Histogenetic Characterization of Giant Cell Tumor of Bone

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Abstract The unpredictable behavior of giant cell tumor (GCT) parallels its controversial histogenesis. Multinucleated giant cells, stromal cells, and CD68⁺ monocytes/macrophages are the three elements that interact in GCT. We compared the ability of stromal cells and normal mesenchymal stromal cells to differentiate into osteoblasts. Stromal cells and mesenchymal cells had similar proliferation rates and lifespans. Although stromal cells expressed early osteogenic markers, they were unable to differentiate into osteoblasts but they did express intracellular adhesion molecule-1, a marker of bone-lining cells. They were unable to form clones in a semisolid medium and unable to promote osteoclast differentiation, although they exerted a strong chemotactic effect on osteoclast precursors. Stromal cells may be either immature proliferating osteogenic elements or specialized osteoblast-like cells that fail to show neoplastic features but can induce the differentiation of osteoclast precursors. They might be secondarily induced

to proliferate by a paracrine effect induced by monocyte-macrophages and/or giant cells. The increased number of giant cells in GCT may be secondary to an autocrine circuit mediated by the receptor activator of nuclear factor κ B.

Introduction

Giant cell tumor of bone (GCT) shows a typical histologic picture [9, 23]. Multinucleated osteoclast-like giant cells (GC), actively resorbing host bone through cathepsin K activity [30], are variably mixed with a mononuclear cell component, among which proliferating spindle-shaped stromal cells (SC) with benign morphologic characteristics [24, 28, 37] and CD14⁺-monocytes/CD68⁺-macrophages are present [21, 29]. Despite its benign histologic appearance, GCT has an unpredictable clinical course. After intralesional or even marginal excision, it locally recurs in 20% to 40% of cases [39], and in less than 5% of cases, GCT may develop histologically benign metastases [3]. GCT may also spontaneously undergo sarcomatous transformation [5], the risk of progression being greatly increased by radiation [6]. The outcome of this lesion cannot be predicted on the basis of histologic or radiographic criteria [9, 23], and in the absence of a clear histogenetic origin, GCT is currently classified among lesions with an uncertain derivation and named after its peculiar morphologic appearance.

A number of studies have explored the histogenesis of GCT with the specific purpose of distinguishing neoplastic elements from reactive cells [13, 14]. As a result of the presence of a remarkably high number of osteoclast-like GC, early researchers suggested GCT was a neoplasm of the osteoclastic lineage, hence the term “osteoclastoma” [7]. However, GC show typical features of normal osteoclasts,

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including calcitonin and vitronectin receptor expression, tartrate-resistant acid phosphatase (TRACP) activity, and lacunar resorption ability [1, 14, 30, 35]. SC were therefore believed the most likely candidate neoplastic elements of GCT, partly based on their ability to grow both in vitro and in vivo [2, 24]. SC exhibit markers of osteogenic differentiation [36, 46] and are able to stimulate osteoclasts [34, 37] through the ligand for receptor activator of nuclear factor κ B (RANKL) [20, 28, 38], a growth factor essential for the recruitment of osteoclasts by osteoblasts under physiological conditions [18]. In agreement with a putative osteogenic origin of SC, osteoid foci may be found both inside and at the periphery of GCT [8, 41], and bone formation, but no evidence of osteoclastogenesis has been observed in immunodeficient mice after subcutaneous injection of GCT tissue or cells [2, 24]. Molecular profiling of GCT has recently demonstrated RANKL is highly expressed in GC but not in SC, suggesting the GC component of GCT is unlikely to be recruited as a result of RANKL production by SC [35]. Thus, neither cytogenetic nor molecular analysis has provided consistent evidence the SC are the neoplastic element. In fact, although telomeric fusions and a reduction in telomeric length are frequent in SC derived from GCT [48], the DNA distribution pattern of GCT is invariably diploid [27, 31], and no deletions or amplifications appear present in SC [35].

Under these circumstances, the hypothesis that SC derive from osteoblast precursors, although proliferating elements are also truly neoplastic and responsible for the differentiation and activation of GC, is questionable. This raises the possibility that GCT derives either from the hematopoietic compartment or from an undefined precursor sharing both stromal and osteoclastic phenotypes [35]. At present, however, the exact nature of GCT, the identity of its neoplastic component, and the relation between its different elements remain as elusive as unpredictable as its clinical behavior.

We explored the transformed nature and the osteoblast-like features of SC from GCT by comparing SC to normal mesenchymal cells (hMSC) and to the transformed Saos-2 cell line for (1) differentiation (expression of osteoblast markers), (2) proliferation (rates), (3) tumorigenicity (growth in independent adhesion condition on semi-solid

medium), (4) induction of chemotaxis of osteoclast precursors, and (5) induction of osteoclast differentiation.

