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Characterisation of the Osteoclastogenic Potential of Human Osteoblastic and Fibroblastic Conditioned Media

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ABSTRACT

Although M-CSF and RANKL are sufficient to promote in vitro osteoclastogenesis, in vivo this is a complex process which requires the action of many signalling molecules and cellular crosstalks. In this work, isolated or combined conditioned media, obtained from human adult skin fibroblast and bone marrow cells, were tested for their osteoclastogenic potential, through an indirect co-culture system, in the absence of recombinant M-CSF and RANKL. Osteoclastogenesis was assessed on human peripheral blood mononuclear cells (PBMC) and CD14+ cell cultures by quantification of total protein content, tartrate-resistant acid phosphatase (TRAP) activity, presence of multinucleated cells positive for TRAP, RT-PCR of TRAP, CATK, CA2, c-myc and c-src and presence of multinucleated cells displaying actin rings, vitronectin and calcitonin receptors. Cultures supplemented with M-CSF and RANKL were used as positive controls. It was observed that the conditioned medium from dexamethasone osteogenic-induced bone marrow cell cultures displayed the highest osteoclastogenic potential, with similar behaviour to that observed in the presence of both M-CSF and RANKL. Comparatively, fibroblastic conditioned medium elicited a slightly lower osteoclastogenic response. Combination of both conditioned media resulted in a significant increase of TRAP activity. On the other hand, conditioned medium from non-osteogenic-induced bone marrow cell cultures presented the lowest osteoclastogenic potential. These results were observed for both PBMC and CD14+ cell cultures, suggesting that fibroblast and osteoblast cells are able to modulate osteoclastogenesis in the absence of physical cell–cell interactions. In addition, osteoclastogenic potential of bone marrow cells increases with their osteoblastic differentiation. J. Cell. Biochem. 109: 205–216, 2010.

KEY WORDS: OSTEOCLASTOGENESIS; CONDITIONED MEDIA; OSTEOBLAST; FIBROBLAST

Osteoclasts are cells specialised in bone resorption, which descend from the monocyte/macrophage lineage. They are multinucleated cells, with typically 4–8 nuclei, formed by fusion of their mononuclear precursors and subsequent differentiation, a process called osteoclastogenesis. It was shown that those precursors are found in bone marrow, as well as in peripheral blood [Suda and Takahashi, 1996]. Bone resorption is initiated by attachment of osteoclasts to bone surface mediated by podosomes, followed by sealing of bone matrix in roughly circular areas (sealing zones). In those sealing zones osteoclasts develop ruffled border membranes, through which they secrete HCl, to promote mineral dissolution, and lytic enzymes, such as cathepsin K, to degrade organic bone matrix [Boyce and Xing, 2008; Vaananen and Laitala-Leinonen, 2008].

Early studies suggested that in vitro osteoclastogenesis was only possible in co-cultures of spleen or bone marrow cells and osteoblasts or other stromal cells [Takahashi et al., 1988]. One decade after, it was identified and characterised one essential osteoclastogenic factor, receptor activator of nuclear factor-κB ligand (RANKL), that showed to be the same as the previous discovered factors osteoclast differentiation factor (ODF) and stromal osteoclast-forming activity (SOFA) [reviewed in Chambers, 2000]. Nowadays, it is accepted that there are two growth factors essential for in vitro osteoclastogenesis,

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monocyte-colony stimulation factor (M-CSF) and RANKL [Boyle et al., 2003].

M-CSF is a homodimeric glycoprotein produced by several cell types, like, for example, granulocytes, endothelial cells, fibroblasts, osteoblasts, lymphocytes and some tumoral cell lines [Kim et al., 2006; Koreny et al., 2006; Fujita and Janz, 2007; Nishiguchi et al., 2007; Kaku et al., 2008]. M-CSF is important for survival of cells from macrophage/osteoclast lineage, as well as for proliferation and fusion of osteoclast precursors [Bekker and Gay, 1990; Yoshida et al., 1990; Lagasse and Weissman, 1997; Pixley and Stanley, 2004]. RANKL, also known as TNF-related activation-inducing cytokine (TRANCE), is a homotrimeric protein, produced by osteoblasts, fibroblasts, bone marrow cells, lymphocytes, among others [Legendre et al., 2003; Walsh et al., 2005; Kim et al., 2006; Sato and Takayanagi, 2006; Fujita and Janz, 2007; Nishiguchi et al., 2007; Hashizume et al., 2008]. RANKL is recognised by receptor activator of nuclear factor-κB (RANK), a surface receptor expressed by osteoclast precursors. This RANK/RANKL interaction activates several signalling pathways which are crucial for osteoclastogenesis [reviewed in Boyce and Xing, 2008] by promoting fusion and activation of osteoclastic precursors and stimulating the expression of several osteoclastic markers, such as tartrate-resistant acid phosphatase (TRAP) and cathepsin K [Boyle et al., 2003; Boyce and Xing, 2008].

Nowadays, most of the in vitro studies with osteoclastic cells involve the utilisation of recombinant M-CSF and RANKL to promote osteoclastogenesis. However, in vivo, this is a complex process that requires the action of several growth factors and cellular interactions [reviewed in Boyce and Xing, 2008; Vaananen and Laitala-Leinonen, 2008]. Among them, the relevance of osteoblast lineage cells on osteoclast formation and development is well documented [Datta et al., 2008]. Also, fibroblasts appear to be important on osteoclastogenesis regulation, and their roles on osteoclast differentiation and function in inflammatory bone joint disorders have been documented [Walsh et al., 2005]. In this context, the aim of the present study is to characterise the osteoclastogenic potential of human osteoblastic and fibroblastic cells in the absence of direct cell–cell interactions. For that, conditioned media from adult osteoblastic and fibroblastic cell cultures, isolated or combined, were tested as potential promoters of osteoclastogenesis of human peripheral blood osteoclast precursors. This is expected to highlight paracrinic mechanisms between these cell types, which in addition to physical cell–cell interactions, are crucial for osteoclast formation and activation in the bone microenvironment. Although osteoclasts descend from the CD14+ monocyte lineage [Vaananen and Laitala-Leinonen, 2008], CD14+ cell isolation from peripheral blood mononuclear cells (PBMC) is time-consuming and requires several cellular manipulations, which can affect their viability/functionality. In order to assess the suitability of PBMC to function as an easily implementable model to address osteoclast issues in vitro, the experiments were performed in both PBMC and in the CD14+ monocyte PBMC subpopulation. Cell cultures performed in the absence or the presence of recombinant M-CSF and RANKL were used as negative and positive control, respectively. Osteoclastogenesis was assessed by several cellular and molecular biology techniques, namely, TRAP activity quantification, RT-PCR analysis of the expression of TRAP, cathepsin K (CATK), carbonic anhydrase 2 (CA2), c-myc and c-src, morphological characterisation and the presence of multinucleated cells positive for actin ring, vitronectin receptor (VNR) and calcitonin receptor (CTR).

