Molecular Immunology 45 (2008) 3330–3337

Contents lists available at ScienceDirect

Molecular Immunology

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

Lipopolysaccharides (LPS) induce the differentiation of human monocytes to osteoclasts in a tumour necrosis factor (TNF) α-dependent manner: A link between infection and pathological bone resorption

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ARTICLE INFO

Article history:
Received 13 February 2008
Received in revised form 23 April 2008
Accepted 24 April 2008

Keywords:
Osteoclasts
Monocytes
LPS
TNFα
Bone infection
Bone resorption
U937

ABSTRACT

The degradation of bone is a serious consequence of persistent bacterial infection, including periodontitis, infection-associated non-unions or osteomyelitis. To test the hypothesis that infection and inflammatory conditions promote the differentiation of monocytes to bone-resorbing osteoclasts, highly purified monocytes, or alternatively, cells of the promyeloid cell line U937, differentiated to monocyte-like cells, were cultivated in the presence of lipopolysaccharides (LPS) for up to 30 days. After 2–4 days, a massive aggregation of the cells was observed, after 15–20 days multinuclear cells with the morphological characteristics of osteoclasts became apparent. These cells expressed the osteoclast-typical proteins tartrate-resistant acid phosphate (TRAcP) and cathepsin K. Moreover, these cells formed resorption pits on calcium phosphate coated cover slips or ivory slices. To test whether the differentiation of the monocytes to osteoclast-like cells was mediated by tumour necrosis factor α (TNFα) secreted by the cells in culture, an antibody directed against TNFα was added together with LPS. Differentiation to osteoclast-like cells was inhibited, suggesting a paracrine effect of locally produced TNFα. In conclusion, we propose that local bacterial infections could create a microenvironment that promotes the generation of bone resorbing cells, which, in turn, could contribute to the infection-associated osteolysis.

1. Introduction

Loss of bone is a serious complication of localised bacterial infections of bones or the adjacent tissue, and is a key feature of caries, periodontitis or osteomyelitis. To establish a link between bacterial infection on one hand and loss of bone on the other, we tested the hypothesis that the infection might create a microenvironment that would promote the generation of osteoclasts, the only cells known so far, to be able to degrade bone.

Osteoclasts are derived from haematopoietic stem cells and share the myeloid precursor cells with monocytes, macrophages, polymorphonuclear neutrophils, and dendritic cells. Exogenous stimuli, notably cytokines, direct the lineage development by activating the appropriate signal transduction pathways and transcription factors (Asagiri and Takayanagi, 2007; Roodman, 2006; Lee and Kim, 2003; Tanaka et al., 2003). Crucial for the development of osteoclasts are colony-stimulating factor (M-CSF-1) and the receptor activator of nuclear factor κb ligand (RANKL), which are provided by stromal cells as shown in mouse bone marrow cell cultures (reviewed in Theill et al., 2002; Teitelbaum, 2000). Stromal cells, however, are not essential for osteoclast generation. Numerous studies showed that cells with the appearance and functional activity of osteoclasts could be generated in vitro by various cytokines, including soluble RANKL, granulocyte-macrophage colony stimulating factor, interleukin (IL)-1 and IL-3, or TNFα (Boyle et al., 2003; Kudo et al., 2002; Matayoshi et al., 1996). Moreover, it was recognised that not only the myeloid precursor cells, but also monocytes and immature dendritic cells can differentiate to osteoclasts when cultured with selected cytokines (Rivollier et al., 2004; Bendre et al., 2003; Danks et al., 2002). Differentiation involves the fusion of the myeloid cells and results in a multinuclear giant cell with distinct morphology and with bone resorbing capacity.

In addition to the physiological process of bone remodelling a pathological loss of bone occurs in association with bone metastasis

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0161-5890/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.molimm.2008.04.022

of tumours, in chronic inflammatory diseases, particularly rheumatoid arthritis, and – as pointed out above – in persistent bacterial infections (Walsh and Gravallese, 2004; Henderson and Nair, 2003; Graves et al., 2001; Plunkett and Rubens, 1999; Nair et al., 1996).

Lipopolysaccharides (LPS), a major component of the cell wall of Gram-negative bacteria, are potent inducers of bone loss, at least in mice (Chung et al., 2006; Sakuma et al., 2000; Dumitrescu et al., 2004; Abu-Amer et al., 1997; Orcel et al., 1993). Moreover, in co-cultures of primary mouse osteoblasts with bone-marrow derived haematopoietic cells, LPS initiated the osteoclast formation, and supported the survival and fusion of osteoclasts via a pathway independent of RANKL (Islam et al., 2007; Zhuang et al., 2007; Doucet and Lowenstein, 2006; Suda et al., 2002). Together, these data suggest an important role of LPS in osteoclastogenesis in mice.

In the present study, we addressed the question whether or not LPS might directly and independently of other exogenous stimuli induce the differentiation of human monocytes to osteoclasts. To exclude the possibility that mediators of other peripheral blood cells, particularly of T cells, contribute the effects seen, highly purified human monocytes were used in addition to the promyeloid cell line U937. U937 can be differentiated in vitro to cells with characteristics of monocytes, e.g. by phorbol 12-myristate 13-acetate (PMA) (Hass et al., 1989; Cannistra et al., 1987).

We now found that in response to LPS in vitro-generated monocyte-like cells and also the isolated monocytes differentiated further to cells with phenotypic and functional characteristics of osteoclasts.

