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ERRATUM

Influence of receptor activator of nuclear factor (NF)-*k*B ligand (RANKL), macrophage-colony stimulating factor (M-CSF) and fetal calf serum on human osteoclast formation and activity

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ORIGINAL PAPER

Influence of receptor activator of nuclear factor (NF)-κB ligand (RANKL), macrophage-colony stimulating factor (M-CSF) and fetal calf serum on human osteoclast formation and activity

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Abstract Human osteoclast (OC) formation and activity was studied in cultures of peripheral blood mononuclear cells (PBMNC) from six healthy donors after stimulation with fetal calf serum (FCS), under the influence of the receptor activator of nuclear factor (NF)-κB ligand (RANKL) and the macrophage-colony stimulating factor (M-CSF). The results showed that selected FCS could stimulate OC formation without any medium supplementation with osteoclastogenic factors. The OC formation, investigated by quantification of multinucleated tartrate-resistant acid phosphatase-positive cells (TRAP+ cells), and the sensitivity of OC progenitors to RANKL and M-CSF, varied widely between individual donors. The OC resorption activity, measured in the ''pit-assay'' on dentine, was strictly dependent on the presence of RANKL and M-CSF in the medium and was also donor dependent. The considerable donor variability should be considered in culture studies investigating, e.g. the interactions of OC with biomaterials or the influence of cytokines, growth factors and drugs on osteoclastogenesis.

Keywords Human osteoclast culture · Donor variability · RANKL · M-CSF

Introduction

Osteoclasts (OC) are highly specialized multinucleated cells, which are responsible for bone resorption. They derive from hematopoietic cells of the monocyte/macrophage lineage. Interactions of OC precursors with osteoblasts/stromal cells are essential for OC differentiation. The most important factors modulating osteoclastogenesis are receptor activator of nuclear factor (NF)-κB ligand (RANKL), a member of the tumor necrosis factor (TNF)-ligand family, and osteoprotegerin (OPG). Both factors are expressed by osteoblasts. RANKL promotes OC development and survival by binding to a membrane-bound receptor, RANK on OC precursors. OPG is a RANKL antagonist, which can inhibit OC formation and bone resorption. Osteoclastogenesis also requires the presence of macrophage-colony stimulating factor (M-CSF), which is also released by osteoblasts. M-CSF binds to the c-fms receptor of early OC precursors modulating their proliferation, differentiation and survival (Boyle et al. 2003).

Generally, for in vitro studies human OC have been generated in co-cultures of hemopoietic cells and osteoblasts or stroma cells (Fujikawa et al. 1996). Since recombinant RANKL protein is commercially available, OC can be generated from human peripheral blood mononuclear cells (PBMNC) in the presence of RANKL and M-CSF, without co-culturing other cells types. OC formation is commonly quantified by the number of plastic adherent multinucleated tartrate-resistant acid phosphatase—positive cells (TRAP+ cells) in cultures of PBMNC (Buckley et al. 2005; Nicholson et al. 2000; Hodge et al. 2004; Husheem et al. 2005; Schilling et al. 2004; Susa et al. 2004; Atkins et al. 2005). However, the expression of TRAP and the multinucleation does not distinguish between formation of non-mature cells (TRAP+ cells) and mature, functionally active osteoclasts. Other markers such as cathepsin K, calcitonin receptor, type II carbonic anhydrase or vitronectin receptor can similar to TRAP, also be regarded as indicators of an OC phenotype.

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Definite evidence for mature OC is the proof of functional activity. OC activity can be assessed in the ''pit-assay'' by measuring the area of resorption lacunae formed in vitro, if PBMNC are cultured on calcified substrates like bone or dentin (Buckley et al. 2005). However, the culture conditions described in the literature differ in many aspects, e.g. the PBMNC density, the concentration of RANKL and M-CSF in the medium, and the supplementation with other factors like transforming growth factor (TGF-ß), tumor necrosis factor (TNF-α), or glucocorticoids (Buckley et al. 2005; Nicholson et al. 2000; Hodge et al. 2004; Husheem et al. 2005; Schilling et al. 2004; Susa et al. 2004). This makes the comparison of different studies extremely difficult. A better characterisation of the OC culture systems is mandatory. Therefore, the aim of the present study was to investigate the influence of different RANKL and M-CSF concentrations on the formation and activity of OC derived from different donors.