MATERIALS AND METHODS

PREPARATION OF CONDITIONED MEDIA FROM FIBROBLAST AND OSTEOBLAST CELL CULTURES

Fibroblast cell cultures. Human adult skin fibroblasts (passages 5–8) were cultured (5×10^4 cell/cm^2) in α-minimal essential medium (α-MEM) containing 10% foetal bovine serum, 100 IU/ml penicillin (Gibco), 2.5 μg/ml streptomycin (Gibco), 2.5 μg/ml amphotericin B (Gibco) and 50 μg/ml ascorbic acid. Cultures were maintained in a 5% CO_2 humidified atmosphere at 37°C. Culture medium was replaced twice a week.

Bone marrow cell cultures. Bone marrow was obtained from patients (25–45 years old) undergoing orthopaedic surgery procedures after informed consent. Bone marrow cells were cultured in α-MEM containing 10% foetal bovine serum, 100 IU/ml penicillin (Gibco), 2.5 μg/ml streptomycin (Gibco), 2.5 μg/ml amphotericin B (Gibco) and 50 μg/ml ascorbic acid. Cultures were maintained in a 5% CO_2 humidified atmosphere at 37°C. Culture medium was replaced twice a week. Primary cultures were maintained for 10/15 days till near confluence. Adherent cells were enzymatically released with 0.04% trypsin and 0.025% collagenase and cultured (5×10^4 cell/cm^2) as described above. Second passage cells were cultured (5×10^4 cell/cm^2) in the presence or in the absence of 10 nM dexamethasone, respectively, +dex and −dex cell cultures.

Conditioned media. After reaching 40–50% confluence, fibroblast and osteoblast cell cultures were maintained for a further 7 days without medium change. Culture medium was collected, centrifuged at 550*g* for 10 min, aliquoted and stored at −20°C. The cell layers were assessed by RT-PCR for the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GADPH), and fibroblast-specific protein 1 (FSP-1), alkaline phosphatase (ALP), bone morphogenetic protein 2 (BMP-2), RANKL and M-CSF.

Conditioned media from human adult skin fibroblasts (conditioned media 1, CM1) and human bone marrow cells cultured in the presence (CM2) or in the absence (CM3) of 10 nM dexamethasone were used as a potential source of M-CSF and RANKL. In order to test the potential effect of foetal bovine serum on osteoclastogenesis, a negative control of conditioned media was performed (CM0), where the conditioned media were prepared exactly in the same way as the others, but in the absence of any cell type.

RT-PCR analysis. RNA was extracted from fibroblast and bone marrow cell cultures after recovering the conditioned media. RNA was isolated with RNeasy® Mini Kit (Qiagen) according to manufacturer's instructions. RNA was quantified by measuring the absorbance of the samples at 260 nm. The expression of GADPH, FSP-1, ALP, BMP-2, M-CSF and RANKL genes was performed by RT-PCR. Half microgram of total cellular RNA from each sample was reverse transcribed and amplified (25 cycles) with the Titan One Tube RT-PCR System (Roche), with an annealing temperature of 55°C. The primers used are listed on Table I. The RT-PCR products were analysed on a 1% (w/v) agarose gel.

TABLE I. Primers Used on RT-PCR Analysis of Cell Cultures Used as a Source of Conditioned Media

Gene	5′ Primer	3′ Primer
GADPH	5′-CAGGACCAGGTTCACCAACAAGT-3′	5′-GTGGCAGTGATGGCATGGACTGT-3′
FSP-1	5′-CTCTGGAGAAGGCCCTGGAT-3′	5′-TTCTTCCTGGGCTGCTTATC-3′
ALP	5′-ACGTGGCTAAGAATGTCATC-3′	5′-CTGGTAGGCGATGTCCTTA-3′
BMP-2	5′-GCAATGGCCTTATCTGTGAC-3′	5′-GCAATGGCCTTATCTGTGAC-3′
M-CSF	5′-CCTGCTGTTGTTGGTCTGTC-3′	5′-GGTACAGGCAGTTGCAATCA-3′
RANKL	5′-GAGCGCAGATGGATCCTAAT-3′	5′-TCCTCTCCAGACCGTAACTT-3′

Densitometric analysis of the bands obtained by RT-PCR was performed with ImageJ 1.41 software.

ISOLATION OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC) AND CD14+ CELLS

The cells were isolated from the blood of healthy donors with 25–35 years old, after informed consent. PBMC were isolated as described previously [Nicholson et al., 2000]. Briefly, blood was diluted with PBS (1:1) and applied on top of Ficoll-Paque™ PREMIUM (GE Healthcare Bio-Sciences). After centrifugation at 400*g* for 30 min, PBMC were recovered and washed twice with PBS. Typically, around 70×10^6 PBMC were obtained for each 100 ml of processed blood.

CD14+ cells were isolated from PBMC. For that, PBMC were incubated with MACS microbeads, conjugated with a monoclonal mouse antibody raised against human CD14, in order to label CD14+ cells. After labelling, CD14+ cells were separated from PBMC with the Magnetic Cell Sorting (MACS; Miltenyi Biotec), according manufacturer's instructions. Cells were eluted with PBS and resuspended in culture medium. The yield of CD14+ cells obtained from PBMC was about 5–10%.

CULTURE AND OSTEOCLASTIC ASSESSMENT OF PBMC AND CD14+ CELL CULTURES

PBMC were seeded at a density of 3×10^6 cells/cm^2 and CD14+ cells at 3×10^5 cells/cm^2. Cells were cultured in α-MEM (Gibco) supplemented with 30% (v/v) human serum (from the same donor where cells were obtained), 2 mM L-glutamine, 100 IU/ml penicillin (Gibco), 2.5 μg/ml streptomycin (Gibco), 2.5 μg/ml amphotericin B (Gibco), in the following experimental conditions: absence of recombinant growth factors and conditioned media (base medium); presence of both recombinant M-CSF (R&D Systems) and RANKL (Insight Biotechnology) (positive control); recombinant M-CSF; recombinant RANKL; isolated CM1-CM3; combined CM1 and CM2–CM3.

M-CSF and RANKL were used at a concentration of 25 and 40 ng/ml, respectively, based on previous published work [Nicholson et al., 2000]. CM1 was used at 10% (v/v) and CM2 and CM3 were used at 20% (v/v). The concentrations of the conditioned media were established from preliminary experiments in which PBMC and CD14+ cell cultures were supplemented with 10–20% and assessed for TRAP activity. In order to maintain the same foetal bovine serum final concentration (approximately 3%) in all experimental conditions, CM0 was included in culture media, when necessary. It was observed that the presence of 0–30% CM0 in culture media did not affect TRAP activity on PBMC and CD14+ cell cultures (Fig. 1). Cultures were maintained in a 5% CO_2 humidified atmosphere at 37°C for 21 days. Culture medium was replaced once a week.

At the end of the culture period, cell cultures were characterised for total protein content and for osteoclastic markers, namely, TRAP activity, RT-PCR and presence of multinucleated cells with actin rings, expressing VNR or CTR.

