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European Journal of Cancer 37 (2001) 629–640

European Journal of Cancer

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Melanoma cells stimulate osteoclastogenesis, c-Src expression and osteoblast cytokines

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Received 7 July 2000; received in revised form 15 November 2000; accepted 15 November 2000

Abstract

Malignant melanomas metastasise to the bone and enhance osteoclast bone resorption. We demonstrated that a 48-h-B16 melanoma cell conditioned media (B16CM) induced osteoclastogenesis in mouse bone marrow cultures, without the requirement of B16 cell-bone marrow cell co-culture. B16 cells transcriptionally expressed detectable levels of *TGFβ1*, *IL-6*, *M-CSF*, *GM-CSF* and *TNFα* mRNAs, albeit to a lower extent compared with levels in osteoblasts, and failed to express *PTHrP*, *OPGL*, *OPG* and *IL-1β*. Interestingly, B16CM greatly upregulated *IL-1β*, *IL-6* and *GM-CSF*, and modestly enhanced *TNFα* and *OPGL* mRNA expression in osteoblasts, suggesting a potential indirect stimulation of osteoclastogenesis via the osteogenic lineage. B16CM barely upregulated c-Fos, but strongly and time-dependently enhanced c-Src expression in the total bone marrow cultures during osteoclast differentiation. Moreover, c-Src expression was enhanced in differentiated and purified osteoclast preparations to higher levels than in stromal cells. In conclusion, melanoma induces osteoclast generation with a paracrine mechanism independent of cell-cell contact, specifically upregulating c-Src in osteoclasts and cytokine expression in osteoblasts.

Keywords: Melanoma; Bone metastasis; Osteoclastogenesis; c-Src; Cytokine; Osteoclast; Osteoblast

1. Introduction

Malignant melanoma is one of the most common tumours in developed countries, and in Europe it is believed to increase annually 4 to 7% [1]. As for other cancers, development of distant metastases is the most important cause for increasing morbidity, progressive treatment failure, and subsequent death in malignant melanomas [2]. Approximately one third to one half of all tumours spread to the bone, the third most common site of metastases, after the liver and the lung. Similar to other tumours, including breast and prostate carcinomas, malignant melanomas frequently metastasise to bone [3–5], where they mostly produce destructive, or osteolytic, lesions [5,6]. Metastases first appear in the bone marrow, then expand in the bone cavity, where the tumour cells are surrounded by active osteoclasts.

Bone destructive metastases are among the most severe complications of malignancy. Patients complain of intractable bone pain, spontaneous fractures, nerve compression syndrome and hypercalcaemia. Once bone metastases are formed, therapy is no longer efficacious, and only pain control as a palliative intervention is, at present, possible. Physiological bone remodelling is maintained by osteoclasts and osteoblasts [7], and bone tissue is rich in cytokines and local factors that, along with systemic hormones, finely regulate the activity of bone cells [8]. Bone metastasis is likely to operate as a third player, which adds new stimulating factors to the bone microenvironment mostly causing extensive osteolysis [4,5,9].

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0959-8049/01/$ - see front matter
PII: S0959-8049(00)00436-6

Bone destruction occurs due to the activity of osteoclasts, multinucleated cells derived from the haematopoietic colony forming unit-granulocyte macrophage (CFU-GM)/monocyte-macrophage family [10]. The mechanism whereby osteolytic lesions occur in bone metastasis is still unclear. However, an increase of both the osteoclast number and activity has been hypothesised according to histopathological findings and animal studies [4]. In this study, we investigated the mechanism by which the melanoma cell line B16 stimulates osteoclastogenesis *in vitro*.

2. Materials and nethods

2.1. Reagents

Cell culture media, serum and reagents were from Gibco (Uxbridge, UK). Sterile glassware was from Falcon Becton Dickinson (Meylan, France). The anti v-Src (AB-1) antibody, also recognising c-Src, was from Oncogene Research Products (Milan, Italy). The anti c-Fos (sc-52), the anti-β-actin (sc-1616) polyclonal antibodies and the horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). 1,25$(OH)_2$vitamin D_3 was kindly donated by Drs Domenico Criscuolo and Mario Piatti, Hoffman LaRoche (Milan, Italy). Reagents for reverse transcriptase-polymerase chain reaction (RT-PCR) were from Promega (Milan, Italy). The Enhanced ChemiLuminescence (ECL) kit was from Amersham Life Science Products (Milan, Italy). Mouse recombinant interleukin-1β (IL-1β), interleukin-6 (IL-6) and granulocyte/macrophage-colony stimulating factor (GM-CSF) were from PeproTech House (London, UK). All other reagents were of the purest grade from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. B16 cell line

This is a murine melanoma cell line with high bone metastatic potential (clone F10; [11]). Cells were purchased from American Type Culture Collection (Rockville, MD, USA) and cultured in Dulbecco's Modified Minimum Essential Medium (DMEM) supplemented with antibiotics and 10% fetal bovine serum (FBS). For experiments, cells were cultured for 48 h in serum-free medium, containing 0.2% fatty acid-free bovine serum albumin (BSA).

2.3. Bone marrow cultures

Primary osteoclasts were differentiated in culture from the bone marrow of newborn CD1 mice by a modification of the method described by David and colleagues [12]. Five- to 7-day-old mice were sacrificed by decapitation, and long bones dissected free from the soft tissues. Bone marrow was flushed from the bone cavity and minced in DMEM supplemented with antibiotics and 10% FBS. Cells were plated in culture dishes and allowed to attach for 24 h in standard medium, at 37°C, in a 95% air/5% CO_2 incubator. Non-adherent cells were then removed by aspiration and extensive washing, then the total adherent cell fraction was cultured up to 10 days in the presence or absence of 10^{-8} M 1,25$(OH)_2$vitamin D_3.

2.4. Osteoblast cultures

Calvaria were removed from 7–9-day old CD1 mice, (Charles River, Milan, Italy) cleaned free from the soft tissue and digested with 1 mg/ml *Clostridium histolyticum* type IV collagenase and 0.025% trypsin for 20 min at 37°C in Hank's buffered solution, with gentle agitation. The procedure was repeated three times and cells from the second and third digestions were plated in Petri dishes and grown to confluence in DMEM supplemented with antibiotics and 10% FCS. At confluence, cells were trypsinised by standard procedures and plated in wells for experiments. The cells obtained with this method showed positivity for alkaline phosphatase activity and expression of the osteoblast markers PTH/parathyroid hormone related peptide (PTHrP) receptor and Osf2/Cbfa1 (osteoblastspecific factor/core binding factor 1) transcription factor, and of bone matrix proteins (osteopontin and bone sialoprotein II).

2.5. Conditioned media

B16 cells were plated into 10 cm diameter culture dishes and grown to confluence. Medium was then replaced with fresh serum-free medium containing 0.2% BSA and cultured for further 48 h. Media were then collected and stored in aliquots at −20°C until use. As controls, we used media prepared as described above from NIH3T3 fibroblasts.