Materials and methods

Osteoclast formation assay

After approval from the ethical committee at the University of Ulm, the Red Cross Blood Bank Baden-Württemberg (Ulm, Germany) provided buffy coats obtained from the blood of 6 healthy anonymous donors. PBMNC were isolated using Histopaque®-1077 (Sigma-Aldrich; centrifugation 30 min at 400 g) and stored in liquid nitrogen. PBMNC of each donor were plated in triplicate in 96-well plates at $5 \times 10^5/cm^2$ in 100 µl of α-Medium (with 2.0 g/ml $NAHCO_3$; Biochrom) containing 10% selected fetal calf serum (FCS; Biochrom) and 2 mM L-Glutamine. Human OC formation in vitro is dependent on the appropriate selection of the FCS lot. Only 1 of 4 FCS lots tested in preliminary experiments supported OC formation. Therefore, this selected FCS was used in the present study. 10 ng/ml of M-CSF and 10 ng/ml of GM-CSF (Chemicon International; USA) were added for the first 24 h to the cultures as short-term treatment with these factors has been shown to stimulate cell proliferation and enhance OC formation (Hodge et al. 2004). After 24 h the medium was replaced by a medium supplemented with various concentrations of RANKL (10–40 ng/ml; Chemicon International, USA) and M-CSF (5–20 ng/ml). The cultures were incubated at 37°C in a humidified atmosphere containing 8.5% CO_2. Medium change was performed twice a week. After 20 days cells were fixed in 4% buffered formaldehyde and TRAP stained (Acid Phosphatase, Leukocyte Kit; Sigma-Aldrich, 387A) according to the manufacturer's instruction. TRAP+ multinucleated cells containing 3 or more nuclei were identified as OC and counted under the light microscope (Olympus IC70) at 400-fold magnification.

Osteoclast resorption assay—pit assay

Dentin (ivory) was kindly provided by the German customs in accordance with the international laws for protection of species. It was sliced with a low-speed, water-cooled diamond saw, sterilised in 70% ethanol and dentin slices ($8 \times 8 \times 0.7$ mm) were placed in 48-well culture plates. 5×10^5 PBMNC/cm^2 were seeded in 50 µl medium on dentin. Cells were allowed to attach for 2 h at 37°C before 200 µl M-CSF and GM-CSF (10 ng/ml each) containing medium were added. After 24 h the medium was removed and cultures were maintained for up to 28 days in 250 µl of medium containing RANKL (20–40 ng/ml) and M-CSF (10–20 ng/ml) with medium changed twice a week. Dentin slices were then washed in phosphate-buffered saline (PBS; Biochrom), ultrasonicated for 5 min to remove adherent cells and stained with 0.1% toluidine blue in PBS. Osteoclastic resorption was quantified by measuring the area of the blue dyed resorption pits with a digital image analysis system (Analysis 5.1, Soft Imaging Systems, Muenster, Germany). The stained dentin surface was imaged with a light microscope (Olympus BX60) at 50-fold magnification, resulting in 20 images for each slice. The digital images were median filtered and preprocessed with an edge enhancement filter. For each image, a grey level histogram was computed and a semi-automatic threshold algorithm depicted the stained area. The percentage of resorption was calculated by dividing the resorbed area by the total area of the imaged dentin surface.

Statistical analysis

Results are presented as mean ± standard deviation (SD) of triplicate cultures. Differences between groups were determined using a Student *t*-test. Statistical significance was set at $P < 0.05$.

Results

To ensure that the generated cells were indeed osteoclasts, we analysed the morphology of the cells, the expression of TRAP and the functional activity in the ''pit assay''. Figure 1A–C shows TRAP+, multinucleated cells after 21 days of cultivation. Resorption lacunae on dentin after 28 days are shown in the Fig. 1D–F.