Protein and TRAP activity quantification. Cellular protein content was quantified by Bradford's method [1976], using bovine serum albumin as a standard. Shortly, PBMC and CD14+ cell cultures were washed twice with PBS and solubilised with 0.1 M NaOH. After addition of Coomassie® Protein Assay Reagent (Fluka) the samples were incubated for 2 min at room temperature and the absorbance was quantified at 600 nm in an ELISA plate reader (Denley, Wellscan WS050).

TRAP activity was assayed using *para*-nitrophenylphosphate (*p*NPP) as a substrate. PBMC and CD14+ cell cultures were washed twice with PBS and solubilised with 0.1% (v/v) Triton X-100. Cellular extracts were incubated with 12.5 mM *p*NPP in 0.04 M tartaric acid and 0.09 M citrate (pH 4.8) for 1 h at 37°C. After addition of 5 M NaOH, the absorbance of the samples was measured at 405 nm in an ELISA plate reader (Denley, Wellscan WS050). Results were expressed as $\mathrm{nmol/min\,mg^{-1}_{protein}}$.

Histochemistry staining of TRAP. PBMC and CD14+ cell cultures were fixed with 3.7% formaldehyde for 10 min and then washed with distilled water. The cells were stained for TRAP with Acid Phosphatase, Leukocyte (TRAP) kit (Sigma), according to manufacturer's instructions. Shortly, after fixation with formaldehyde cells were incubated with naphthol 0.12 mg/ml AS-BI, in the presence of 6.76 mM tartrate and 0.14 mg/ml Fast Garnet GBC, for 1 h at 37°C in the dark. Cell layer was washed, stained with haematoxylin and visualised by light microscopy. Cells multinucleated (4–8 nuclei) and positive for TRAP were counted from three replicas from three independent experiments.

RT-PCR analysis. PBMC cultures were assessed for the expression of GADPH, TRAP, CATK, CA2, c-myc and c-src genes by RT-PCR. The RT-PCR analysis was performed as described for the fibroblastic and osteoblastic cultures. The primers used are listed in Table II.

Visualisation of multinucleated cells with actin rings, VNR and CTR by confocal laser scanning microscopy (CLSM). PBMC cultures were washed twice with PBS and fixed with 3.7% (v/v) *para*-formaldehyde for 15 min. After fixation, cells were permeabilised for 5 min with 0.1% (v/v) Triton X-100 and stained for actin with 5 U/mL Alexa Fluor® 647-Phalloidin (Invitrogen), for nucleus with 500 nM propidium iodide and for VNR and CTR with 50 μg/ml mouse IgGs anti-VNR and IgGs anti-CTR (R&D Systems), respectively. Detection of IgGs anti-VNR and IgGs anti-CTR was performed with 2 μg/ml Alexa Fluor® 488-Goat anti-mouse IgGs.

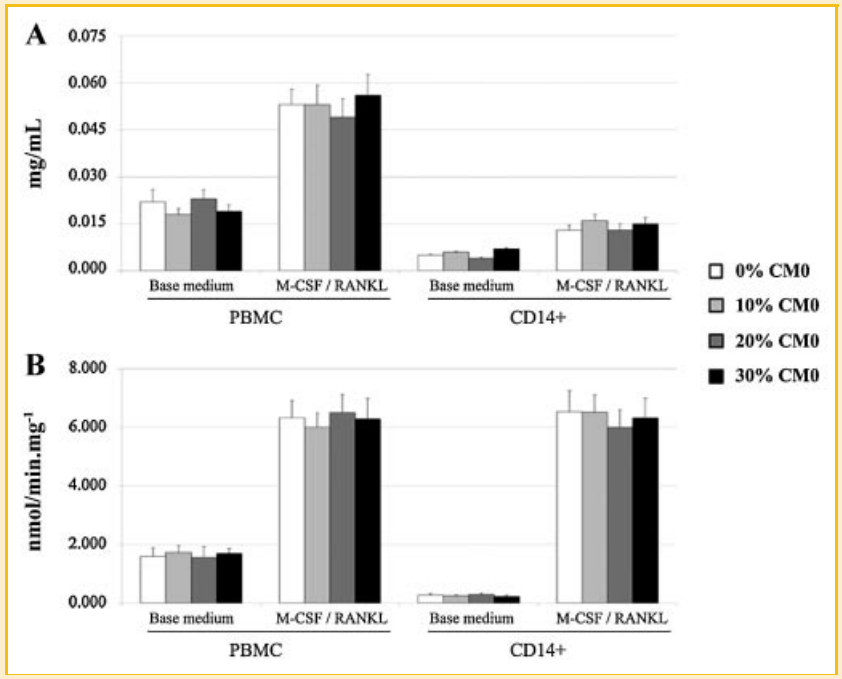

Fig. 1. Total protein content (A) and TRAP activity (B) of PBMC and CD14+ cell cultures maintained for 21 days in the absence or presence of both recombinant M-CSF and RANKL, and in the absence or presence of 10–30% CM0. TRAP activity was normalised with total protein content of cell cultures.

STATISTICAL ANALYSIS

Results presented in this study were obtained from three separate experiments using cell cultures from different patients. There were three replicates for each experimental situation. Densitometric values obtained for RT-PCR products are the means of three different gel analyses. Groups of data were evaluated using a two-way analysis of variance (ANOVA), and no significant differences in the pattern of the cell behaviour were found. Statistical differences found between control and experimental conditions were determined by Bonferroni's method. Values of $P \leq 0.05$ were considered significant.

RESULTS

CHARACTERISATION OF CELL CULTURES USED AS A SOURCE OF THE CONDITIONED MEDIA

Conditioned media from fibroblast and osteoblast cell cultures were used as potential promoters of the osteoclastic differentiation of human PBMC and CD14+ cells. After recovering the conditioned media, cell cultures were characterised by RT-PCR for the expression of the housekeeping gene GADPH and FSP-1, ALP, BMP-2, M-CSF and RANKL genes (Fig. 2A). The densitometric analysis of the gel bands is shown in Figure 2B. The values obtained for each lane were normalised for the corresponding GADPH value.

GADPH was expressed by every cell culture. Fibroblasts expressed high levels of FSP-1 and the highest levels of M-CSF; they also expressed ALP and RANKL, but BMP-2 expression was practically absent. Bone marrow cells supplemented with dexamethasone showed the highest expression level of ALP, BMP-2 and RANKL, and those cultured in the absence of the glucocorticoid displayed lower expression of these genes. No expression of FSP-1 was observed on both bone marrow cell cultures.

