs on osteoclast differentiation.

1. Introduction

A beneficial effect of pregnancy on clinical symptoms has been observed in several Th1-mediated autoimmune diseases (reviewed in Ref. [1]).

Pregnancy is viewed as a Th2-predominant state, although several Th1-related cytokines are vital to early pregnancy [2]. Th2 cytokines are associated with the down-regulation of Th1 cytokines and may confer protection from Th1-mediated autoimmune diseases.

Placental derived-immunoregulatory factors have been described so far [3–6]. We have previously reported that rat placental culture supernatant improves clinical symptoms in an experimental model of arthritis in rats [7].

Placental extracts (PE) have been traditionally used to treat chronic inflammatory diseases in Oriental medicine. It was reported that PE alleviated the arthritic symptoms in adjuvant-induced arthritis [8]. Furthermore, it was described that PE inhibited IL-1β- induced bone resorption in mouse, as measured by ^{45}Ca release [9], and protected cartilage from degradation, in experimental osteoarthritis [10].

Bone resorption is performed by osteoclasts, multinucleated giant cells, originated mainly from monocytes. They undergo a series of differentiation steps: acquire specific markers such as tartrate-resistant acid phosphatase (TRAP), fuse to multinucleated giant cells, and polarize upon contact with bone [11]. Essential

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http://dx.doi.org/10.1016/j.placenta.2012.12.015

signals for osteoclast differentiation are macrophage colony-stimulating factor (M-CSF) and Receptor Activator of Nuclear factor (NF)-κB (RANK)- ligand (RANKL) [12]. The induction of the transcription factor Nuclear Factor of Activated T cells (NFAT) plays a major role [13,14].

Based on the protective role of pregnancy during arthritis, we ask whether protein extracts obtained from mouse placenta at different days of pregnancy could modulate osteoclast differentiation of monocyte precursors.

2. Methods

2.1. Animals

BALB/c mice, 5 wk of age, were maintained at our Institutional Animal Care Facility. All procedures were conducted under consent of the Committee on the Ethics of animal experiments of "Instituto de Estudios de la Inmunidad Humoral Prof. R. Margni", in accordance with guidelines of EU Directive 2010/63/EU.

2.2. Isolation of placental tissue

Conceptuses were collected from timed-pregnant mice on embryonic days E7.5 and E17.5 (vaginal plug on day 0.5). Five mice were employed per group, and tissue from each mouse was processed separately. Fetus and decidua basalis were discarded from conceptuses and the remaining placental tissue was isolated. To note is that the developing placenta is not readily separated from the decidual components at day E7.5.

2.3. Preparation of isogeneic placental extracts

The placental tissue was washed twice in Tris buffered saline, (TBS), pH 7.4 and homogenized manually in TBS containing protease inhibitor cocktail (Roche, Buenos Aires, Argentina). The homogenate was exposed to 4 freeze–thaw cycles and after centrifugation (6000 g, 20 min, 4 °C) the supernatant was collected and sterilized with 0.2 micron filters. Total protein concentration was determined by the Bradford Assay (Bio Rad Laboratories, CA, USA). The aqueous placental extracts (PEs) were aliquoted and stored at −80 °C.

2.4. Cell cultures

The murine monocyte cell line RAW 264.7 differentiates into osteoclast like cells in the presence of RANKL and M-CSF [15]. Cells were grown in α-minimum essential medium (Gibco BRL, Rockville, MD, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Natocor, Córdoba, Argentina), 1% (v/v) penicillin–streptomycin solution, 1 mM sodium pyruvate, 2 mM L-glutamine, and 0.1 mM non-essential amino acids (all from Gibco) at 37 °C in a humidified atmosphere of 5% CO_2. For osteoclastogenesis experiments, cells were seeded in either 100 mm^2 culture plates or 24 well plates at a density of 2.5×10^6/plate or 5×10^3/well respectively, and cultured during the indicated times in the presence or absence of 100 ng/ml of RANKL and 20 ng/ml of M-CSF (both from Chemicon, MA, USA). Concentrations of RANKL and M-CSF were as previously described [15].

To asses the effect of PE on osteoclast differentiation, cells were pre-cultured for 1 h in the presence or absence of 50 μg/ml of either, PE obtained at E7.5 (PE7) or at E17.5 (PE18), before RANKL and M-CSF treatment and were present throughout the experiments. The concentration of PE used was selected as the maximal concentration at which cell viability of RAW 264.7 was not affected (Fig. 1).

2.5. Tartrate-resistant acid phosphatase (TRAP) staining

Cells plated at $5 \times 10^3/cm^2$ were cultured in the presence or absence of RANKL, M-CSF, and PEs, during 5 days, with a medium change on the third day. TRAP staining was performed using a commercially available kit (Sigma Aldrich, St. Louis, MO, USA). Cells were visualized in a light microscope. The number of TRAP Positive Multinucleated Cells (TPMC, osteoclast like-cells) per microscopic field was determined, out of 25 fields for each condition.