Materials and Methods

We analyzed the SC behavior in terms of osteoblast differentiation, proliferation, tumorigenicity, and induction of osteoclast chemotaxis and differentiation. For this purpose, three different types of cells were compared. In particular, five different SC cultures isolated from GCT tissues were compared with two hMSC cultures and one osteoblast-like tumor cell line. GCT tissue samples, obtained from five patients undergoing surgical excision (Table 1), were partly processed for histology, partly snap-frozen in liquid nitrogen, and stored at -80°C for molecular analysis and partly used to obtain primary cultures. In all cases, histologic evaluation confirmed the typical features of GCT [23]. In Case 4, however, both size and number of GC were remarkably less represented than in other cases. To obtain cell cultures, tumor specimens were minced into small fragments, placed in culture dishes, cultured in Iscove's Modified Dulbecco's Medium (IMDM; Invitrogen, Carlsbad, CA) plus 10% fetal bovine serum (FBS; Mascia Brunelli, Milan, Italy), 2 mM L-glutamine, 100 U/mL penicillin (Invitrogen), and 100 mg/mL streptomycin (Invitrogen) and incubated at 37°C in a 5% CO_2 humidified atmosphere. At confluence, adherent cells were subcultured, whereas nonadherent cells and tumor debris were discarded. GC were present only at the first passage, whereas SC were further amplified. Only SC at the fifth to sixth passage were used. hMSC were isolated by density gradient from patients undergoing hip surgery, as previously described [32], and maintained in α -Modified Minimum Essential Medium (Sigma, St Louis, MO) plus 10% FBS, 2 mM L-glutamine, 100 mM ascorbic acid-2 phosphate, and 1% penicillin-streptomycin. Human peripheral blood monocytes (PBMC) were obtained from fresh buffy coats (AVIS, Bologna, Italy) layered over Ficoll (Sigma). Mononuclear cells were extracted and seeded in Dulbecco's Modified Eagle's Medium High Glucose (DME/HIGH; Euroclone, Milan, Italy) plus 10%

Table 1. Clinical characteristics of five giant cell tumors of bone

Case	Age (years)	Gender	Site	Grade	Remarks
GCT-1	53	Male	Distal femur	3	Recurrence
GCT-2	33	Female	Distal femur	3	Primary; pathologic fracture
GCT-3	51	Male	Distal radius	2	Primary
GCT-4	17	Female	Distal femur	3	Primary
GCT-5	25	Female	Proximal tibia	2	Recurrence

GCT = giant cell tumor.

FCS (Hyclone, Milan, Italy) [15]. To obtain osteoclast cultures, the medium was replaced after 1 hour with a differentiating medium (RANKL 30 ng/mL and macrophage colony-stimulating factor [M-CSF] 25 ng/mL; Peprotech, Rocky Hill, NJ). After 7 days, TRACP activity was evaluated by cytochemistry (Acid Phosphatase Leukocyte Assay; Sigma). TRACP-positive cells containing three or more nuclei were considered osteoclasts. Saos-2 osteosarcoma cells (ATCC, Manassas, VA) were maintained in IMDM plus 10% FBS, 2 mM L-glutamine, and 1% penicillin and streptomycin at 37°C in a 5% CO₂ humidified atmosphere.

Cells were fixed, stained with acridine orange or Hoechst 33258 (6 and 1.25 mg/mL, respectively; Sigma) and observed by fluorescence microscopy. Two of the authors (SA, MS) independently determined the percentage of binuclear cells from a total of 300 counted cells.

SC, hMSC, and Saos-2 were seeded in osteoblast-differentiating medium (100 µM ascorbic acid-2 phosphate and 10⁻⁸ M dexamethasone; Sigma) and, at confluence, either fixed to determine ALP activity (kit Number 86R; Sigma) or further cultured with 10 mM β-glycerophosphate-containing medium to evaluate their potential of osteogenic differentiation. After 2 weeks, cells were fixed

and stained with 2% alizarin red (Sigma) to identify mineral nodule formation.

To evaluate mRNA transcription of stromal cell-derived factor-1 (SDF-1), intercellular adhesion molecule-1 (ICAM-1), and RANKL, total RNA was isolated from tissue samples or from untreated semiconfluent adherent cells (SC and hMSC). To assess the transcription of the Core binding factor a-1 (Cbfa1), osteocalcin, and Type I collagen, SC and hMSC were seeded in differentiating medium, and total RNA was obtained at medium change (T0) and after 14 days (T1). mRNA from cell cultures or from pulverized tissue (Mikro-Dismembrator; B Braun Biotech International, Melsungen, Germany), were extracted by TRIzol RNA isolation reagent (Invitrogen), reverse-transcribed into cDNA (Advantage RT-for-PCR Kit; Clontech Laboratories, Palo Alto, CA), and amplified as follows: denaturation at 94°C (5 minutes), 30 cycles of denaturation (94°C, 30 seconds), annealing (30 seconds), extension (72°C, 30 seconds), and final extension at 72°C (7 minutes). Bands were quantified by a dedicated software (Quantity one; Biorad, Hercules, CA). We used various primers and annealing temperatures (Table 2).