PROTEIN CONTENT AND TRAP ACTIVITY IN PBMC AND CD14+ CELL CULTURES

Human PBMC and CD14+ cells were cultured in different experimental conditions, in order to analyse the osteoclastogenic potential of some conditioned media. After 21 days of culture in the presence of isolated or combined fibroblastic or osteoblastic

TABLE II. Primers Used on RT-PCR Analysis of PBMC Cultures

Gene	5′ Primer	3′ Primer
GADPH	5′-CAGGACCAGGTTCACCAACAAGT-3′	5′-GTGGCAGTGATGGCATGGACTGT-3′
TRAP	5′-ACCATGACCACCTTGGCAATGTCTC-3′	5′-ATAGTGGAAGCGCAGATAGCCGTT-3′
CATK	5′-AGGTTCTGCTGCTACCTGTGGTGAG-3′	5′-CTTGCATCAATGGCCACAGAGACAG-3′
CA2	5′-GGACCTGAGCACTGGCATAAGGACT-3′	5′-AAGGAGGCCACGAGGATCGAAGTT-3′
c-myc	5′-TACCCTCTCAACGACAGCAG-3′	5′-TCTTGACATTCTCCTCGGTG-3′
c-src	5′-AAGCTGTTCGGAGGCTTCAA-3′	5′-TTGGAGTAGTAGGCCACCAG-3′

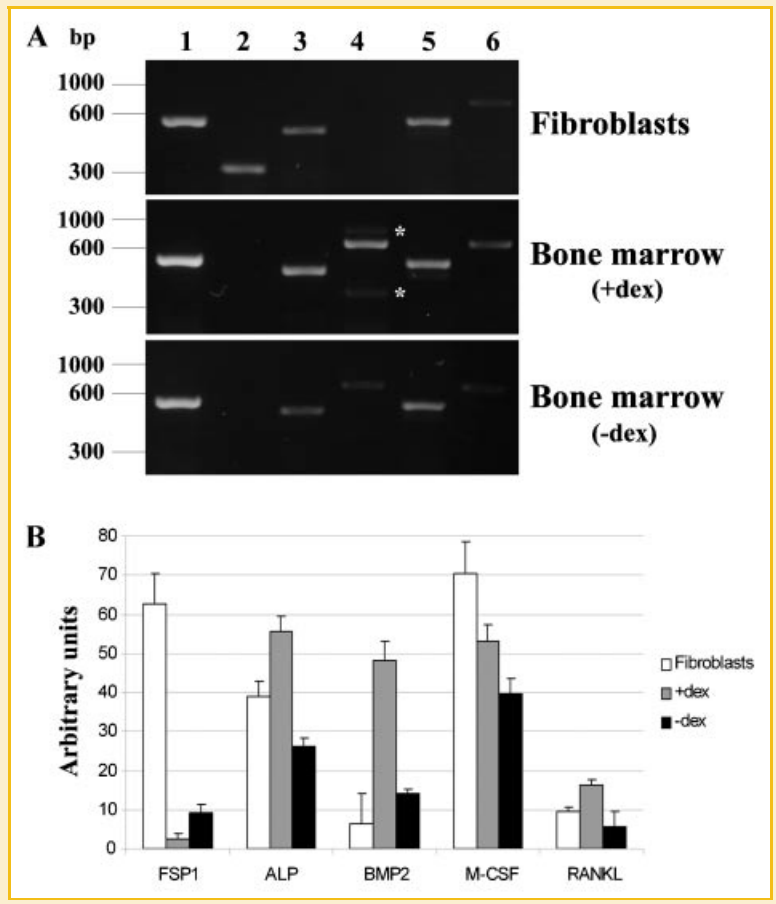

Fig. 2. Molecular characterisation of fibroblast and osteoblast cell cultures from which conditioned media were obtained. A: After recovering conditioned media, cell cultures were analysed by RT-PCR for GADPH (lane 1), FSP-1 (lane 2), ALP (lane 3), BMP-2 (lane 4), M-CSF (lane 5) and RANKL (lane 6) gene expression. B: Densitometric analysis of the RT-PCR products. The values obtained for each band were normalised with GADPH. Values are the means of three gel analyses of the bands obtained on RT-PCR. *Unspecific RT-PCR products.

conditioned media, cell cultures were assessed for total protein content and TRAP activity. Cultures were also performed in the absence (base medium) or presence of either one or both (positive control) recombinant M-CSF and RANKL. Results are shown in Figures 3 and 4.

Total protein content. PBMC cultures performed in the absence of growth factors showed relatively low amount of protein, suggesting a low cell number (Fig. 3A, group I). Supplementation with both M-CSF and RANKL (Fig. 3A, group II) increased significantly the protein content (about 125%). Compared with the positive control, cultures supplemented with M-CSF revealed approximately the same cellular protein amount. However, supplementation with RANKL resulted in lower values, with about 70% of the amount obtained for positive control (Fig. 3A, group II). Cultures supplemented with isolated or combined conditioned media presented approximately the same protein content (Fig. 3A, groups III and IV, respectively). In addition, values were similar to those found in the positive control.

When compared with PBMC cultures, CD14+ cultures displayed lower amounts of protein for all tested conditions (Fig. 3B). However, comparing the protein content determined for the different conditions, the general pattern was similar for both PBMC and CD14+ cell cultures.

TRAP activity. PBMC cultures performed in base medium showed little TRAP activity, with about 63% less enzyme activity when compared with positive control (see Fig. 4A, groups I and II). Cultures supplemented with just recombinant M-CSF or RANKL (Fig. 4A, group II) showed a somehow high value for TRAP activity, being about 66% and 74% of the value determined for positive control. As shown in Figure 3, group III, cultures supplemented with CM2 displayed approximately the same behaviour than that observed with the supplementation with both recombinant M-CSF and RANKL. The presence of CM1 or CM3 resulted in a lower performance, with values for TRAP activity of 72% or 64% of the one obtained for the positive control, respectively. Cultures supplemented with combined CM1 and CM2 showed higher TRAP activity than the one obtained for isolated CM1 or CM2, and, in addition, an increase of about 37%, compared with positive control. On the other hand, combination of CM1 with CM3 did not alter significantly the effect of each conditioned medium alone.

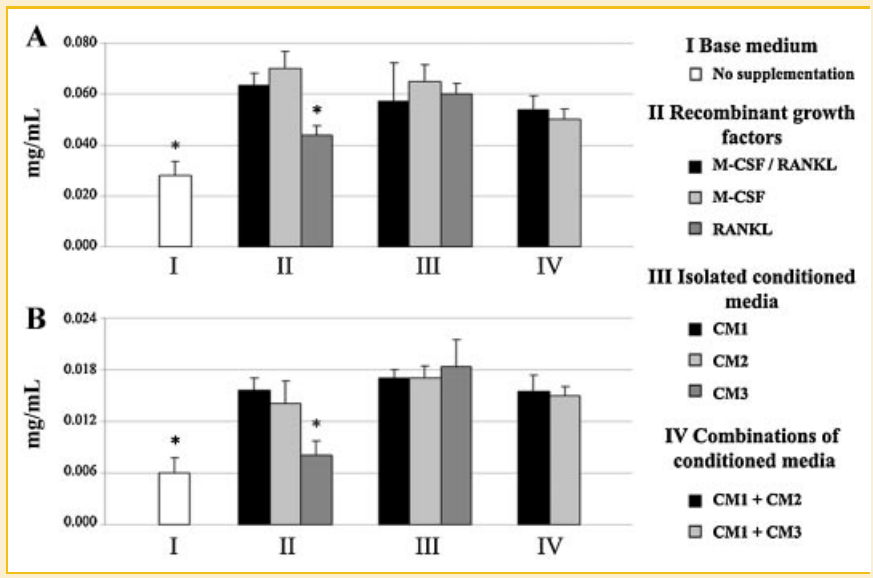

Fig. 3. Total protein content of PBMC (A) and CD14+ (B) cell cultures. Cells were cultured for 21 days in base medium (without any supplementation) or in the presence of one or both recombinant M-CSF and RANKL, or in the presence of isolated or combined conditioned media. Cellular protein content was quantified by Bradford's method. *Significantly different from the positive control.