2.6. Cell viability

The WST-1 tetrazolium salt was employed under manufacturer's instruction (Roche, Buenos Aires, Argentina). Optical density was measured at 450 nm in a microplate reader (Multiscan EX, Thermo Fischer Scientific, Rockford, IL, USA).

2.7. RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Cells were cultured in the presence or absence of stimuli, for 48 h. Total RNA was isolated from the cells with TriZol isolation reagent (Invitrogen, Grand Island, NY, USA) and transcripts encoding mouse *Cathepsin K* and β-*actin* were analyzed by semiquantitative RT-PCR. Primers for PCR amplification were: *Ctpsk* forward, 5′-GAGGGCCAACTCAAGAAGAA -3′; reverse, 5′- GCCGTGGCGTTATACATACA -3′; *Actinb* forward, 5′-GTCGACAACGGCTCCGGCA-3′; reverse, 5′-GTCAGGTCCCGGCCAGCCA-3′. Amplified cDNAs were separated by agarose gel electrophoresis, and bands were visualized by ethidium bromide staining.

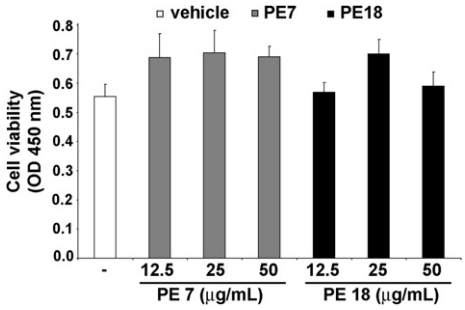

Fig. 1. Cell viability of RAW 264.7 cells cultured with extracts of placental tissue obtained from mouse at day 7.5 (PE7) and 17.5 (PE18) of pregnancy. Cells were seeded in a 96 well plate at $1 \times 10^4/cm^2$ and cultured during 48 h with 0, 12.5, 25, and 50 μg/ml of PE7 (open bars) or PE18 (closed bars). WST-1 tetrazolium salt reagent was added to cultures during the last 90 min. Optical density was measured at 450 nm. PEs showed no effect on the viability of the cells. Data are expressed as mean ± SD from triplicate cultures. Shown is a representative experiment of three performed.

2.8. Gelatin zymography

Cells were seeded in either 1% or 0% fetal bovine serum-containing culture medium and cultured for 48 h in the presence or absence of stimuli. Conditioned medium was analyzed for MMP-9 activity by gelatin substrate gel electrophoresis [16], and quantified by densitometric analysis using the NIH Image Program.

2.9. Cell lysis and NFATc1 immunoblot analysis

Macrophages were cultured in the presence or absence of RL + M, during 96 h. To asses the role of PEs on NFAT expression, cells were precultured with either PE7 or PE8 for 1 h before RANKL plus M-CSF (RL + M) stimuli.

Macrophages were lysed in a hypertonic buffer and NFATc1 was assessed in the total extracts by immunoblot as described [17].

2.10. Plasmid constructs and transient transfection assays

The luciferase reporter plasmid NFAT/AP1 luc was as described [18]. Null *Renilla* was from Promega (Madison, WI, USA). Cells were transfected by the FuGene HD method (Roche Applied Sciences, Penzberg, Germany) in accordance with the manufacturer's instructions. Forty eight hours later, the cells were exposed to vehicle or placental extracts (PE7, PE18) for 1 h and then treated with RL + M for 24 h. Cells were lysed according to the instructions of the Dual Glo Luciferase assay kit (Promega), and luciferase activity was measured in a luminometer (Victor X Plate Reader, PerkinElmer, Waltham, MA, USA). Results were normalized to a *Renilla* luciferase internal control.

2.11. Cytokine assays

Cytokine-specific ELISA (BD Biosciences, San Jose, CA) was used for detecting IL-4, IL-6, IL-10, IL-12 and IFN-γ concentrations in PE7 and PE8, according to the manufacturer's instructions.

PE7 and PE18 were assessed for TGF-β by immunoblot. Placental extracts were resolved by 12% SDS-PAGE under reducing conditions. Proteins transferred to nitrocellulose membranes were immunoblotted with goat polyclonal anti-TGF-β or goat polyclonal anti- β-actin antibodies, and then incubated with a peroxidase labeled secondary antibody (all from Santa Cruz Biotechnology, Delaware, CA, USA). Bound antibodies were detected by ECL (Pierce).

2.12. Inhibition studies

Anti-IL-10R neutralizing antibody (BD Biosciences) was added to cells 30 min before incubation with PEs, and RL + M. Anti-TGF-β blocking antibody was incubated for 30 min with PEs and then, antibody-treated PEs were added to macrophages and cultured for 30 min before stimulation with RL + M. Medium and stimuli were replaced at day 3. After 5 days of culture cells were stained for TRAP. Metalloprotease (MMP) activity was assessed by gelatin zymography after 2 days of culture.