We assessed cell growth rates by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) bromide

Table 2. Primers and annealing temperatures used for reverse transcriptase-polymerase chain reaction analysis

Primer sequence (5'-3')	NCBI sequence viewer (accession number)	Product size	Annealing temperature
β-actin	NM_001101	838 bp	65°C
5'-ATCTGGCACCACCTTCTACAATGAGCTGCG-3'			
5'-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3'			
Cbfa1	NM_004348	306 bp	57°C
5'-CCAGCCACCTTTACTTACAC -3'			
5'-AGCGTCAACACCATCATTTCT-3'			
Osteocalcin	NM_199173	250 bp	55°C
5'-AGCGAGGTAGTGAAGAGA-3'			
5'-AGGGGAAGAGGAAAGAAG-3'			
SDF-1	NM_001033886	229 bp	62°C
5'-AGCCAACGTCAAGCATCTCA-3'			
5'-CCTTTTCTGGGCAGCCTTTC-3'			
Type I collagen	NM_000088	523 bp	54°C
5'-CCCACCGACCAAGAAAC-3'			
5'-CACCATCCAAACCACTGA-3'			
ICAM-1	NM_000201	395 bp	57°C
5'-TCATCACTGTGGTAGCAGCC-3'			
5'-GTCTTGCTCCTTCTCTTGG-3'			
RANKL	NM_003701	122 bp	54°C
5'-CGTCGCCCTGTTCTTCTA-3'			
5'-GAGTTGTGTCTTGAAAATCTGC-3'			

Cbfa1 = Core binding factor a-1; SDF-1 = stromal cell derived factor-1; ICAM-1 = intracellular adhesion molecule-1; RANKL = receptor activator of nuclear factor κB.

colorimetric assay. Cells were seeded (7,000 cells/cm² for Saos-2 and SC or 18,000 cells/cm² for hMSC) in duplicate in complete medium. After 3, 8, and 13 days, MTT (5 mg/mL; Sigma) was added and incubated for 3 hours at 37°C. After medium removal, dye crystals were solubilized by DMSO and the absorbance (540 nm) was read (Cytofluor-2350 fluorimeter; Millipore, Billerica, MA). This experiment was repeated twice. For colony-forming unit assay, we seeded cells in duplicate (30 cells/cm²) in complete IMDM. After 1 to 2 weeks, medium was discarded, and cells were fixed and stained with crystal violet dye (0.2%). Colonies with more than 10 cells were counted. This experiment was repeated three times.

We seeded cells in complete medium and fixed at semiconfluence, permeabilized with Hepes-Triton X-100, incubated with the Ki67 monoclonal antibody (DAKO, Glostrup, Denmark) [40, 42] and with a FITC-conjugated secondary antibody (DAKO) and counterstained with Evans Blue dye (Sigma). The percentage of Ki67-positive cells was calculated out of a total of 300 cells.

To analyze the *in vitro* replicative lifespan, we split SC or hMSC at a 1:3 or 1:2 ratio at early or late passages, respectively, and maintained in complete medium. The population doubling (PD) number was calculated by the count/split method. For the anchorage-independent growth assay, cells were plated in a semisolid medium (IMDM plus 10% FBS plus agar 0.33%; Sigma). Colonies were counted after 5 to 6 days. For both assays, cells were incubated at 37°C in a 5% CO₂ atmosphere.

The chemotactic activity of SC on CD14⁺-monocytes was analyzed by a migration assay. CD14⁺ PBMC were added to the higher chamber of a transwell with adherent SC or hMSC, or no cells (control), in the lower chamber. We seeded SC and hMSC in duplicate in a 48-well plate (60,000 cells/well). After 24 hours, medium was changed, polycarbonate filters (Costar, San Francisco, CA) with a pore size of 8.0 µm were added, and CD14⁺-monocytes were seeded in the upper compartment (30,000 cells/well). CD14⁺ cells were obtained from PBMC by immunomagnetic separation (Automated Magnetic Cell Sorting; Miltenyi Biotech, Bergisch Gladbach, Germany) [22] using CD14 antibody-conjugated magnetic particles (CD14 MicroBeads; Miltenyi Biotech). After 24 hours, the filter was removed and migrated cells were fixed, stained with a crystal-violet solution, and counted. The experiment was repeated twice.

We determined the induction of osteoclast-mediated resorption activity by performing cocultures of PBMC with SC or hMSC. These were seeded (1500 cells/cm²) on a layer of PBMC that had been previously (1 hour before) isolated and seeded on a cell culture plate coated with europium-conjugated collagen (OsteoLyse Assay kit; Lonza Group, Basel, Switzerland). After 10 days, released

europium-conjugated collagen fragments were detected by a time-resolved fluorescence plate reader (Wallac Victor; Perkin Elmer, Waltham, MA). To evaluate the secretion of the isoform 5b of TRACP (TRACP 5b), a specific marker of differentiated osteoclasts [16], PBMC were cocultured with SC or hMSC, as described for the OsteoLyse assay, and after 14 days, TRACP 5b activity was quantified in the supernatant by the BoneTRAP Assay (SBA-Sciences, Oulu, Finland).

As a result of the low number of experiments, we presumed the data were not normally distributed. Therefore we used the Mann-Whitney U-test to evaluate the difference between SC cells and untreated hMSC for ICAM-1 mRNA expression and clonal efficiency or between SC or hMSC or Saos-2 and the control condition for chemotaxis, TRACP 5b, and collagen degradation assays. We used StatViewTM 5.0.1 software (SAS Institute Inc, Cary, NC) for all analyses.