2.6. Acid and alkaline phosphatase activities

Cells were fixed in 3% paraformaldehyde in 0.1 M cacodylate buffer for 15 min, then extensively washed in the same buffer. Tartrate-resistant acid phosphatase (TRAP) and alkaline phosphatase activities were detected histochemically using the Sigma kits nos. 386 and 85, respectively. For biochemical assays, cells were lysed in 0.1% sodium dodecyl sulphate (SDS) containing protease inhibitors, and TRAP activity was evaluated in aliquots using the Sigma kit 104-ATL. Either kits were used according to the manufacturer's instruction.

2.7. Bone resorption

Bone slices were cut from adult bovine cortical bone using a Buhler Isomet 2000 precision saw and rinsed in

acetone, cleaned by ultrasonification (3×10 min in distilled water) and both sides sterilised overnight under ultraviolet (UV) light. Bone marrow cells or differentiated osteoclasts were plated on bone slices and incubated for 2 to 10 days, according to the experimental design, in the presence of the factors as indicated in the 'Results' section. At the end of incubation, cells were removed by ultrasonication (3×10 min), sections were stained with toluidine blue, pits were enumerated and the pit index was computed according to Caselli and colleagues [13].

2.8. RT-PCR

RNA was prepared using the acid phenol technique [14]. For RT-PCR, 1 μg of total RNA was reverse transcribed using Moloney Murine Leukemia Virus (M-MLV) RT and the equivalent of 0.1 μg was added to PCR reactions. These were carried out in a final volume of 20 μl buffer containing 200 μM of 2′-deoxynucleoside 5′-triphosphates (dNTPs), 1.5 mM $MgCl_2$, 10 pM of each primer and 1 unit Taq-DNA-polymerase. PCR conditions were as follows:

- Parathyroid hormone related peptide (*PTHrP*), 35 cycles; 95°C (1 min), 55°C (1 min) and 72°C (1 min).
- Interleukin 1β (*IL-1β*), 30 cycles, 94°C (1 min), 50°C (1 min), 72°C (1 min).
- Interleukin 6 (*IL-6*), 35 cycles; 95°C (1 min), 65°C (1 min) and 72°C (1 min).
- Macrophage-colony stimulating factor (*M-CSF*), 30 cycles, 94°C (40 s), 50°C (45 s), 72°C (1 min).
- Granulocyte, macrophage-colony stimulating factor (*GM-CSF*), 35 cycles, 94°C (1 min), 55°C (30 s), 72°C (30 s).
- Tumour necrosis factor α (*TNFα*), 30 cycles, 94°C (15 s), 58°C (15 s), 72°C (30 s).
- Transforming growth factor β-1 (*TGFβ*-1), 32 cycles, 94°C (1 min), 58°C (30 s), 72°C (30 s).
- Osteoprotegerin ligand (*OPGL*), 30 cycles, 94°C (1 min), 65–60–55–50–45°C (10, 5, 5, 5, 5 cycles, respectively), 72°C (1 min).
- Osteoprotegerin (*OPG*), 28 cycles, 94°C (30 s), 55°C (30 s), 72°C (30 s).
- Glyceraldehyde phosphate dehydrogenase (*GAPDH*), 25 cycles, 95°C (1 min), 55°C (2 min), 72°C (1 min).

The following primer pairs were used to amplify fragments of mouse *PTHrP* [15], *IL-1β* (GeneBank Accession No. NM8361), *IL-6* (GeneBank Accession No. X54542), *M-CSF* [16], *GM-CSF* [17], *TNFα* [18], *TGFβ*-1 [19], *OPGL* [20], *OPG* [20] and *GAPDH* [21]:

PTHrP	forward 5′-TGGTGTTCCTGCTCAGCTA-3′ reverse 5′-CCTCGTCGTCTGACCCAAA-3′
IL-1β	forward 5′-ATGGCAACTGTTCCTGAACTCAAGT-3′ reverse 5′-CAGGACAGGTATAGATTCTTTCCT-TT-3′
IL-6	forward 5′-ATGAAGTTCCTCTCTGCAAGAGACT-3′ reverse 5′-CACTAGGTTTGCCGAGTAGATCTC-3′
M-CSF	forward 5′-GAATCTTCACTGGGCACTAAC-3′ reverse 5′-CTTCCCATATGTCTCCTTTCC-3′
GM-CSF	forward 5′-ATGTGGCTGCAGAATTTACT-3′ reverse 5′-TTGTGTTTCACAGTCCGTTTCC-3′
TNFα	forward 5′-GCAGGTCTACTTTGGAGTCATTGC-3′ reverse 5′-TCCCTTTGCAGAACTCAGGAATGG-3′
*TGFβ*1	forward 5′-GTGGCTTCTAGTGCTGACGCC-3′ reverse 5′-TGCCGTACAACTCCAGTGACG-3′
OPGL	forward 5′-GGTCGGGCAATTCTGAATT-3′ reverse 5′-GGGAATTACAAAGTGCACCAG-3′
OPG	forward 5′-AAAGCACCCTGTAGAAAACA-3′ reverse 5′-CCGTTTTATCCTCTCTACACTC-3′
GAPDH	forward 5′-CACCATGGAGAAGGCCGGGG-3′ reverse 5′-GACGGACACATTGGGGGTAG-3′

PCR amplified fragments were resolved by 1.8% agarose/ethidium bromide gel electrophoresis and observed under UV light.

2.9. Western blotting

Cells were lysed with 0.1% SDS containing protease inhibitors. 30 to 50 μg cell protein aliquots were then resolved by 8% SDS-polyacrylamide gel electrophoresis (PAGE), blotted onto nitrocellulose filters, probed with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies. Bands were detected by ECL.

2.10. Densitometric analysis

Band areas were analysed by scanning densitometry, using the Molecular Analyst software for the Bio-Rad Laboratories (Haercules, CA) model 670 scanning densitometer, and each value expressed as arbitrary densitometric units. Normalisation was performed using as

internal controls the reference genes *GAPDH* for PCR and *β-actin* for western blotting.

2.11. Statistics

Data are expressed as means±standard errors of the mean (SEM) versus control of three independent experiments performed in triplicate. Statistical significance was computed by the one-way analysis of variance (ANOVA). A P value <0.05 was conventionally considered statistically significant.

3. Results

3.1. Osteoclast generation by vitamin D_3

Bone marrow adherent cells in culture progressively proliferated, and proliferation was reduced by 10^{-8} M $1,25(OH)_2$vitamin D_3. This mixed population was initially formed by stromal cells, and cells morphologically indistinguishable from monocyte-macrophages. Approximately 50% of stromal cells were positive for the osteoblast marker alkaline phosphatase, indicating their osteogenic phenotype. By day 6, several multinucleated disc-shaped cells appeared, and increased with time up to 10 days. In the absence of $1,25(OH)_2$vitamin D_3, cultures became confluent, with the majority of cells belonging to the stromal lineage.