In a general way, values obtained for TRAP activity on PBMC (Fig. 4A) and CD14+ (Fig. 4B) cell cultures were similar for all tested conditions. However, there are two significant exceptions. Compared to positive control, TRAP activity of CD14+ cell cultures performed in the absence of recombinant growth factors was almost negligible and those performed in the presence of isolated M-CSF and RANKL was about 32% and 36%, respectively. Those values are in clear contrast with the ones obtained for PBMC cultures.

PRESENCE OF MULTINUCLEATED CELLS POSITIVE FOR TRAP

To confirm the presence of osteoclastic cells, PBMC and CD14+ cell cultures were stained for TRAP and for nuclei. Multinucleated cells (4–8 nuclei) positive for TRAP were counted for each culture

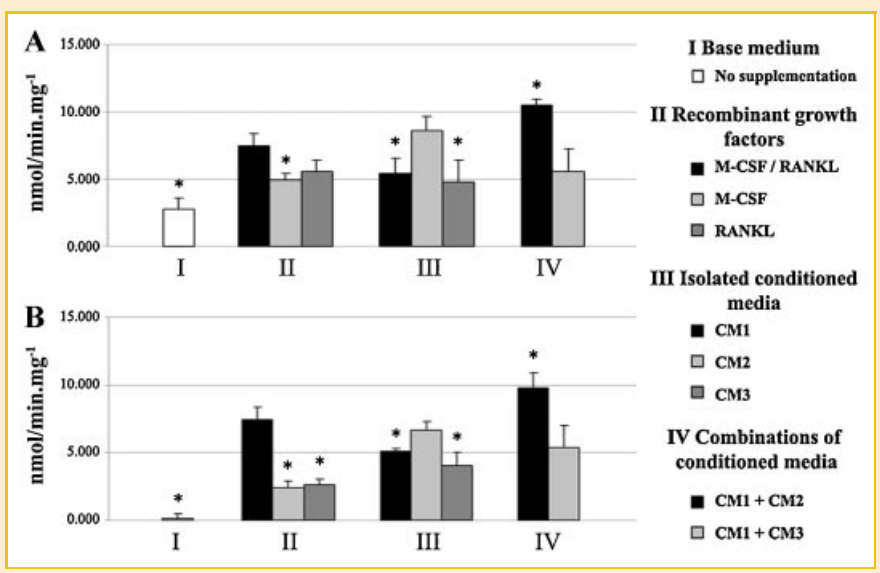

Fig. 4. TRAP activity of PBMC (A) and CD14+ (B) cell cultures. Cells were cultured for 21 days in base medium (without any supplementation) or in the presence of one or both recombinant M-CSF and RANKL, or in the presence of isolated or combined conditioned media. TRAP activity was quantified by *p*NPP hydrolysis assay and normalised with total protein content. *Significantly different from the positive control.

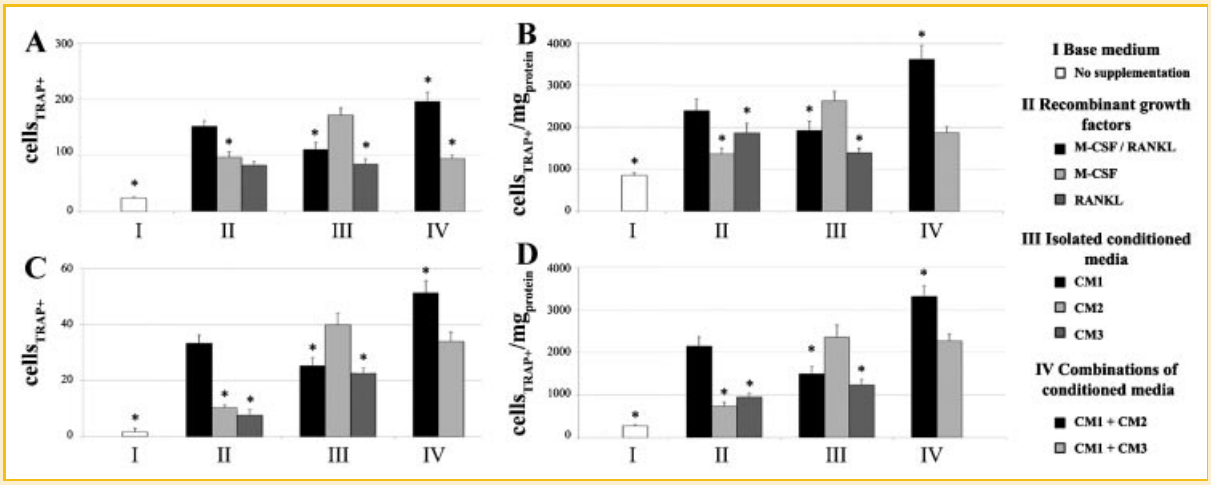

Fig. 5. TRAP staining of PBMC (A, B) and CD14+ (C, D) cell cultures performed in the absence or presence of isolated or combined recombinant M-CSF and RANKL, or in the presence of isolated or combined CM1–CM3. Multinucleated cells positive for TRAP were counted (A, C) and the value obtained was normalized for total protein content (B, D). *Significantly different from the positive control.

condition tested. The values obtained are presented in Figure 5A,C. Those values were normalised for total protein content of cultures and represented in Figure 5B,D.

PBMC cultures performed in base medium (Fig. 5A, group I) revealed the presence of less osteoclastic cells than the other conditions (about 16% of the value obtained for positive control). The presence of isolated recombinant M-CSF or RANKL (Fig. 5A, group II) increased the amount of multinucleated TRAP-positive cells by about fourfold, although the value was significantly lower than that observed in the presence of both recombinant factors (63% and 54%, respectively). Supplementation with CM2 elicited a response similar to positive control. Cultures performed in the presence of CM1 or CM3 displayed a lower number of osteoclastic cells than the cultures performed in the presence of both recombinant M-CSF and RANKL (72% and 55%, respectively). Supplementation with combined CM1 + CM2 increased the amount of osteoclastic cells in culture, when compared with isolated CM1 or CM2 supplementation or even with positive control (about 29% higher). On the other hand, combination of CM1 + CM3 did not alter significantly the amount of multinucleated cells TRAP positive in culture, when compared with CM1 or CM3 alone.