Results

SC cultures were composed of mononuclear spindle-shaped and polygonal cells (Fig. 1A–E), whereas hMSC were homogeneous cultures of flattened cells (Fig. 1F). In SC cultures only, binuclear cells were also observed (20%–27%), mostly polygonal.

All osteoblastic markers but Type I collagen were consistently expressed in GCT tissue samples (Fig. 2A). SC showed variable levels of ALP activity and a mild mineralization activity, remarkably less than hMSC (Table 3). We detected very low levels of *Cbfa1* and osteocalcin, and Type I collagen mRNA were detected in SC under basal conditions, whereas in hMSC, at the same time point, *Cbfa1* mRNA was already evident (Fig. 2B–D). After osteoblast differentiation induction (T1), mRNA for *Cbfa1* and osteocalcin were generally increased, whereas Type I collagen did not remarkably change. On the contrary, in stimulated hMSC (T1), *Cbfa1* decreased, whereas both osteocalcin and Type I collagen increased. To further verify the identity of SC, the mRNA of ICAM-1 was evaluated. At T0, SC showed a higher ($p = 0.0495$) expression of ICAM-1 than either unstimulated or stimulated hMSC (Fig. 2E).

The proliferation rate of SC was less (MTT assay $p = 0.0495$, Ki67 assay $p = 0.0209$) than that of Saos-2 but similar to that of hMSC (Fig. 3A–B). Still, SC had a more extended lifespan compared with hMSC. In fact, they were all maintained in continuous culture for 13 to 26 PD, whereas hMSC reached their senescence after 10 PD.

With regard to tumorigenesis, SC cells showed a higher ($p = 0.0039$) clonal efficiency compared with hMSC (Fig. 3C). Neither SC nor hMSC were able to form

Fig. 1A–F In vitro morphologic features of stromal cells (SC) and human mesenchymal stromal cells (hMSC) are shown. Cell morphology was assessed in SC-1 (A), SC-2 (B), SC-3 (C), SC-4 (D), SC-5 (E), and hMSC (F) after acridine orange staining of adherent cells. SC cultures were composed of spindle-shaped and polygonal (arrows) cells, whereas hMSC were homogeneous cultures of flattened cells. In SC cultures, binuclear cells were also observed (stain, acridine orange; original magnification, $\times 10$; bar = 50 μm).

colonies in soft agar in contrast to the high number of colonies (190) formed by Saos-2 cells.

All SC but SC-4 induced a considerable migration of CD14⁺ monocytes (SC-1, 2, 5, $p = 0.0495$), whereas hMSC did not exert any effect (Fig. 4A). The chemotactic effect of SC on monocytes did not appear related to the synthesis of the chemoattractive factor SDF-1, because SC-1 and SC-3 that induced cell migration did not express SDF-1 mRNA (Fig. 4B).

hMSC induced higher ($p = 0.0209$) levels of TRACP 5b and collagen degradation compared with untreated PBMC (control). However, when PBMC were cocultured with SC, no increase in osteoclast formation or collagen resorption activity was detected in comparison to hMSC (Fig. 5A–B). RANKL was expressed at high mRNA levels in all GCT tissue samples but one (GCT-4, interestingly

showing only a few small GC on histology), but was almost undetectable in all SC isolated from the corresponding tissue (Fig. 5C).

Discussion

GCT is a challenging histologically benign bone lesion that recurs rarely, although definitely originates “benign” metastases and frequently transforms to sarcoma after radiation. In the absence of a clear histogenetic origin, GCT is named after its peculiar histologic appearance. The typical morphologic description is that of a benign mononuclear SC lesion with abundant benign osteoclast-like giant cells. Immunohistochemical and molecular studies of GCT tissues demonstrate two populations of SC, one

Fig. 2A–E The expression of osteoblastic lineage-related genes is shown. The mRNA expression of osteoblast-related genes was analyzed in giant cell tumor (GCT) tissues, in untreated stromal cells (SC), or in human mesenchymal stromal cells (hMSC) (T0) and in SC or in hMSC after 14 days in differentiating medium (T1) (hMSC, $n = 3$). Gel electrophoresis results for GCT tissues (A); gel electrophoresis results and semi-quantitative analysis for SC and hMSC for core binding factor a-1 (Cbfa1) (B), osteocalcin (C), Type I collagen (D), and intracellular adhesion molecule-1 (E). In the upper panel, a representative image of the amplification product is shown, whereas in the lower panel, specific bands were quantified by dedicated software for densitometric evaluation. Each amplified product of the corresponding gene was normalized to β -actin signals determined in parallel for each sample (B–E); otherwise, only a representative image of the amplification product as a single panel is shown (A).