Characterisation of the cultures in the presence or absence of $1,25(OH)_2$vitamin D_3, demonstrated that the hormone induced generation of cells belonging to the osteoclast lineage. Multinucleated and the mononuclear cells with monocyte-macrophage phenotype were TRAP-positive assessed by histochemical analysis (Fig. 1a). The biochemical assay revealed that, parallel to the appearance of osteoclasts, TRAP activity significantly increased in culture in comparison with the

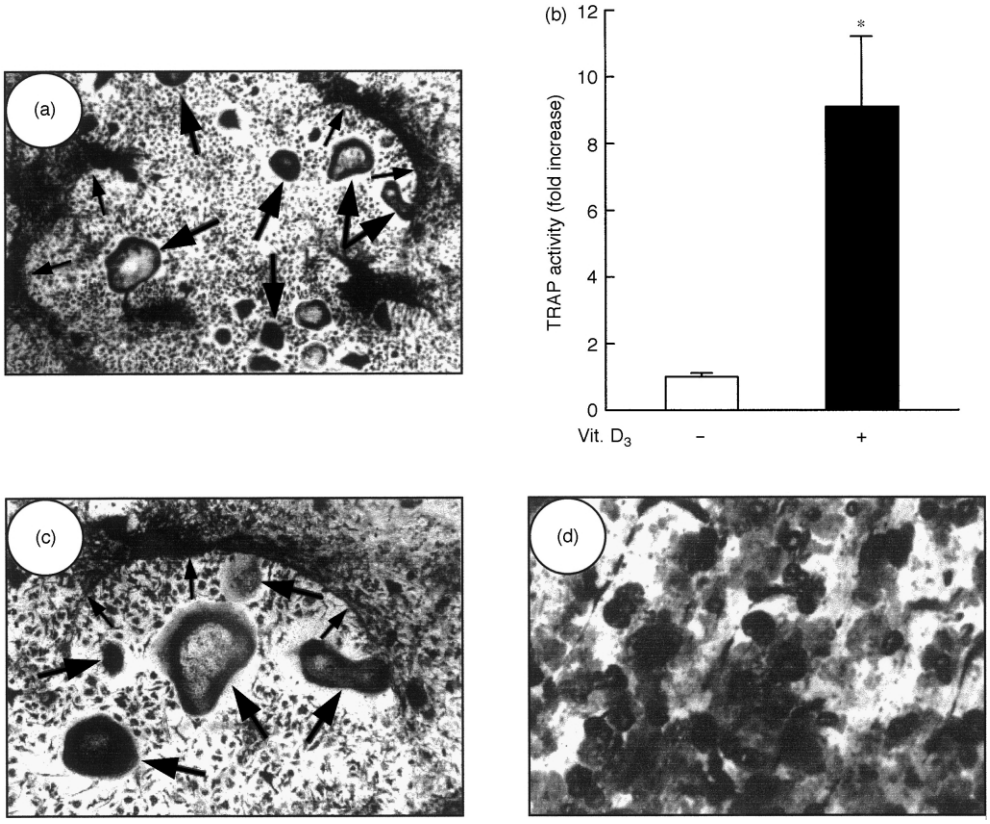

Fig. 1. Osteoclast characterisation. (a) Ten-day bone marrow cultures incubated in the presence of 10% fetal bovine serum (FBS) and 10^{-8} M $1,25(OH)_2$vitamin D_3 were fixed and histochemically stained for tartrate-resistant acid phosphatase (TRAP) activity. The figure shows several TRAP-positive multinucleated osteoclasts (large arrows) as well as a layer of stromal cells (small arrows). Magnification 100×. (b) In parallel cultures incubated with or without 10^{-8} M $1,25(OH)_2$vitamin D_3, TRAP activity was evaluated biochemically. Note a significant increment of enzyme activity in the presence of the hormone. Data represent fold increase of TRAP activity versus control and are expressed as the mean±SEM of three independent experiments. $^*P<0.05$. (c) Enlargement of the field shown in (a) to highlight the area where stromal cells are spontaneously detaching (small arrows). Several mononuclear TRAP-positive cells, which are assumed to represent putative osteoclast precursors, are intermingled with the multinucleated osteoclasts (large arrows). Magnification 300×. (d) Ten-day bone marrow cultures incubated in the presence of 10^{-8} M $1,25(OH)_2$vitamin D_3 to differentiate osteoclasts and their putative precursors, were purified free from stromal cells, trypsinised, transferred onto bone sections and further incubated for 48 h. Sections were then cleaned free from osteoclasts by ultrasonication and resorption pits (dark areas) were stained with toluidine blue. Magnification 400×.

untreated counterpart (Fig. 1b). The multinucleated cells expressed the vitronectin receptor, retracted in response to calcitonin, and efficiently resorbed bone [22], confirming their osteoclastic phenotype (data not shown). In addition, we observed that the osteoclast population could be purified >80% when the cultures were established at high cell density (plating the total bone marrow from 1.5 mice/cm^2). This allowed the stromal cells to overgrow, forming a superconfluent layer which spontaneously detached (Fig. 1c) and could be flushed off from the culture dish. No enzymatic treatment was required. After removal of the stromal cells, a monolayer of multinucleated and mononuclear TRAP-positive cells could be appreciated, with <20% of the stromal cells left. Detachment of these osteoclastic cells from the culture dish by incubation with 0.05%

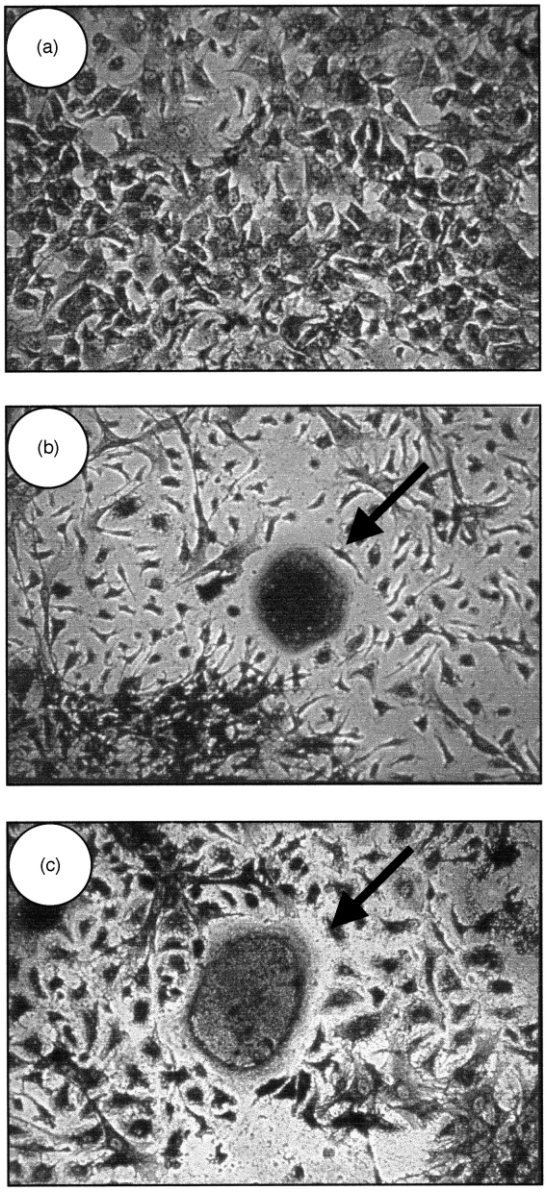

Fig. 2. Osteoclast generation by B16 conditioned medium. Bone marrow adherent cells were incubated in medium containing 3% FBS for 10 days (a) alone, (b) with 10^{-8} M 1,25(OH)$_2$vitamin D$_3$, or (c) with 48-h conditioned medium (1:4) from B16 melanoma cells. Cultures were stained for TRAP activity. Note that no osteoclasts were formed in the absence of 1,25(OH)$_2$vitamin D$_3$ or B16 melanoma cell conditioned medium, whereas osteoclast (arrow in band C) formation can be appreciated in the presence of these factors. Magnification 800×. Similar results were observed in three independent experiments.