All multinucleated cells expressed high levels of TRAP. The intensity of TRAP-staining did not vary between the

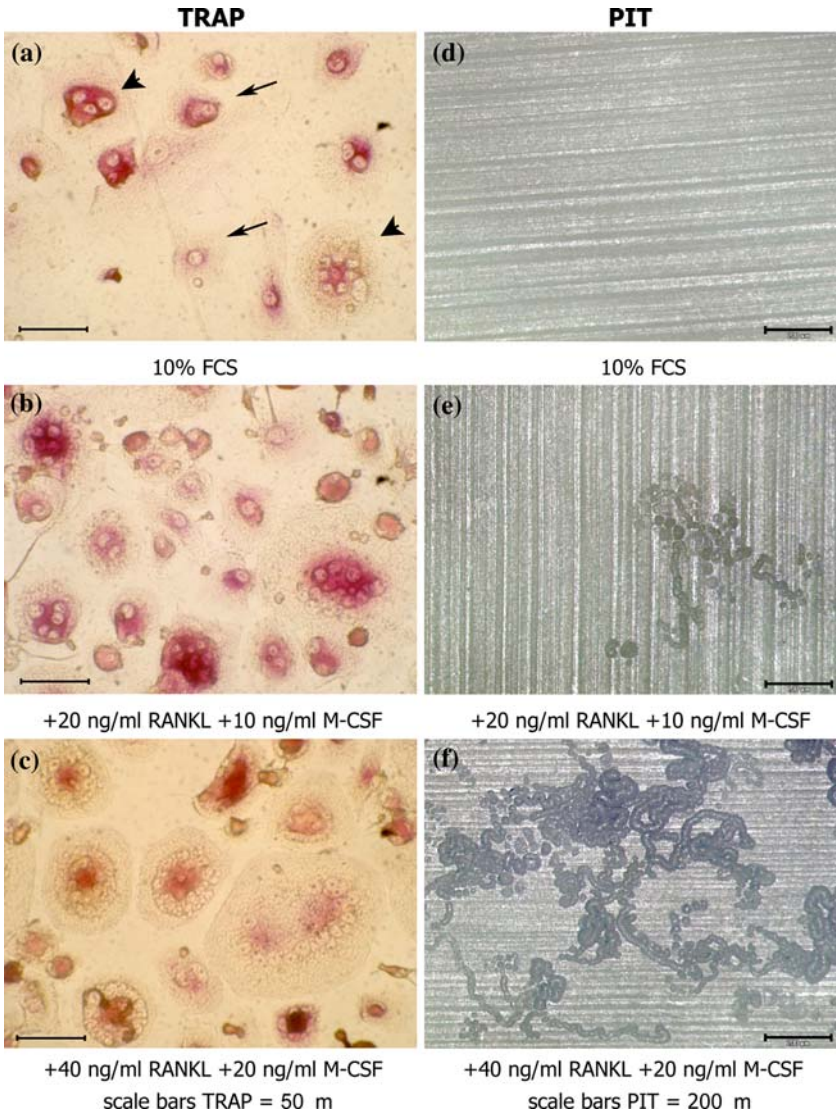

Fig. 1 Photomicrographs of TRAP-stained cells on plastic after 21 days of cultivation (**A**–**C**) and of toluidine blue-stained resorption lacunae (pits) formed on dentin after 28 days of culture (**E**–**F**). All multinucleated cells express TRAP (red). Multinucleated TRAP+ osteoclasts with 3 and more nuclei (indicated by arrowheads) were generated in the presence of 10% FCS (**A**), with 20 ng/ml RANKL and 10 ng/ml M-CSF (**B**) or with 40 ng/ml RANKL and 20 ng/ml M-CSF. Large osteoclasts containing more than 10 nuclei were formed in the presence of 40 ng/ml RANKL and 20 ng/ml M-CSF (**C**). TRAP+ cells with less than 3 nuclei (monocytes/macrophages) are indicated by arrows. Dentin was not resorbed in presence of 10% FCS (**D**). Small resorption pits were formed in presence of 20 ng/ml RANKL and 10 ng/ml M-CSF (**E**). Large pits were formed in presence of 40 ng/ml RANKL and 20 ng/ml M-CSF (**F**)

culture conditions. In the presence of 10% FCS or 20 ng/ml RANKL and 10 ng/ml M-CSF TRAP+ cells mostly contained less than 10 nuclei (Fig. 1A, B). At the concentration of 40 ng/ml RANKL and 20 ng/ml M-CSF the formation of numerous TRAP+ cells containing more than 10 nuclei was frequently observed (Fig. 1C). The TRAP+ cells were much larger compared to the culture with no or lower concentration of RANKL and M-CSF present.