2. Material and methods

2.1. Cells and cell culture conditions

U937 were obtained from ATCC (American Type Culture Collection, Manassas, USA). The cells were cultivated in RPMI–1640 cell culture medium (Invitrogen, Karlsruhe, Germany), supplemented with 10% FCS (PAN Biotech GmbH, Aidenbach, Germany) and 1% penicillin/streptomycin (Invitrogen) at 37 °C at 5% CO_2 in 160 ml cell culture flasks (Nunc, Wiesbaden, Germany). For differentiation, the cells were cultivated in 24-well plates (Nunc) at a density of 5×10^4 cells/ml/well and stimulated with PMA (Sigma–Aldrich, Seelze, Germany) at a concentration of 0.1 μg/ml for 2 days (days −2 to day 0). Then LPS from Salmonella enterica serotype minnesota (Sigma–Aldrich) was added at a concentration of 1 μg/ml, and culture was continued for up to 30 days. The medium was renewed at days 7, 14, 21, and 28, and LPS was added at these time points. For comparison, the differentiated cells were stimulated with tumour necrosis factor (TNF) α (3 ng/ml) (obtained from Immunotools Friesoythe, Germany). For functional inhibition of TNFα, two monoclonal antibodies to TNFα were used, one, purchased from R&D Systems (clone 6401) was used in a final concentration of 0.1 μg/ml, the other "Remicade" (Essex Pharma GmbH, München, Germany) in a final concentration of 1 mg/ml. The antibodies were added at day 0 together with the LPS. Inhibition of osteoclast formation was assessed by day 14 morphologically and by the functional resorption assays.

Monocytes were isolated from the peripheral blood of healthy donors (informed consent was obtained and the institutional guidelines were observed). The blood was layered on Ficoll (Linaris, Wertheim, Germany), centrifuged for 20 min at 2500 rpm, the mononuclear cell fraction was harvested and monocytes were obtained by adherence to plastic dishes. The cell fraction contained approximately 95% monocytes, as judged by cytofluorometry using an antibody to CD14 (AbD Serotec Düsseldorf, Germany). For differentiation to osteoclasts, the monocytes were cultivated in RPMI-1640, supplemented with FCS and penicillin/streptomycin in the presence of either LPS or TNFα.

2.2. Staining of cells for TRAcP

The protocol and kit from Sigma was used.

2.3. Detection of cathepsin K

Cells were fixed with 4% PFA solution for 5 min at room temperature, then incubated with an antibody to cathepsin K (Medicorp, Montreal, Canada) (6 μl) for 1 h. Following washing, binding of the antibody was visualised using the APAAP kit (DakoCytomation, Glostrup, Denmark). For comparison, mouse IgG was used.

2.4. Determination of bone resorption

Calcium phosphate-coated plates were obtained from BD biosciences (BD BioCoat™ Osteologic™, San Jose, USA) and resorption was determined by staining according to the protocol supplied by BD Biosiences (Technical Bulletin # 444). The plates were examined microscopically and photographed using a Canon EOS 350D digital camera. The resorbed areas in relation to the whole area were quantified using the Image J programme developed by Wayne Rasband (www.rsb.info.nih.gov/ij/). Six parallel plates were quantified a resorption was calculated as % of the whole plate.

Ivory was obtained from the Bundesamt für Umweltschutz (Bonn, Germany). The ivory was sliced in discs, about 500 μm thick using a low-speed saw (Leica SP 1600; Leica Microsystems, Wetzlar, Germany). The cells were cultivated on the ivory slices for 14–20 days. Resorption cavities were visualised by staining with toluidine blue. In brief, the slices were washed in PBS, incubated for 10 min with 5% sodium hypochlorite to remove the cells, and incubated with toluidine blue solution (20%) for 40 s (toluidine blue solution: 1 g of toluidine blue (Serva, Heidelberg, Germany) in sodium hydrogen carbonate, 3 g in 120 ml aqua dest. and 80 ml of glycerol (Roth, Karlsruhe, Germany)). The slices were examined using an inverted microscope (Leica DM IL).

2.5. RT-PCR

Total RNA was isolated from cultivated, stimulated U937, using High Pure RNA Isolation Kit (Roche, Mannheim, Germany), at days −2, 0, 2, 6, 9, 15, 20 and 30. cDNA was generated using the AMV Synthesis Kit (Roche). RT-PCR was performed using the primers and conditions listed below (Table 1). The primers were synthesised by GbR Hermann, Denzlingen, Germany. The RT-PCR products were visualised using a 2% agarose gel (MP Agarose, Roche) containing 1 μl/ml SYBER green (Invitrogen, OR, USA). As molecular markers, the markers II and V from Roche were used.

2.6. Determination of TNFα and expression of TNFα receptors expression

U937 were differentiated with PMA as described above and then cultivated further with LPS (1 μg/ml). Supernatants were harvested at days −2, 0, 1, 2 and 4 and accumulation of TNFα in the supernatant was determined by ELISA (R&D Systems, Wiesbaden, Germany). To quantify TNFα and TNFαR1 and TNFαR2 specific mRNA 10^6 cells, harvested at the respective days, suspended in 400 μl of lysis buffer from the MagnaPure mRNA Isolation Kit I (RAS, Mannheim, Germany) containing 1% (w/v) DTT. The mRNA was isolated with the MagnaPure-LC device using the mRNA-I standard protocol and reversely transcribed using AMV-RT and oligo-(dT)