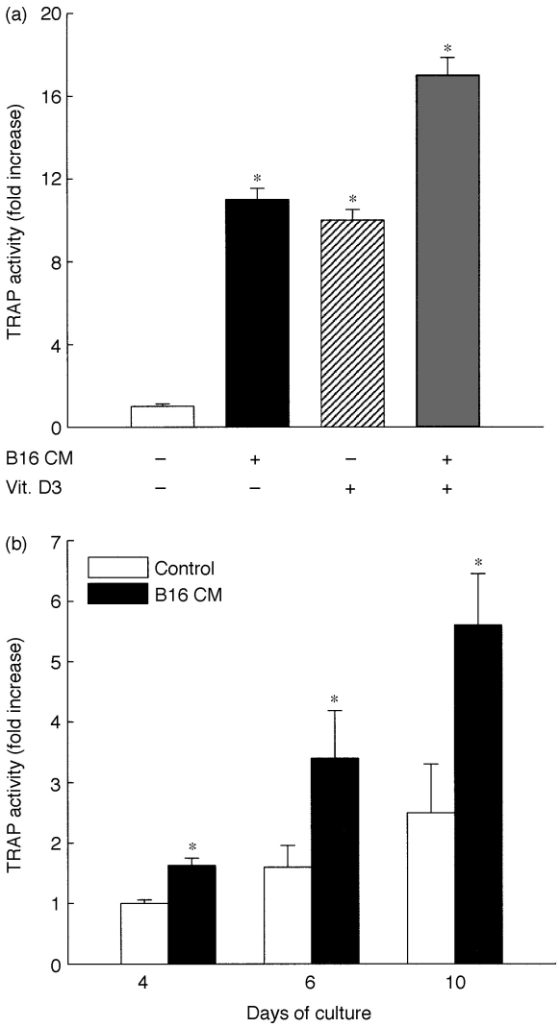

Fig. 3. Biochemical assay for TRAP activity. (a) Bone marrow adherent cells were incubated for 10 days in medium containing 3% FBS, with or without B16 conditioned medium (B16 CM) or 1,25(OH)$_2$vitamin D$_3$ (Vit. D3), as indicated on abscissa. TRAP activity was evaluated biochemically and expressed as fold increase versus untreated controls. Data are the mean±standard error of the mean (SEM) of three independent experiments. *$P<0.01$ and **$P<0.001$ versus control. (b) Time-course of TRAP activity. Bone marrow adherent cells were incubated in medium containing 3% FBS and 48-h conditioned medium (1:4) from B16 melanoma cells (B16 CM) for the time indicated on abscissa, with medium replenishment every other day. TRAP activity was evaluated biochemically and expressed as fold increase versus untreated controls at 4 days of culture. Data are the mean±SEM of three independent triplicate experiments.*$P<0.05$ versus the control of the same day.

trypsin/0.02% ethylene diamine tetraacetic acid (EDTA) at 37°C for 5 min and replating onto bone slices resulted in efficient pit formation (Fig. 1d).

3.2. Osteoclast generation by B16 melanoma cell conditioned medium

To test whether B16 cells affected osteoclast differentiation, we first established bone marrow cultures in which the percent of FBS was progressively reduced from 10 to 1%. This was done in order to minimise the effects of serum factors that could modify the responses to the factors released by the melanoma cells. We observed that, although reduced, osteoclastogenesis could be clearly induced by 1,25$(OH)_2$vitamin D_3 in low serum (Fig. 2b), and that 3% FBS still allowed proper cell growth (Fig. 2a). Therefore, we selected this concentration for the subsequent experiments where conditioned media from B16 cell cultures were tested. To obtain conditioned media, B16 confluent cells were starved in serum-free, 0.2%BSA-containing medium for 48 h, then the conditioned media were collected and used diluted 1:4 with fresh medium to a final FBS concentration of 3%. Bone marrow cultures were then established in parallel with or without melanoma conditioned media, and with or without 1,25$(OH)_2$vitamin D_3. Using this experimental design, we observed that cultures treated with melanoma conditioned media were able to undergo osteoclastogenesis (Fig. 2c).

In these cultures, we measured TRAP activity and observed that, compared with control, it was increased by the B16 conditioned media to a similar extent as by 1,25$(OH)_2$vitamin D_3. Some additive effect (approximately 1.6 fold) could be observed when both conditioned media and 1,25$(OH)_2$vitamin D_3 were used in combination (Fig. 3a). The effect was time-dependent, as a progressive increase of TRAP activity was observed in the B16 conditioned medium-treated cultures from day 4 to 10 of culture (Fig. 3b). Enumeration of TRAP-positive giant cells demonstrated that B16 CM induced an increase of multinucleated osteoclasts relative to untreated cultures similar to that induced by 1,25$(OH)_2$vitamin D_3. Again, when B16 CM and 1,25$(OH)_2$vitamin D_3 were used in combination, an additive effect could be observed (Fig. 4). In order to evaluate the specificity, we treated the bone marrow cultures with medium conditioned by NIH3T3 fibroblasts (NIH3T3 CM) prepared as per the B16 CM. In

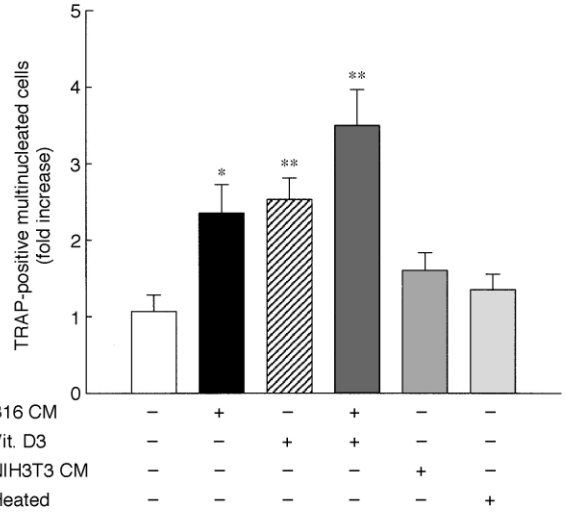

Fig. 4. Estimation of osteoclast number. Bone marrow adherent cells were incubated in medium containing 3% FBS, with or without B16 conditioned medium (B16 CM) or 1,25$(OH)_2$vitamin D_3 (Vit. D3), as indicated on abscissa. Cultures were also incubated with NIH3T3 conditioned media (CM), as well as with B16 CM previously inactivated by heating at 57°C for 30 min (heated B16 CM). TRAP activity was evidenced histochemically, then TRAP-positive multinucleated cells were enumerated and expressed as fold increase versus untreated controls. Data are the mean±SEM of three independent experiments. *$P < 0.01$ and **$P < 0.001$ versus control.