The amount of multinucleated cells positive for TRAP observed on CD14+ cell cultures was lower than the one observed on PBMC cultures, for all tested conditions (Fig. 5C). However, the general pattern observed in the different conditions was qualitatively identical on PBMC and CD14+ cell cultures, with two major exceptions. Once again, the difference between positive control and cultures performed in base medium, or in the presence of isolated M-CSF or RANKL, was higher for CD14+ cell cultures than for PBMC cultures.

The normalisation of the number of multinucleated cells TRAP positive with total protein content of cell cultures (Fig. 5B,D) revealed a pattern similar to the one obtained in Figure 5A,C, respectively. The only exception involves the cultures performed in the presence of M-CSF or RANKL. In fact, unlike that observed with the non-normalised data, RANKL supplementation elicited an osteoclastogenic response higher than the one observed with M-CSF supplementation. This behaviour was observed for both PBMC and CD14+ cell cultures.

RT-PCR ANALYSIS OF OSTEOCLASTIC CULTURES

PBMC cultures performed in the presence of both recombinant M-CSF and RANKL, or in the presence of isolated or combined fibroblastic and osteoblastic conditioned media, were analysed by RT-PCR for the expression of GADPH, osteoclastic markers TRAP, CATK and CA2, osteoclastic differentiation factor c-myc, and osteoclastic activation factor c-src genes. The results are presented in Figure 6A. The densitometric analysis of the gel bands is shown in Figure 6B. The values obtained for each lane were normalised for the corresponding GADPH value.

All the cultures expressed the genes analysed. GADPH expression was high for all the PBMC cultures. Positive control and CM2-supplemented cultures displayed a similar pattern of gene expression. Cultures performed in the presence of CM1 revealed just a slight decrease in the expression of the analysed genes (about 10–20%), when compared to positive control. Combination of CM1 + CM2 elicited a high level of osteoclastic genes expression, being about 10–20% higher than cultures performed in the presence of both M-CSF and RANKL. Cultures supplemented with CM3 or CM1 + CM3 revealed the lowest expression of all the genes analysed, except for TRAP, whose expression was not much different from the other tested conditions. Compared to positive control, gene expression was decreased by about 17% for TRAP, 42% for CATK and CA2 and 75% for c-myc and c-src.

PRESENCE OF ACTIN RINGS AND EXPRESSION OF VNR AND CTR IN PBMC CULTURES

Cultures were also analysed by CLSM in order to observe some osteoclastic characteristics, namely, multinucleated cells, actin rings and cells positive for VNR and CTR. As shown in Figure 7A, it was

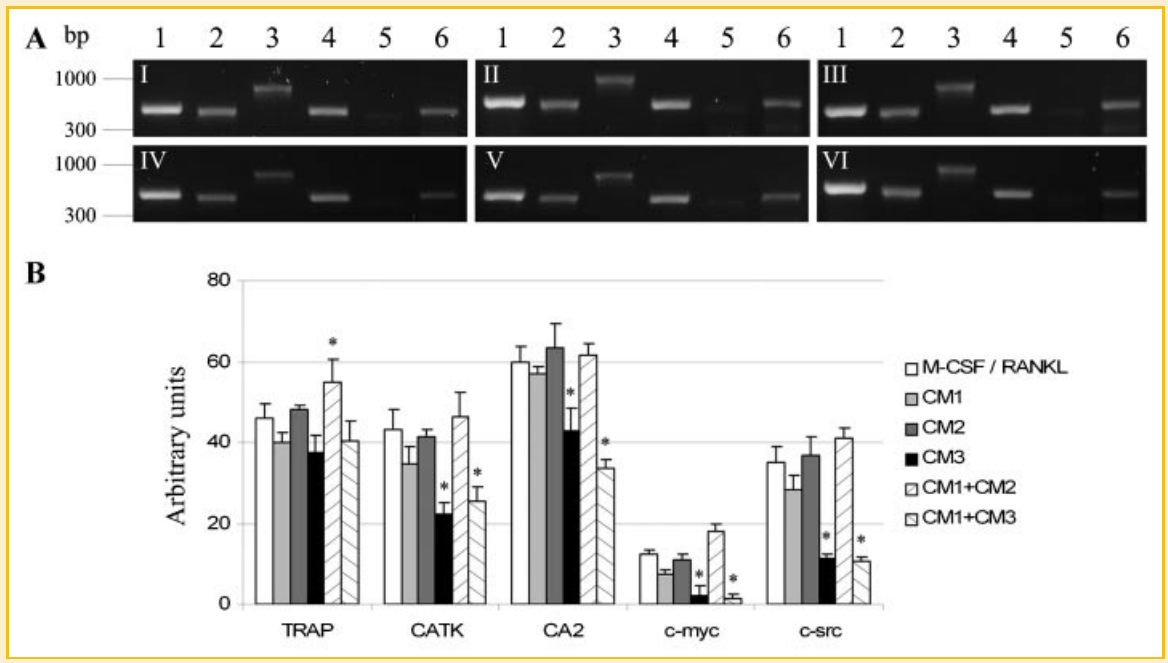

Fig. 6. RT-PCR analysis of PBMC cultures (A) performed for 21 days in the presence of both M-CSF and RANKL (I), or in the presence of isolated (II–IV) or combined CM1–CM3 (V and VI). The RT-PCR bands were analysed by gel densitometry and normalised with GADPH (B). Values are the means of three independent gel analyses for each RT-PCR product. *Significantly different from the positive control.

possible to visualise multinucleated cells with actin rings and positive for VNR and CTR. CLSM observation was in agreement with the results obtained for TRAP activity. In fact, cell cultures supplemented with CM2 and CM1 + CM2 showed a higher number of cells displaying osteoclastic features (Fig. 7B).

DISCUSSION

Osteoclastogenesis is initiated with fusion of osteoclast precursors and subsequent differentiation into mature osteoclast. This complex process requires the action of several growth factors and cellular interactions [reviewed in Boyce and Xing, 2008; Vaananen and Laitala-Leinonen, 2008]. Among them, mechanisms involving interactions with osteoblasts and fibroblasts play a relevant role [Walsh et al., 2005; Datta et al., 2008]. The present work analyses the osteoclastogenic potential of these cell types in an indirect co-culture system, that is, in the absence of physical cell interactions. Conditioned media from adult osteoblastic and fibroblastic cell cultures were used to promote osteoclastogenesis of human peripheral blood osteoclast precursors. Osteoclastic cells are descendent of the CD14+ monocyte lineage present in the peripheral blood and, in the present work, two cell populations were tested, PBMC and CD14+ cells. Once CD14+ cells are obtained from PBMC, their isolation is technically more complex and time-consuming, which can affect the cellular viability/activity. Furthermore, although PBMC contains CD14+ cells, it is a heterogeneous population which includes also other cell types that can modulate osteoclastogenesis. PBMC and CD14+ cultures performed in the absence or the presence of M-CSF and RANKL, the two growth factors used to promote osteoclastogenesis in vitro [Nicholson et al., 2000], were used as negative and positive controls, respectively.