Table 3. Alkaline phosphatase and mineralization activity of SC and hMSC

Cell cultures	Alkaline phosphatase activity	Mineralization
GCT-1	–	–
GCT-2	+	–
GCT-3	–	+
GCT-4	+++	++++
GCT-5	+	–
hMSC	+ / +++	++++

The intensity and extent of staining was scored as follows: no staining (–), very weakly positive or less than 25% stained cells (+), weakly positive or 25% to 50% stained cells (++), moderately positive or 50% to 75% stained cells (+++), and strongly positive or greater than 75% stained cells (++++); SC = stromal cells; hMSC = human mesenchymal stem cells; GCT = giant cell tumor.

consisting of proliferating spindle-shaped cells that present markers of the osteoblastic lineage [19, 36], whereas the other population consists of polygonal cells, which stain for CD14⁺/CD68⁺ monocyte/macrophage antigens [29]. SC proliferate in vitro and are currently being considered as truly neoplastic elements of GCT that are able to activate osteoclast precursors and are responsible for GC formation. We therefore explored the potential role of SC from GCT as the neoplastic element by comparing their in vitro growth, expression of osteoblast markers, and ability to influence osteoclast activity with those of hMSC and Saos-2 osteosarcoma cells.

For this purpose, we isolated and in vitro amplified SC from five GCT fresh tissue samples. While these five cases reflected the spectrum of clinical and pathologic variability

Fig. 3A–C Proliferation rate and colony-forming unit (CFU) efficiency of stromal cells (SC) are shown. Cell proliferation, Ki67 index, and clonal efficiency were evaluated in SC and compared with human mesenchymal stromal cells (hMSC) or Saos-2 cells. (A) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay (hMSC $n = 2$) (A); Ki67 index (hMSC $n = 2$) (B); number of colonies formed after 1 to 2 weeks (hMSC $n = 3$) (C). The proliferation rate of SC is comparable to that of hMSC, whereas CFU is higher.

featured by GCT, including history (primary versus recurrence), imaging characteristics (active versus aggressive lesion), and typical histologic heterogeneity, the data reflects only five cases and might not represent data from a much larger sample. Another limit of this study is the fact that cells for in vitro culture did not always maintain the characteristics of SC observed in the original tissue samples. To partially overcome this inherent limitation of cell cultures, we also performed molecular analysis on tissue samples.

The hypothesis that SC derive from osteoblast precursors is based on the observation that they express early

osteogenic markers [19, 35, 46] and that the expression of these markers may be further induced by bone morphogenetic protein 2 [19]. To verify the differentiation level of this osteoblastic component of GCT, we compared SC and hMSC in terms of morphology, in vitro growth characteristics, and osteogenic differentiation. A 20% to 27% binuclear cell component [27] was present in SC but not in hMSC. Under basal conditions, SC and hMSC showed similar levels of ALP activity, but SC did not express Cbfa1, an early marker of osteoblastic differentiation that is always expressed in hMSC [26]. Cbfa1 is a member of the RUNX family of transcription factors with a highly restricted tissue expression pattern in bone. Its expression is crucial for osteogenic differentiation and bone formation [11], because it regulates the transcription of several osteoblast-specific genes, including osteocalcin and Type I collagen [17, 25]. After osteogenic induction, both Cbfa1 and osteocalcin mRNA were increased in SC. This effect was less evident for osteocalcin and opposite for Cbfa1 in hMSC. On the contrary, both Type I collagen mRNA and the ability to form mineral nodules remained almost null in SC as compared with hMSC. These data indicate that although SC express early osteogenic markers, their differentiation to a classic fully osteoblastic phenotype cannot be achieved even after appropriate induction. To verify if SC might be considered similar to another cell type, the so-called “bone lining cell” [12], expressing very low levels of Type I collagen and osteocalcin and known to contribute to bone collagen degradation, we analyzed the mRNA for ICAM-1, the expression of which is increased in this osteoblast subtype [12, 43]. Interestingly, ICAM-1 plays a pivotal role in osteoclastogenesis, because osteoblasts expressing ICAM-1 support bone resorption activity [44, 45]. mRNA semiquantitative analysis for ICAM-1 suggests this adhesion molecule is expressed in tissue samples of GCT, and SC tend to show a higher expression of ICAM-1 as compared with both undifferentiated hMSC (T0) and differentiated osteoblasts (T1) [26]. The hypothesis that SC are similar to the bone lining cell phenotype deserves additional investigation.

Although SC are generally now considered the neoplastic element of GCT, their transformed nature has never been supported by consistent morphologic evidence, in vitro studies, cytogenetic analysis, or molecular findings [27, 31, 35, 48]. We therefore compared the in vitro growth characteristics of SC and hMSC and observed a similar proliferation rate, although SC showed a higher colony-forming ability and an extended, but limited, lifespan. Considering the substantially normal in vitro proliferation rate of SC in contrast to the relatively high number of mitoses observed in GCT tissue specimens, it is reasonable to propose the existence of a mitogenic factor in the GCT microenvironment that is lost in the in vitro conditions.

Fig. 4A–B Chemotaxis of $CD14^+$ monocyte induced by stromal cells (SC) is shown. The ability of SC to chemoattract $CD14^+$ human blood monocytes and the mRNA expression of the chemoattractive factor stromal cell derived factor-1 (SDF-1) were compared with those of human mesenchymal stromal cells (hMSC). Number of

migrated cells after coculture with SC or hMSC ($n = 3$) (A); gel electrophoresis results for SDF-1 in SC, hMSC, and in giant cell tumor tissues (B). SC induced considerable migration of $CD14^+$ monocytes, although this ability was not dependent on SDF-1 production.