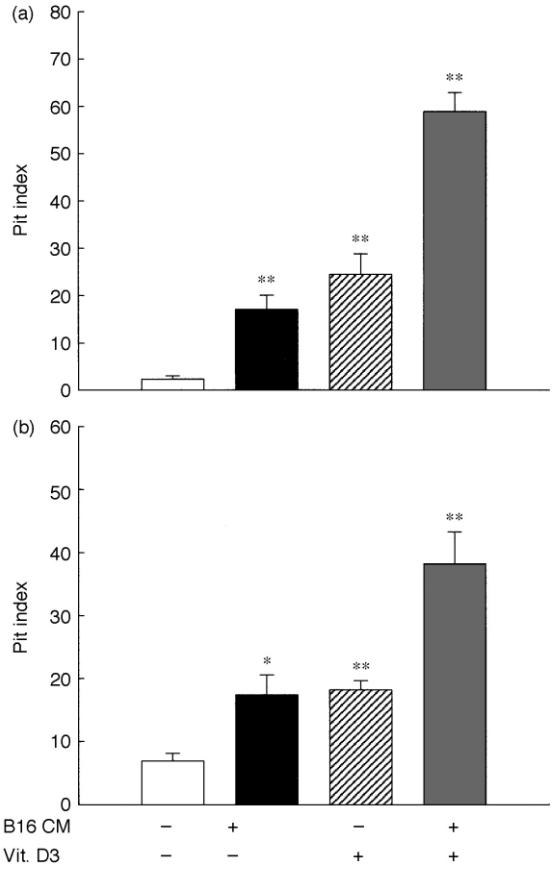

Fig. 5. Pit assay. (a) Bone marrow cultures were established in the presence of bone slices and treated as indicated throughout the osteoclastogenesis time (10 days). (b) Osteoclasts were first differentiated from bone marrow by 1,25 $(OH)_2$vitamin D_3 as indicated in methods, then trypsinised, plated onto bone slices and treated as indicated for 48 h. At the end of incubation, bone slices were cleaned free from cells by ultracentrifugation and stained with toluidine blue. Pits were enumerated and the pit index computed according to Caselli and colleagues [13]. Data are the mean±SEM of three independent experiments. *$P < 0.01$ and **$P < 0.001$ versus control.

this circumstance, we did not observe a significant increase in osteoclast number relative to the controls (Fig. 4). Interestingly, heat-inactivated B16 CM obtained by incubation at 57°C for 30 min, failed to stimulate osteoclast formation relative to the untreated cultures (Fig. 4). These results strongly suggest that heat-sensitive, melanoma cell-specific paracrine factors are likely to contribute to the melanoma-dependent induction of osteoclastogenesis, and that direct tumour cell–bone cell contact is not mandatory.

We next evaluated by the pit assay whether B16 CM was able to stimulate bone resorption. To accomplish this, bone marrow cultures were established in the presence of bone slices, and the incubation with the test agents was proceeded throughout the osteoclastogenesis time (10 days). With this experimental design, we observed that B16 CM stimulated bone resorption, similar to $1,25(OH)_2$vitamin D_3. Again, an additive effect was noticed when B16 CM and $1,25(OH)_2$vitamin D_3 were added together (Fig. 5a). Similar results were obtained using osteoclasts previously differentiated with $1,25(OH)_2$vitamin D_3, then plated onto bone slices and incubated with B16 CM for 48 h (Fig. 5b). NIH3T3 CM and heat-inactivated B16 CM had no effect on the pit index (data not shown).

3.3. Transcriptional expression of paracrine factors

Among the numerous cell-derived bone-seeking factors, PTHrP, IL-1β, IL-6, M-CSF, GM-CSF, TGFβ-1,