TRAP+ cell formation was observed in 5 of 6 tested donors (Fig. 2). The number of TRAP+ cells in cultures supplemented with 10% FCS but without any additional factors varied in donor No. 1, 2, 3 and 5 between 69 ± 46 and 466 ± 20 cells/well. In donor No. 4 and 6, less than 10 TRAP+ cells per well were observed. The addition of 10 ng/ml RANKL and 5 ng/ml of M-CSF to the culture medium only significantly increased the number of TRAP+ cells in donors No. 3 and 6. Higher concentrations of RANKL and M-CSF did not further increase OC formation. In one case (donor No. 1), even a decrease in OC number was observed in the presence of the highest concentration of RANKL and M-CSF. This effect was associated with an increase in cell area observed in this donor, indicating that small TRAP+ cells fused to form larger cells when exposed to the higher concentration of growth factors.

In contrast to TRAP+ cell formation, the formation of resorption pits on dentine slices (OC activity) was not observed in cultures supplemented with FCS alone. The resorption activity in donor No. 1, 2, 3 and 5 was strictly dependent on the concentration of the added RANKL and M-CSF. No OC activity was observed in donor No. 4 and 6 (Table 1). In these donors, the TRAP+ cell formation was also very low. The cell response to RANKL and M-CSF is exemplarily presented for donor No. 2 in Fig. 3. At least

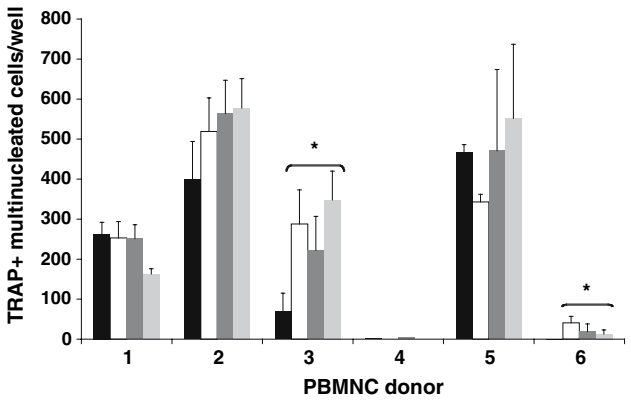

Fig. 2 Effect of growth factors on osteoclast formation of peripheral blood mononuclear cells (PBMNC) from different donors. Human osteoclasts quantified as TRAP+ multinucleated cells per well were generated from PBMNC in medium containing 10% fetal calf serum (FCS) alone or in medium supplemented with different concentrations of RANKL and M-CSF: black columns—10% FCS alone; white columns—10 ng/ml RANKL and 5 ng/ml M-CSF; grey columns—20 ng/ml RANKL and 10 ng/ml M-CSF; light grey columns—40 ng/ml RANKL; 20 ng/ml M-CSF. *indicates a significant difference ($P < 0.05$) to the culture with FCS alone

Table 1 Osteoclast activity measured in the pit assay on dentin slices in the presence of RANKL (40 ng/ml) and M-CSF (20 ng/ml)

Blood donor	% resorbed area/dentin slice (mean ± SD; $n = 3$)
1	2.0 ± 0.1
2	10.7 ± 3.0
3	8.2 ± 5.5
4	0
5	5.3 ± 1.3
6	0

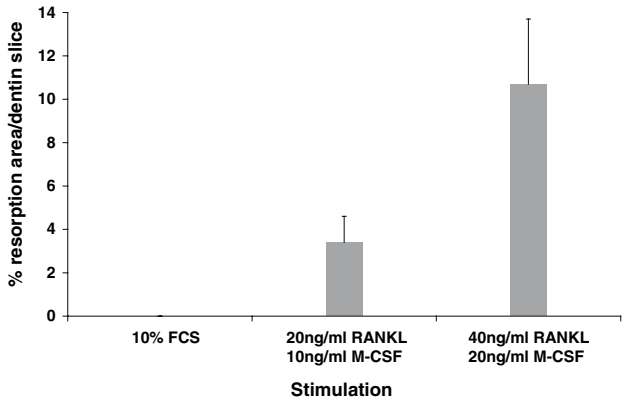

Fig. 3 Pit assay: Quantitation of dentin resorption (% resorption per dentine slice) in osteoclast culture of PBMNC from donor No. 2 stimulated with 10% FCS alone or with different concentrations of RANKL and M-CSF

20 ng/ml of RANKL and 10 ng/ml of M-CSF were needed to activate dentin resorption. In the presence of 20 ng/ml RANKL and 10 ng/ml M-CSF only small resorption pits were formed (Fig. 1E). Resorption lacune that formed in the presence of 40 ng/ml RANKL and 20 ng/ml M-CSF were much larger (Fig. 1F).