Fig. 5A–C Osteoclast differentiation and activity induced by stromal cells (SC) are shown. The isoform 5b of tartrate-resistant acid phosphatase (TRACP) and collagen resorption activity were evaluated in coculture of peripheral blood monocytes with SC and human mesenchymal stromal cells (hMSC). The expression of receptor activator of nuclear factor κB (RANKL) mRNA was also analyzed in SC, hMSC, and giant cell tumor tissues (hMSC $n = 2$). For TRACP 5b assay (A), and Osteolyse assay (B), p was calculated for the difference between SC and hMSC. (C) Gel electrophoresis results for RANKL. SC did not induce osteoclast activity or differentiation.

When considering another characteristic of tumor cells, that is the lifespan extent [33], this was only slightly higher in SC (3–16 PD more than in hMSC), an extent that is insufficient to attribute SC an immortalized or transformed phenotype [4]. Moreover, although SC showed a higher colony-forming unit ability compared with hMSC, they were not able to form colonies in a semisolid medium, an in vitro index of cell transformation as shown by Saos-2 osteosarcoma cells. None of these data appear to support the hypothesis that SC are transformed cells, but rather indicate they simply proliferate in vitro for a similar number of passages as hMSC.

Current opinion suggests SC in GCT induce the recruitment, the differentiation, and the activation of GC

through RANKL-mediated paracrine stimulation [36, 37]. In agreement with previous observations [29], we found SC are able to effectively induce osteoclast precursor chemotaxis, although this activity was not mediated by SDF-1. In this study, however, SC were not able to induce osteoclast differentiation at a higher extent than hMSC, and, accordingly, they did not express RANKL mRNA. It is important to emphasize osteoclast may spontaneously differentiate from their precursors [10], although appropriate differentiation stimuli are generally used to amplify this phenomenon [37]. The finding of high RANKL levels in all but one corresponding GCT tissue sample is in agreement with a previous report showing that in GCT, RANKL mRNA and protein are more highly expressed in GC than

Fig. 6A–B Histogenesis of giant cell tumor (GCT) is shown. Hypothetical models of interaction between stromal cells (SC) and giant cells (GC) in GCT. Neoplastic osteoblast-like stromal cells induce osteoclast activation and differentiation through receptor activator of nuclear factor κ B (RANKL) secretion (A). Multinucleated GC/monocytes promote osteoclast activation and differentiation as a result of an autocrine RANKL-mediated circuit, in turn inducing hyperplastic proliferation and activation of osteoblast-like intracellular adhesion molecule-1-positive “bone lining cells” (B).

in SC, suggesting the existence of an autocrine circuit in the monocyte-osteoclast cell component [35].

Taken together, these observations are inconsistent with the currently accepted model that in GCT, the secretion of RANKL by a putative neoplastic, osteoblast-like SC is primarily responsible for the formation of GC (Fig. 6A) [47]. On the contrary, based on our data, although we analyzed only five GCT samples that might not be entirely representative, we propose SC could be hyperplastic rather than truly neoplastic elements, exhibiting some but not all characteristics of osteogenic cells. Accordingly, SC could be secondarily induced to proliferate as a result of a paracrine effect induced by other cell types that are present in GCT tissues but not in *in vitro* conditions such as monocyte-macrophages and GC. The increased formation of GC might be attributed to an autocrine circuit mediated by RANKL and activated by an unknown stimulus (Fig. 6B).

Besides some differences in the *in vitro* growth characteristics, SC showed most, but not all, markers of the osteoblastic lineage, as previously reported [19, 36]. However, SC were unable to fully differentiate *in vitro*. Moreover, they did not induce osteoclast differentiation but exerted a strong chemotactic effect on osteoclast precursors. These findings open alternative perspectives on the role of the different cell components of GCT as a basis for understanding the nature of this lesion.

References

1. Athanasou NA, Bliss E, Gatter KC, Heryet A, Woods CG, McGee JO. An immunohistological study of giant-cell tumour of bone: evidence for an osteoclast origin of the giant cells. *J Pathol.* 1985;147:153–158.
2. Bauer FC, Urist MR. The reaction of giant cell tumors of bone to transplantation into athmic mice: including observations on osteoinduction in the host bed. *Clin Orthop Relat Res.* 1981;159:257–264.
3. Bertoni F, Present D, Sudanese A, Baldini N, Bacchini P, Campanacci M. Giant-cell tumor of bone with pulmonary metastases: six case reports and a review of the literature. *Clin Orthop Relat Res.* 1988;237:275–285.
4. Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, Harley CB, Shay JW, Lichtsteiner S, Wright WE. Extension of life-span by introduction of telomerase into normal human cells. *Science.* 1998;279:349–352.
5. Brien EW, Mirra JM, Kessler S, Suen M, Ho JK, Yang WT. Benign giant cell tumor of bone with osteosarcomatous transformation ('dedifferentiated' primary malignant GCT): report of two cases. *Skeletal Radiol.* 1997;26:246–255.
6. Boriani S, Sudanese A, Baldini N, Picci P. Sarcomatous evolution of giant cell tumor. *Ital J Orthop Traumatol.* 1986;12:203–211.
7. Cameron JAP, Marsden ATH, Lumpur K. Malignant osteoclastoma. *J Bone Joint Surg Br.* 1952;34:93–96.
8. Campanacci M, Baldini N, Boriani S, Sudanese A. Giant-cell tumor of bone. *J Bone Joint Surg Am.* 1987;69:106–113.
9. Campanacci M, Giunti A, Olmi R. Giant-cell tumours of bone. A study of 209 cases with long-term follow-up in 130. *Ital J Orthop Traumatol.* 1975;1:153–180.
10. D'Amelio P, Grimaldi A, Pescarmona GP, Tamone C, Roato I, Isaia G. Spontaneous osteoclast formation from peripheral blood