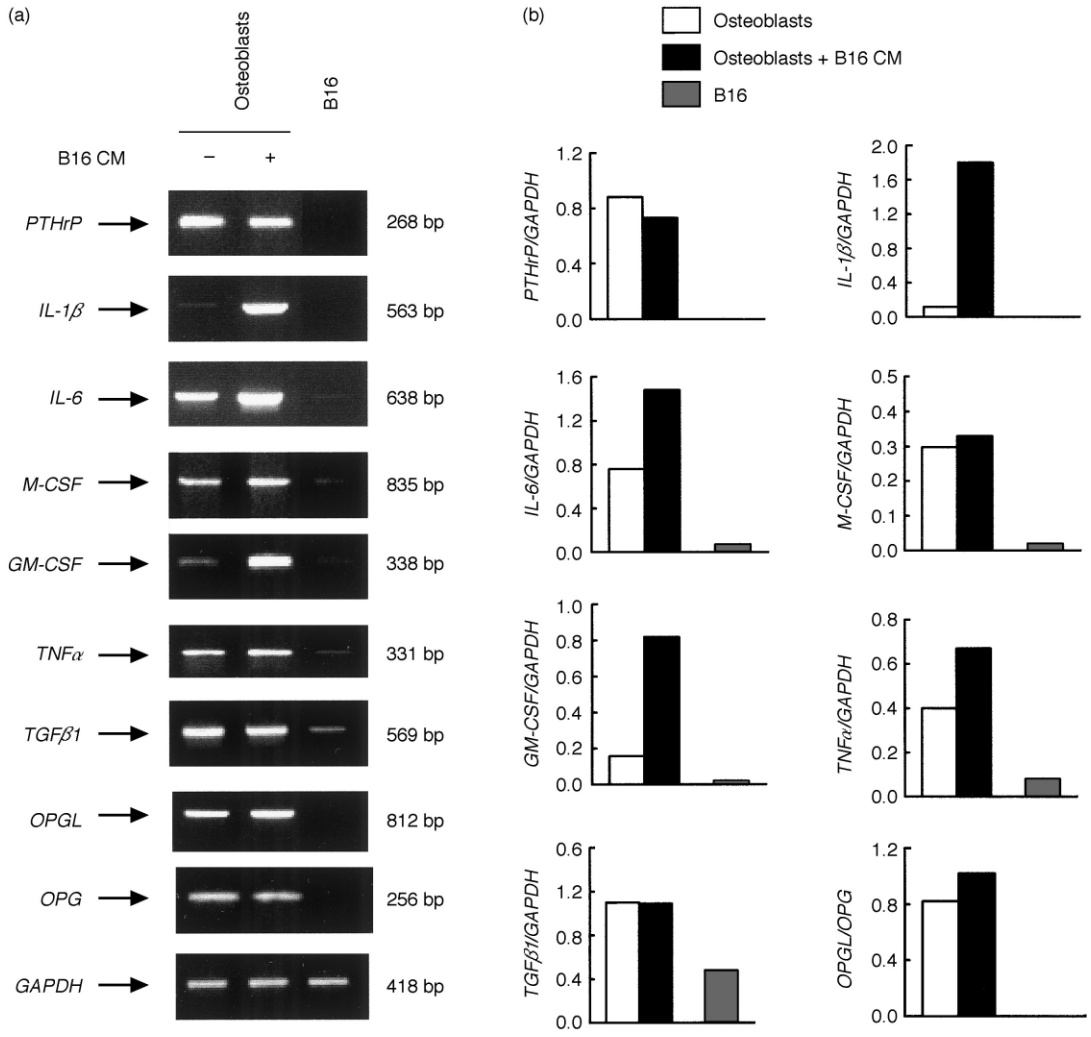

Fig. 6. Transcriptional expression of parathyroid hormone related peptide (*PTHrP*), interleukin-1β (*IL-1β*), interleukin-6 (*IL-6*), macrophage-colony stimulating factor (*M-CSF*), granulocyte, macrophage-colony stimulating factor (*GM-CSF*), tumour necrosis factor α (*TNFα*), transforming growth factor β1, (*TGFβ1*), osteoprotegerin ligand (*OPGL*) and osteoprotegerin (*OPG*) and glyceraldehyde phosphate dehydrogenase (*GAPDH*). (a) B16 cells were grown in conditions identical to those used for obtaining the conditioned medium. Confluent osteoblasts were incubated in 3% FBS-containing Dulbecco's Modified Eagle Medium (DMEM) with or without B16 cell conditioned medium (1:4) for 24 h. Cells were lysed, the RNA extracted and reverse-transcribed. The equivalent of 0.1 μg RNA was subjected to a polymerase chain reaction (PCR) reaction. (b) Densitometric analysis of the PCR shown in (a). Note that in the last panel, the *OPGL*/*OPG* ratio is represented. Similar results were observed in three independent experiments.

TNFα, OPGL and OPG are likely to play pivotal roles in regulating osteoclastogenesis, and have been referred to as potent modulators of tumour-induced osteolytic lesions [23–28]. Therefore, we tested whether B16 cells expressed these molecules by RT-PCR. For this series of experiments, we used as reference cells primary osteoblasts, which are known to release these cytokines and support osteoclastogenesis. Fig. 6 demonstrates that B16 cells failed to express detectable levels of *PTHrP*, *IL-1β*, *OPGL* and *OPG* mRNAs, and showed faint expression of *IL-6*, *M-CSF*, *GM-CSF* and *TNFα* mRNAs. The expression of *TGFβ*-1 was instead clearly apparent, but still lower than in primary osteoblasts. These results suggest that none of these factors is a 'strong' candidate to directly support osteoclast generation. However, B16 conditioned media could stimulate the expression of the paracrine factors in the osteogenic cells. To explore this possibility, we treated confluent osteoblasts with B16 conditioned medium, diluted 1:4 in 3% FBS-containing medium, for 24 h. RT-PCR showed no regulation of *PTHrP*, *M-CSF*, *TGFβ*-1 and *OPG*, and a modest upregulation of *OPGL* and *TNFα*. In contrast, *IL-1β*, *IL-6* and *GM-CSF* were strongly enhanced (Fig. 6), indicating that osteoblasts could be targeted by B16 conditioned medium and prompted to synthesise regulators of osteoclastogenesis.

3.4. Regulation of osteoclast generation by IL-1β, IL-6 and GM-CSF

In order to address whether the osteoblast factors upregulated by B16 CM could contribute to the regulation of osteoclastogenesis, we established bone marrow cultures and treated them for 10 days with mouse recombinant IL-1β (10 ng/ml), IL-6 (10 ng/ml) and GM-CSF (0.5 ng/ml). We observed that, while IL-1β and IL-6 induced osteoclastogenesis, GM-CSF was without effect (Fig. 7). In a pit assay, IL-1β and IL-6 also enhanced bone resorption (by 4.25- and 3.53-fold, respectively, $n=3$, $P<0.01$; data not shown).

3.5. c-Fos and c-Src protein expression

We further investigated whether in the bone marrow cultures melanoma conditioned media affected the expression of c-Fos and c-Src, the products of two proto-oncogenes which have been observed as being relevant for the osteoclast function. The *c-Fos* gene encodes for a member of the activator protein-1 (AP-1) transcription factor. This gene is critical for osteoclast differentiation [29], and its targeted disruption prevents osteoclastogenesis in mice [30]. The c-Fos protein was upregulated approximately 1.5-fold in bone marrow cultures incubated with 10^{-8} M $1,25(OH)_2$vitamin D_3 (Fig. 8a). In the cultures where the melanoma conditioned media were tested, a similar, but slightly smaller,

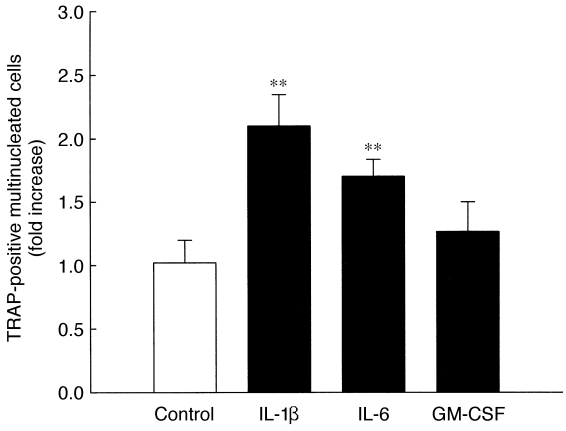

Fig. 7. Osteoclast generation by cytokines. Bone marrow adherent cells were incubated in medium containing 3% FBS and mouse recombinant IL-1β (10 ng/ml), IL-6 (10 ng/ml) or GM-CSF (0.5 ng/ml) for 10 days. TRAP activity was evidenced histochemically, then TRAP-positive multinucleated cells were enumerated and expressed as fold increase versus untreated controls. Data are the mean±SEM of three independent experiments. $*P<0.001$ versus control.

increase in c-Fos expression was observed (Fig. 8b), indicating that this gene is modestly modulated by melanoma-derived paracrine factors. The *c-Src* gene has been shown to be critical for the bone resorbing activity, in that loss of function mutations of *c-Src* in mice impair osteoclast function [31]. This protein was upregulated by $1,25(OH)_2$vitamin D_3 (Fig. 8a). Similarly, melanoma conditioned media increased c-Src expression in the bone marrow cultures in parallel to osteoclast generation (Fig. 8b).