Fibroblast and osteoblast cell cultures were established in standard culture conditions, with bone marrow cells being cultured in the absence or in the presence of 10 nM dexamethasone, a classic in vitro osteogenic inducer [Chen and Feldman, 1979; Pereira et al., 2009]. Upon recovery of the conditioned media, analysis of gene expression of the cell layer by RT-PCR showed some representative phenotypic differences among them. Fibroblasts showed expression of FSP-1, a fibroblastic marker [Okada et al., 1998] and no expression of BMP-2. Also, some expression of ALP was observed. Although being considered a classic in vitro osteoblastic marker, expression of ALP by fibroblasts was already demonstrated in previous reports [Weiss et al., 1989; Galicka et al., 2002]. As expected, dexamethasone-treated bone marrow cell culture showed the highest expression level for the osteoblastic genes analysed, namely, ALP and BMP-2. Non-supplemented bone marrow cell cultures (−dex) also expressed ALP, but almost no BMP-2 expression was observed, which indicates a lower degree of osteoblastic differentiation [Marie et al., 2002]. Moreover, all the cultures expressed M-CSF and RANKL, which is in line with their potential involvement on osteoclastogenesis. However, although M-CSF expression was higher in fibroblastic cells, RANKL expression was higher in +dex cultures. Bone marrow cell cultures performed in the absence of dexamethasone displayed the lower expression of M-CSF and RANKL.

PBMC and CD14+ cell cultures performed in base medium (with human serum, but no further supplementation) presented low protein content, a parameter that would provide, with some reserves, information regarding the number of the adherent cell population.

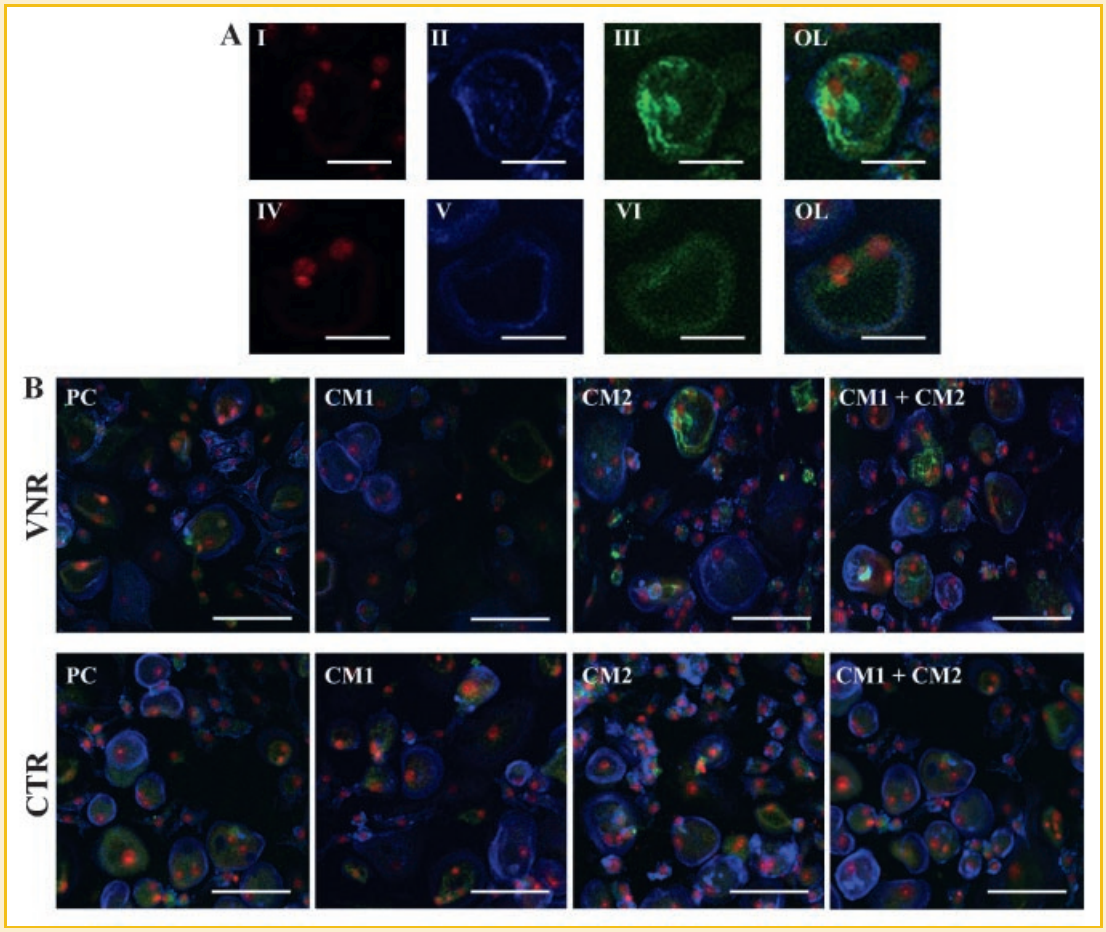

Fig. 7. PBMC cultures visualised by CLSM after 21 days of culture. A: Representative images: osteoclast stained red for nuclei (I and IV), blue for actin (II and V), and green for vitronectin receptors, VNR (III) or calcitonin receptors, CTR (VI); OL, overlay. B: CLSM visualisation of cells cultured in the presence of recombinant M-CSF and RANKL (positive control, PC), CM1, CM2, or CM1 + CM2. White bars represent 60 μm (A) or 100 μm (B). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

This observation suggests that human serum supplementation per se was not sufficient to guarantee a high rate of cell number. Combination of M-CSF and RANKL increased significantly total protein content. Interestingly, M-CSF alone gave a similar response, which is in agreement with its reported role in osteoclastogenesis, by increasing osteoclastic precursor cell survival [Pixley and Stanley, 2004]. Comparatively, supplementation with RANKL alone resulted in lower protein content, most probably because this growth factor appears to be mainly engaged with later events on osteoclast differentiation [Boyle et al., 2003; Boyce and Xing, 2008]. Cultures supplemented with the fibroblast and osteoblast conditioned media, isolated or combined, presented similar protein content to that of the positive control or M-CSF alone, suggesting the presence of molecules important for cell survival/proliferation.

In both PBMC and CD14+ cell cultures, supplementation with M-CSF or RANKL induced the expression of TRAP, which was further increased in the presence of both factors (positive control). Supplementation with conditioned medium from fibroblast cell cultures (CM1) resulted in slightly lower values, but in the presence of conditioned medium from (+dex) bone marrow cell cultures (CM2) TRAP activity was similar to positive control. Additionally, the combination of CM1 and CM2 further increased TRAP activity. In all tested conditions, TRAP activity was mainly associated with the presence of multinucleated cells. It is worthwhile to note that the expression of TRAP on the monocyte CD14+ cell population was totally dependent on the presence of recombinant growth factors or conditioned media, as the cultures maintained in base medium presented negligible values of TRAP activity. However, PBMC were able to synthesise some TRAP in the absence of any supplementation. Once osteoclasts descend from the CD14+ monocyte lineage, and CD14+ cells are isolated from PBMC, this observation suggests that the PBMC population contains cell types other than CD14+ lineage cells that also express TRAP and/or have a role in promoting the expression of this enzyme by CD14+ cells. Further studies are needed to clarify this issue. In fact, although TRAP activity is a characteristic feature of osteoclastic cells, the presence of other cell types that also express TRAP, for instance, macrophages, cannot be excluded [Datta et al., 2008].