- mononuclear cells in postmenopausal osteoporosis. *FASEB J*. 2004;19:410–412.
11. Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell*. 1997;89:747–754.
 12. Everts V, Delaissè JM, Korper W, Janses DC, Tighelaar-Gutter W, Safting P, Beertsen A. The bone lining cell: its role in cleaning Howship's lacunae and initiating bone formation. *J Bone Miner Res*. 2002;17:77–90.
 13. Gamberi G, Benassi MS, Bohling T, Ragazzini P, Molendini L, Sollazzo MR, Merli M, Ferrari C, Magagnoli G, Bertoni F, Picci P. Prognostic relevance of C-myc gene expression in giant-cell tumor of bone. *J Orthop Res*. 1998;16:1–7.
 14. Goldring SR, Roelke MS, Petrisson KK, Bhan AK. Human giant cell tumors of bone identification and characterization of cell types. *J Clin Invest*. 1987;79:483–491.
 15. Granchi D, Amato I, Battistelli L, Avnet S, Capaccioli S, Papucci L, Donnini M, Pellacani A, Brandi ML, Giunti A, Baldini N. In vitro blockade of receptor activator of nuclear factor-kb ligand prevents osteoclastogenesis induced by neuroblastoma cells. *Int J Cancer*. 2004;111:829–838.
 16. Halleen JM, Tiitinen SL, Ylipahkala H, Fagerlund KM, Väänänen HK. Tartrate-resistant acid phosphatase 5b (TRACP 5b) as a marker of bone resorption. *Clin Lab*. 2006;52:499–509.
 17. Harada H, Tagashira S, Fujivara M, Ogawa S, Katsumata T, Yamaguchi A, Komori T, Nakatsuka M. Cbfa1 isoforms exert functional differences in osteoblast differentiation. *J Biol Chem*. 1999;274:6927–6928.
 18. Hofbauer LC, Khosla S, Dunstan CR, Lacey DL, Boyle WJ, Riggs BL. The roles of osteoprotegerin and osteoprotegerin ligand in the paracrine regulation of bone resorption. *J Bone Miner Res*. 2000;15:2–12.
 19. Huang L, Teng XY, Cheng YY, Lee KM, Kumta SM. Expression of preosteoblast markers and Cbfa1 and Osterix gene transcripts in stromal tumour cells of giant cell tumour of bone. *Bone*. 2004;34:393–401.
 20. Huang L, Xu J, Wood JD, Zeng MH. Gene expression of osteoprotegerin ligand, osteoprotegerin, and receptor activator of NF-kB in giant cell tumor of bone. *Am J Pathol*. 2000;156:761–767.
 21. Huang TS, Green AD, Beattie CW, Das Gupta TK. Monocyte-macrophage lineage of giant cell tumor of bone. Establishment of a multinucleated cell line. *Cancer*. 1993;71:1751–1760.
 22. Husheem M, Nyman JK, Vääräniemi J, Vaananen HK, Hentunen TA. Characterization of circulating human osteoclast progenitors: development of in vitro resorption assay. *Calcif Tissue Int*. 2005;76:222–230.
 23. Jaffe JL, Lichtenstein L, Portis RB. Giant cell tumor of bone: its pathologic appearance, grading, supposed variants and treatment. *Arch Pathol*. 1940;30:993–1031.
 24. Joyner CJ, Quinn JM, Triffitt JT, Owen ME, Athanasou NA. Phenotypic characterisation of mononuclear and multinucleated cells of giant cell tumour of bone. *Bone Miner*. 1992;16:37–48.
 25. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell*. 1997;89:755–764.
 26. Kulterer B, Friedl G, Jandrositz A, Sanchez-Cabo F, Prokesch A, Paar C, Scheideler M, Windhager R, Preisegger KH, Trajanoski Z. Gene expression profiling of human mesenchymal stem cells derived from bone marrow during expansion and osteoblast differentiation. *BMC Genomics*. 2007;8:70–84.
 27. Kusuzaki K, Takeshita H, Murata H, Hashiguchi S, Nozaki T, Emoto K, Ashihara T, Hirasawa Y. Relationship between binucleated and multinucleated cells in giant cell tumor of bone. *Anticancer Res*. 2000;20:2463–2468.
 28. Lau YS, Sabokbar A, Gibbons CL, Giele H, Athanasou N. Phenotypic and molecular studies of giant-cell tumors of bone and soft tissue. *Hum Pathol*. 2005;36:945–954.
 29. Liao TS, Yurgelun MB, Chang SS, Zhang HZ, Murakami K, Blaine TA, Parisien MV, Kim W, Winchester RJ, Lee FYL. Recruitment of osteoclast precursors by stromal cell derived factor-1 (SDF-1) in giant cell tumor of bone. *J Orthop Res*. 2005;23:203–209.
 30. Lindeman JHN, Hanemaaijer R, Mulder A, Dijkstra S, Suzhai K, Bromme D, Verheijen JH, Hogendoorn PC. Cathepsin K is the principal protease in giant cell tumor of bone. *Am J Pathol*. 2004;165:593–601.
 31. Mankin HJ, Connor JF, Schiller AL, Perlmutter N, Alho A, McGuire M. Grading of bone tumors by analysis of nuclear DNA content using flow cytometry. *J Bone Joint Surg Am*. 1985;67:404–413.
 32. Marletta G, Ciapetti G, Satriano C, Perut F, Salerno M, Baldini N. Improved osteogenic differentiation of human marrow stromal cells cultured on ion-induced chemically structured poly-epsilon-caprolactone. *Biomaterials*. 2007;28:1132–1140.
 33. Mathon AN, Lloyd AC. Cell senescence and cancer. *Nat Rev Cancer*. 2001;1:203–213.
 34. Miyamoto N, Higuchi Y, Takima M, Ito M, Tsurudome M, Nishio M, Kawano M, Sudo A, Uchida A, Ito Y. Spindle-shaped cells derived from giant-cell tumor of bone support differentiation of blood monocytes to osteoclast-like cells. *J Orthop Res*. 2000;18:647–654.
 35. Morgan T, Atkins GJ, Trivett MK, Johnson SA, Kansara M, Schlicht SL, Slavin JL, Simmons P, Dickinson I, Powell G, Choong PF, Holloway AJ, Thomas DM. Molecular profiling of giant cell tumor of bone and the osteoclastic localization of ligand for receptor activator of nuclear factor kappaB. *Am J Pathol*. 2005;167:117–128.
 36. Murata A, Fujita T, Kawahara N, Tsuchiya H, Tomita K. Osteoblast lineage properties in giant cell tumors of bone. *J Orthop Sci*. 2005;10:581–588.
 37. Nicholson GC, Malakellis M, Collier FM, Cameron PU, Holloway WR, Gough TJ, Gregorio-King C, Kirkland MA, Myers DE. Induction of osteoclasts from CD14-positive human peripheral blood mononuclear cells by receptor activator of nuclear factor kappaB ligand (RANKL). *Clin Sci*. 2000;99:133–140.
 38. Nishimura M, Yuasa K, Mori K, Miyamoto N, Ito M, Tsurudome M, Nishio M, Kawano M, Komada H, Uchida A, Ito Y. Cytological properties of stromal cells derived from giant cell tumor of bone (GCTSC) which can induce osteoclast formation of human blood monocytes without cell to cell contact. *J Orthop Res*. 2005;23:979–987.
 39. O'Donnell RJ, Springfield DS, Motwani HK, Ready JE, Gebhardt MC, Mankin HJ. Recurrence of giant cell tumors of the long bones after curettage and packing with cement. *J Bone Joint Surg Am*. 1994;76:1827–1833.
 40. Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. *J Cell Physiol*. 2000;182:311–322.
 41. Schuffstall RM, Gregory JE. Osteoid formation in giant cell tumours of bone. *Am J Pathol*. 1953;29:1113–1131.
 42. Scotlandi K, Serra M, Manara C, Maurici D, Benini S, Nini G, Campanacci M, Baldini N. Clinical relevance of Ki-67 expression in bone tumors. *Cancer*. 1995;75:806–814.
 43. Tanaka Y, Maruo A, Kujii K, Nomi M, Nakamura T, Eto S, Minami Y. Intercellular adhesion molecule 1 discriminates functionally different populations of human osteoblasts: characteristic involvement of cell cycle regulators. *J Bone Miner Res*. 2000;15:1912–1923.