To explore whether B16 cells directly modulated osteoclast c-Src, we first differentiated the osteoclasts by vitamin D_3 as described earlier, then purified them by flushing off the stromal cells, and finally treated the purified osteoclast population for 48 h with the B16 cell conditioned medium. As shown in Fig. 9, in these circumstances western blotting analysis demonstrated a greater upregulation of c-Src expression in the purified osteoclasts versus stromal cells. These data indicate that B16 cells can be capable of paracrine stimulation of c-Src in the osteoclast lineage, and rule out the possibility that increased c-Src levels observed during osteoclastogenesis were merely due to increments in osteoclast numbers.

4. Discussion

In this work, we have demonstrated that melanoma cells induce osteoclast generation with a mechanism that is independent of cell–cell contact, specifically upregulating the levels of the c-Src tyrosine kinase. Tumour-induced osteoclast generation is likely to be due to indirect, osteoblast-mediated, stimulation via the IL-1β and IL-6 pathways.

To date, there have been limited *in vitro* investigations aimed at dissecting the mechanisms involved in cancer-induced osteolytic lesions. Clohisy and colleagues [32] have shown the osteoclastogenic potential of breast carcinoma conditioned medium *in vitro*. Quinn and colleagues [33] have employed macrophages isolated from primary breast carcinomas and found that they could be prompted to develop into an osteoclast-like phenotype by cocultures with UMR 106 rat osteoblast-like cell line in the presence of $1,25(OH)_2$vitamin D_3. Ono and colleagues [34] showed that the BALB/c-MC breast carcinoma cell line stimulates osteoclastogenesis, but that tumour cell-bone marrow cell contact is strictly required. Pederson and colleagues [24] have established the pattern of stimulation of osteoclast bone resorption *in vitro* by a breast cancer cell line, and Grano and colleagues [35] have shown that the same cell line stimulates IL-6 production by osteoclasts. Other studies used

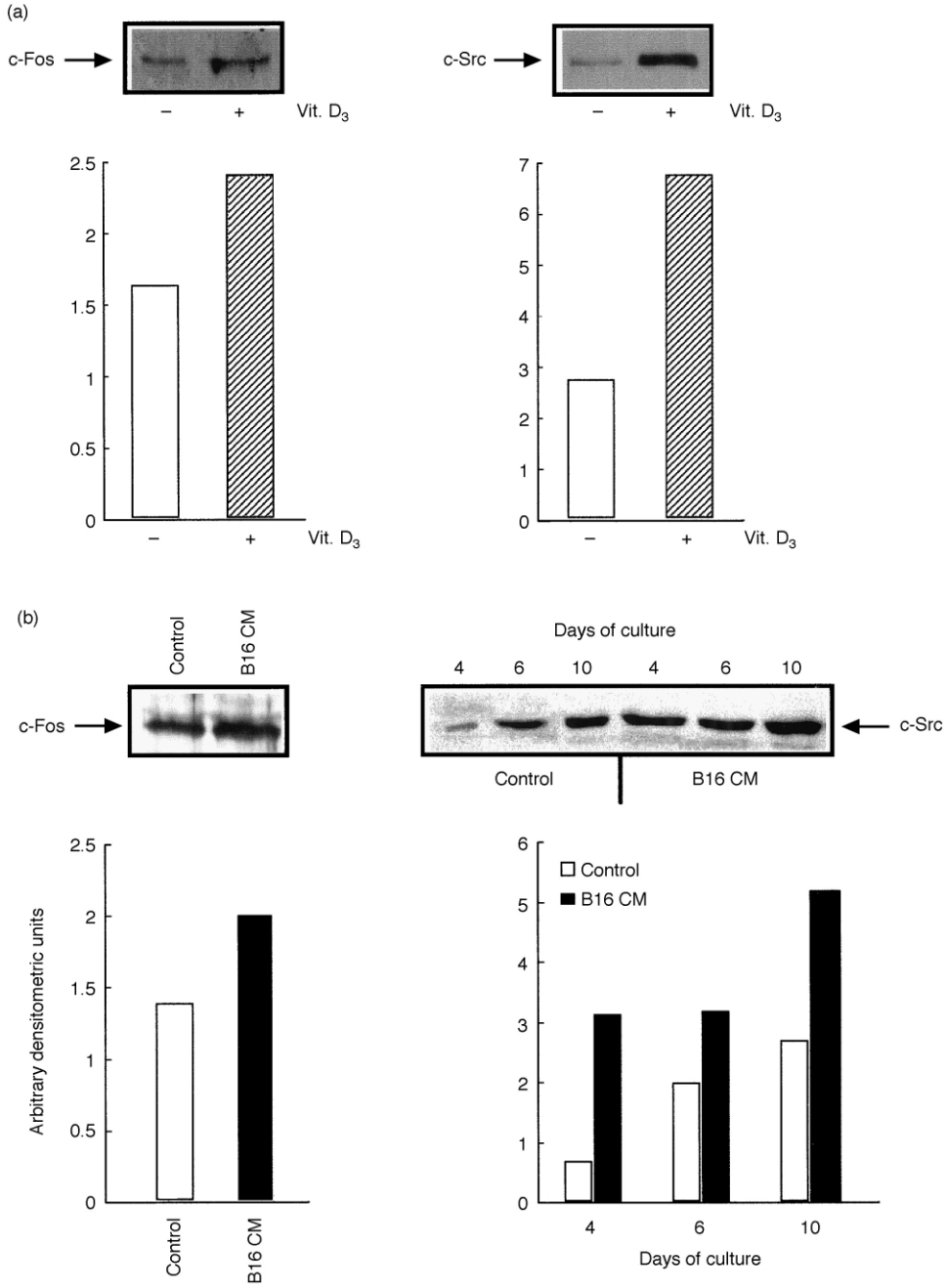

Fig. 8. Analysis of c-Fos and c-Src expression. Osteoclasts were generated *in vitro* in the presence of (a) 10^{-8} M $1,25(OH)_2$vitamin D_3 (Vit. D3) or (b) 48-h conditioned medium (1:4) from B16 melanoma cells (B16 CM). c-Fos and c-Src expression was then evaluated by western blot analysis. Antibody dilution, primary 1:200; secondary 1:5000. Protein loading: 50 μg. In (a) expression was evaluated after 10 days. In (b) c-Fos expression was evaluated after 10 days and c-Src expression was evaluated at the indicated days of culture. Similar results were observed in three independent experiments.