To further characterise the osteoclastic cultures, PBMC cultures were analysed by RT-PCR for the expression of TRAP, CATK, CA2, c-myc and c-src. In general, compared to the positive control, expression of these genes was similar in the presence of CM1, CM2

and CM1 + CM2, but significantly lower following supplementation with CM3 and CM1 + CM3, especially for c-myc and c-src. Once c-myc seems to be important for osteoclast differentiation, and c-src for mature osteoclast activation [Zhao et al., 2007], these results clearly suggest a low degree of osteoclast differentiation and activation in these conditions. The molecular characterisation of osteoclastic cultures allowed a more precise analysis of the effects of conditioned media on osteoclastogenesis. Considering just the expression of TRAP, it seems that it is not possible to differentiate significantly CM1, CM3 or CM1 + CM3 supplementations, from the other conditions. However, molecular analysis of other osteoclastic genes revealed evident differences among the cultures. Taken together, although TRAP activity is routinely used as a marker for osteoclast differentiation/activation, the present results suggest that conclusions withdrawn from this parameter must be carefully considered and should be complemented with the expression analysis of other relevant markers. In that context, it is noteworthy to highlight that c-myc and c-src appear as good choices to increase sensibility to the experimental data.

PBMC cultures were also analysed by CLSM for the formation of actin rings, and the expression of VNR and CTR established osteoclastic markers [Vaananen and Laitala-Leinonen, 2008]. The results proved the presence of osteoclasts in the cultures and also corroborated the results obtained for TRAP activity and RT-PCR analysis, once cultures supplemented with CM2 or CM1 + CM2 showed a high number of osteoclastic cells, similar to that observed in the positive control.

The present work showed that conditioned media from bone marrow cells cultured in the presence (CM2) or absence (CM3) of dexamethasone had different effects on TRAP activity and gene expression of osteoclast-associated markers. In fact, CM2 elicited a response identical to the positive control, but CM3 showed a poorer performance. This difference was also evident when CM2 or CM3 were combined with CM1. Once dexamethasone is an osteoblast differentiation promoter [Chen and Feldman, 1979], the results obtained clearly show that osteoblastic differentiation is required in order to have an increase on osteoclast formation and activation. This observation is in agreement with the fact that dexamethasone promotes an increase of RANKL mRNA expression, on osteoblasts, by two- to fourfold [Hofbauer et al., 1999]. Also, it is worthwhile to note that conditioned medium from fibroblastic cultures elicited an osteoclastogenic response only slightly lower than that observed for the positive control or the conditioned medium from the osteogenic-induced bone marrow cell cultures. This observation is in line with the potential role of fibroblast in the regulation of osteoclastogenesis in vivo. In fact, there are several reports about the effect of fibroblastic cells on bone metabolism, and, more precisely, on osteoclast differentiation/activation. However, they are usually associated to inflammatory joint disorders, such as rheumatoid arthritis, among others [Udagawa et al., 2002; Walsh et al., 2005; Sato and Takayanagi, 2006]. The exact role of this cell type in the process, in the absence of such disorders, remains unclear. Our results clearly reveal that, in non-pathological conditions, fibroblasts have the potential to modulate osteoclastogenesis.

Taken together, TRAP activity, RT-PCR analysis and CLSM visualisation of the cultures showed that the conditioned media from fibroblast and osteoblast cell cultures elicited osteoclastogenesis of mononuclear precursor cells. In addition, results suggested additive/synergistic effects of osteoclastogenic factors arising from the two cell types. These observations corroborate a potential involvement of fibroblasts and osteoblasts on regulation of osteoclast formation and function, as it was proposed before [Udagawa et al., 2002; Walsh et al., 2005; Sato and Takayanagi, 2006]. In the present study, the positive osteoclastogenic effect could be related with the potential presence of M-CSF and RANKL on the conditioned media. In fact, as demonstrated by RT-PCR, the fibroblastic and osteoblastic cultures used to prepare the conditioned media displayed expression of both factors. These data are in agreement with many published studies regarding M-CSF and RANKL expression by fibroblasts and osteoblasts [Legendre et al., 2003; Boyce and Xing, 2008; Hashizume et al., 2008]. The present differences observed in M-CSF and RANKL expression were somehow in line with the osteoclastogenic potential of the conditioned media. However, it is also expected that some conditioned media contain other molecules that have a positive effect on osteoclastogenesis, such as IL-1, IL-6, IL-17, TNF-α and VEGF, for example [Udagawa et al., 1995; Holt et al., 1996; Jimi et al., 1998; Lam et al., 2000; Ragab et al., 2002; Kim et al., 2005]. Regarding this, it is known that the osteoblasts and the fibroblasts are able to express a large variety of signalling molecules, including those mentioned above [Udagawa et al., 1995; Holt et al., 1996; Legendre et al., 2003; Motokawa et al., 2005; Hashizume et al., 2008]. Molecular analysis of the different conditioned media is warranted in order to investigate the presence and the concentration of relevant molecules. Regarding previous studies, few analysed the effect of conditioned media on osteoclast formation and activation. It was shown that conditioned medium from osteoblasts subjected to mechanical stress can be used to promote osteoclastogenesis [Rucci et al., 2007] and, also, that conditioned media derived from fibroblasts, either treated or not with cytokines, stimulated the release of calcium from bone organ cultures [Cochran and Rouse, 1993].

In conclusion, it was demonstrated that conditioned media from adult fibroblastic and osteoblastic cell cultures were able to promote osteoclastogenesis of peripheral blood precursor cells, corroborating the involvement of these cell types on osteoclast formation and activation. Conditioned medium obtained from osteogenic-induced bone marrow cell cultures displayed similar behaviour to that of recombinant M-CSF and RANKL. In addition, the combination of conditioned media from osteoblast and fibroblast cell cultures resulted in increased expression of some osteoclastic markers. Although supplementation with recombinant M-CSF and RANKL allows more controlled conditions to study osteoclastogenesis in vitro, conditioned media supplementation displays the effect of a variety of other molecules known to be important for osteoclast formation and function. So, in addition to a clear economic benefit, the utilisation of conditioned media from fibroblast and osteoblast cells has the advantage to mimic complex cellular interactions relevant in bone homeostasis. In addition, results showed that PBMC cultures, in the tested conditions, displayed similar osteoclastogenic behaviour to that observed for CD14+ cell cultures. This suggests that PBMC population might be a suitable model for osteoclastogenesis studies, offering the clear advantage that its isolation is

technically less complex and time-consuming, which can help to preserve cellular viability/activity of osteoclastic precursors.

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