44. Tanaka Y, Mine S, Hanagiri T, Hiraga T, Morimoto I, Figdor CG, van Kooyk Y, Ozawa H, Nakamura T, Yasumoto K, Eto S. Constitutive up-regulation of integrin-mediated adhesion of tumor-infiltrating lymphocytes to osteoblasts and bone marrow-derived stromal cells. *Cancer Res.* 1998;58:4138–4145.
45. Tanaka Y, Morimoto I, Nakano Y, Okada Y, Hirota S, Nomura S, Nakamura T, Eto S. Osteoblasts are regulated by the cellular adhesion through ICAM-1 and VCAM-1. *J Bone Miner Res.* 1995;10:1462–1469.
46. Wulling M, Delling G, Kaiser E. The origin of the neoplastic stromal cell in giant cell tumor of bone. *J Cancer Res Clin Oncol.* 2001;34:983–993.
47. Zheng MH, Robbins P, Xu J, Huang L, Wood DJ, Papadimitriou JM. The histogenesis of giant cell tumour of bone: a model of interaction between neoplastic cells and osteoclasts. *Histol Histopathol.* 2001;16:297–307.
48. Zheng MH, Siu P, Papadimitriou JM, Wood DJ, Murch AR. Telomeric fusion is a major cytogenetic aberration of giant cell tumors of bone. *Pathology.* 1999;31:373–378.