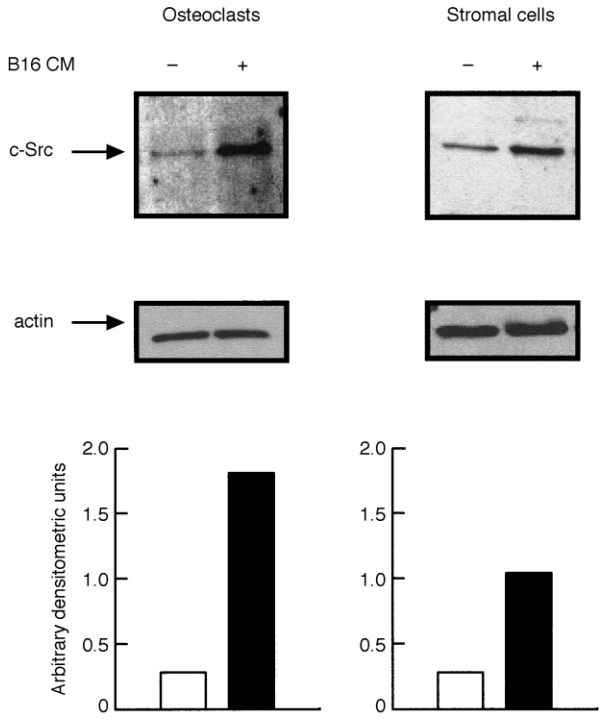

Fig. 9. Induction of c-Src expression in differentiated osteoclasts. Bone marrow adherent cell cultures were performed at high cell density and osteoclasts were differentiated in the presence of 10^{-8} M $1,25(OH)_2$vitamin D_3 for 7 days. Osteoclasts and their putative mononuclear precursors were then purified free from stromal cells (purity >80%), and treated with a 48-h B16 conditioned media (B16 CM), diluted 1:4, for a further 48 h. In parallel experiments, stromal cells were obtained from bone marrow cell cultures incubated, in the absence of $1,25(OH)_2$vitamin D_3, and treated with B16 cell conditioned media as described for the osteoclasts. Cells were then lysed and cell protein subjected to western blotting analysis as described in Fig. 8. Protein loading: 30 μg. Similar results were observed in three independent experiments.

in vivo approaches in nude mice which, although allowing a fine morphological and histochemical characterisation of the lesions [36,37], and providing important information particularly regarding the therapeutic effects of bisphosphonates [38], did not allow analysis at the cellular and molecular levels.

Physiological osteoclast generation occurs only if osteogenic cells establish direct contact with the osteoclast precursors via cell adhesion molecules and membrane bound OPGL [29,39]. In addition, osteoblasts secrete paracrine factors [23,40,41] that regulate osteoclast development and function. This was the case also in our bone marrow cultures, in which cells positive for the osteoblast marker alkaline phosphatase were mixed with the osteoclast precursors. Ono and colleagues [34] have shown that breast cancer cells stimulate osteoclast formation only if cocultured with bone cells. Our experimental model clearly revealed that for melanoma this is not a critical event, and that paracrine stimulation induces osteoclastogenesis. However, our data seem to rule out direct involvement of PTHrP, OPGL, OPG and IL-1β as paracrine regulators produced by melanoma. *IL-6*, *M-CSF*, *GM-CSF*, *TNFα* and *TGFβ*-1 were all expressed by the B16 cells, however their levels were low relative to those in osteoblasts. According to this observation, we believe that it is unlikely that, at least in our hands, these cytokines have a direct role in the osteogenic potential of this cell line. Interestingly, however, we found that B16 conditioned media enhanced the transcription of *IL-1β*, *IL-6*, *GM-CSF* and, to a lesser degree, *OPGL* and *TNFα* in osteoblasts. Therefore, we can speculate that one of the mechanisms employed by B16 to stimulate osteoclastogenesis is indirect, via the enhancement of cytokine expression by osteoblasts. In confirmation, addition of mouse recombinant IL-1β and IL-6 to the bone marrow cultures enhanced osteoclastogenesis, whereas GM-CSF had no effect. We have to point out that our results are in contrast with those recently published by Chikatsu and colleagues [42], who observed that B16 cells induced osteoclast generation only when cocultured with bone marrow cells, with a mechanism which appeared to depend on OPGL/OPG regulation. The reason for this discrepancy is at present unknown. However, our study clearly indicates that alternative pathways may contribute to the osteoclastogenesis potential of B16 melanoma cells.

We also demonstrated that B16 cell conditioned media faintly upregulated c-Fos, but greatly enhanced c-Src levels. c-Src increments seem to be due to an effect of the conditioned media on osteoclast gene expression, and not merely to the fact that our cultures were progressively enriched in osteoclasts. This gene was apparently affected by B16 cell conditioned medium in the stromal lineage as well, but to a lesser extent (~3.7-fold versus ~6.5-fold in osteoclasts). In our opinion, this does not subtract from the results, due to the osteoclastogenesis supporting role of these cells. We thus believe that the *c-Src* proto-oncogene may represent an important target for melanoma paracrine regulation of osteoclast function, and that this finding may be of great relevance in view of a therapeutical application of c-Src inhibitors for the prevention of osteolytic metastases. In fact, it is clear from *c-Src* knockout studies in mice that, despite the fact that this gene is abundantly expressed in several cell types including neurones [43] and platelets [44], the only obvious phenotype develops in the skeleton due to poor osteoclast activity [31,45], and, in our hands, to altered osteoblast function [46]. This may be due to compensation for the loss of *c-Src* by other members of the *c-src* family in other cell types, except the bone cells. Therefore, inhibition of c-Src would be predicted to affect bone without altering the metabolism of other organs, making c-Src an elective candidate for therapeutical interruption of bone resorption [47]. Inhibition of bone resorption by c-Src inhibitors could in turn reduce the malignant mass within the bone micro-

environment. In fact, it has been proposed that a reciprocal stimulation is established between osteoclastic cells and metastasis [4,5]. Due to activation of osteoclasts, a number of cytokines and growth factors are produced or released from the bone matrix, and stimulate the growth of tumour cells within the bone microenvironment [48]. Recent evidence in animal models has revealed that inhibition of bone resorption by bisphosphonates reduces the incidence and the expansion of bone metastases [37,49]. This is, therefore, considered a tool, not only to decrease morbidity due to osteolysis, but also to retard tumour dissemination within the bone. According to our results, a similar approach could be predicted to be successful for c-Src inhibitors [47].

In conclusion, we provided evidence that one of the conditions determining the spreading of osteolytic lesions by murine melanoma relates to the capability of this tumour to stimulate osteoclastogenesis, and that paracrine modulation, and not cell–cell contact, represents the underlying mechanism. We also demonstrated that melanoma may influence osteoclast generation via osteoblasts. Finally, we showed that melanoma upregulates c-Src, a non-receptor tyrosine kinase central to bone cell function, that can be regarded as a potential target for the pharmacological control of bone metastases.

Acknowledgements

The excellent technical work of Mr Giancarlo Sciortino and Mr Luigi Pellegrino is gratefully acknowledged. This study was supported by grants from the 'Associazione Italiana per la Ricerca sul Cancro' (AIRC) and from the 'Ministero dell'Università e della Ricerca Scientifica e Tecnologica-Università dell' Aquila-Cofinanziamento 1997' to AT, and a grant from 'Ministero della Sanità' to the 'Istituto Dermopatico dell'Immacolata' to T.F. Dr Marina Brama is the recipient of a fellowship from 'Fondazione Italiana per la Ricerca sul Cancro' (FIRC).

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