Discussion

Our study demonstrates that selected FCS could stimulate TRAP+ cell formation without any additional medium supplementation with osteoclastogenic factors. FCS contains an undefined mixture of hormones, growth factors, nutrients and many other molecules, with a considerable degree of interbatch variation. It cannot be excluded that RANKL and M-CSF are present in FCS in concentrations sufficient to support TRAP+ cell formation. FCS also may contain several other growth and differentiation factors (e.g., TGF-ß, TNF-α, Il-3, Il-6) which, together with RANKL and M-CSF, could influence the osteoclastogenesis (Husheem et al. 2005; Susa et al. 2004; Atkins et al. 2005; Leffert 1974; Quinn and Gillespie 2005).

The formation of TRAP+ cells in the presence of FCS alone varied widely between individual donors and was not generally increased under the influence of RANKL and M-CSF. A marked and significant effect of RANKL and M-CSF on TRAP+ cell formation was observed in only one donor (No. 3). Other authors also reported high donor variations in OC generation. Susa et al. found considerable individual variations in TRAP+ multinucleated cell generation and OC activity in cell cultures supplemented with RANKL, M-CSF, TGF-ß1 and dexamethasone (Susa et al. 2004). The effect of FCS alone on OC cultures has not been reported in this study. The reasons for the different ability of donors to form TRAP+ cells and active OC remain unknown. One reason for donor variations in osteoclastogenesis could be the variable composition and number of different subpopulations of OC progenitors in PBMNC samples derived from different donors. This could influence the capacity of individual PBMNC populations to form OC. PBMNC are a heterogeneous cell population containing monocytes, lymphocytes and other blood cells. The monocyte fraction, from which macrophages, osteoclasts, endothelial and dendritic cells are derived, is again heterogeneous. It has been reported that CD14, CD11b and CD61 positive monocytes have OC forming capacity (Husheem et al. 2005). CD14 positive monocytes have the highest potential to differentiate into bone-resorbing OC and are therefore, considered to be the most likely OC precursor candidate (Nicholson et al. 2000; Husheem et al. 2005). Another reason for the observed donor variation might be the different sensitivity of OC progenitor sub-

populations to RANKL, M-CSF and/or to other osteoclastogenesis modulating factors present in FCS. Additionally, hematopoietic cells and cells of the immune system present in PBMNC population could express various factors, e.g. GM-CSF, Il-6, and TGF-ß1, which might additionally influence OC formation and activity (Quinn and Gillespie 2005).

In contrast to TRAP+ cell formation, the OC activity evaluated in the ''pit assay'' on dentin was not observed in cultures supplemented with FCS alone. Similar results were reported by Faust et al. (1999). In this study, under simple culture conditions in presence of FCS alone without addition of growth factors, cytokines, steroids or stroma cells the OC bone resorption activity was very low and did not correspond to the large number of TRAP+ cells obtained. In the present study, medium supplementation with RANKL and M-CSF was necessary to induce active OC in four of six donors studied (No. 1, 2, 3 and 5). However, the formation of TRAP+ cells in these donors could be induced with FCS alone. Apparently, abundant TRAP+ cell formation could be induced with selected FCS. However, for the induction of functional OC, medium supplementation with high concentration of RANKL and M-CSF is mandatory. These results suggest that formation of the premature TRAP+ cells and the development of active OC might require different concentrations of RANKL and M-CSF.

In two of the six donors (No. 4 and 6), active OC could not be induced even after medium supplementation with RANKL and M-CSF and also the TRAP+ cell formation was extremely low. This could be due to the donor specific composition of PBMNC population containing low number of osteoclastic progenitors or progenitor subpopulations with low differentiation potential.

It can be concluded that formation of TRAP+ multinucleated cells could be induced by a selected FCS without medium supplementation with RANKL and M-CSF. The induction of mature, active OC was strictly dependent on the presence of high concentration of RANKL and M-CSF. Both OC formation and activity was donor dependent. The considerable donor variability in osteoclastogenesis should be considered in OC culture studies investigating, e.g. the role of osteoclasts in the resorption process of biomaterials or the influence of cytokines, growth factors and drugs on OC formation and activity.

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