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Bone remodelling and tumour grade modifications induced by interactions between bone and swarm rat chondrosarcoma

E. Grimaud[1], C. Damiens[1], A.V. Rousselle[1,2], N. Passuti[1,2], D. Heymann[1,3] and F. Gouin[1,2]

[1]Pathophysiology of Bone Resorption Laboratory, Medicine Faculty, Nantes Cedex 1, France,

[2]Department of Orthopaedic Surgery, Hôtel-Dieu Hospital, place A. Ricordeau, Nantes Cedex 1, France and

[3]Histology-Embryology Laboratory, Medicine Faculty, Nantes, France

Summary. Chondrosarcoma is currently defined as a malignant cartilage tumour arising de novo or within a pre-existing benign cartilage tumour. Chondrosarcoma can be surgically resected, but all grades have significant rates of local recurrence. The purpose of the present study was to develop an animal intraosseous chondrosarcoma model simulating the progression of human chondrosarcoma and elucidating its behaviour and biology. An intraosseous Swarm rat model was designed to assess interactions between bone and chondrosarcoma. A comparison of tumour grading was carried out according to transplantation site. The effects of chondrosarcoma cells (SRC cells) on the mineralisation capacities of osteoblasts and on osteoclast differentiation were studied in relation to modifications observed *in vivo* at the cellular level. Transplantation of Swarm rat chondrosarcoma within bone marrow or contiguous to induced periosteal lesions led to extensive bone remodelling with trabecular bone rarefaction and periosteal apposition. Transplantation in close contact to bone but without any periosteal lesion had no effect on bone, suggesting that bone healing factors interact with tumour development. With the intramedullary model, the development of tumours of different grade confirms that bone environment is an important factor in malignancy. A decrease of bone nodule formation was noted after co-cultures of SRC cells with rat bone marrow, but there was no modification of osteoclast differentiation after cultures of total rabbit bone cells with SRC cells. These data reveal the importance of interactions between bone environment and tumour in inducing bone remodelling and variations in tumour malignancy.

Key words: Swarm rat chondrosarcoma, Bone remodelling, Grading, Malignancy

Offprint requests to: Dr D. Heymann, Laboratoire de Physiopathologie de la Résorption Osseuse, EE 99-01, Faculté de Médecine, 1 rue Gaston Veil, 44035 Nantes cedex 1, France. Fax: + 33 (0) 2 40 41 28 60. e-mail: dominique.heymann@sante.univ-nantes.fr

Introduction

Chondrosarcoma, identified by Lichtenstein and Jaffe as a malignant bone tumour clearly distinct from osteosarcoma (Lichtensein and Jaffe, 1942), is currently defined as a malignant cartilage tumour arising de novo or within a pre-existing benign cartilage tumour. Human chondrosarcomas, which represent less than 10% of primary malignant bone tumours, are classified as low, intermediate or high grade on the basis of histological and cytological features (O'Neal and Ackerman, 1952). Chondrosarcoma can be surgically resected, but all grades have significant rates of local recurrence (Gitelis et al., 1981). Histological examination alone is usually inadequate to differentiate low-grade chondrosarcoma from benign cartilaginous tumours such as enchondroma or osteochondroma. However, other detection techniques [e.g. p53 expression or cytofluorometric DNA ploidy analysis (Terek et al., 1998; Kusuzaki et al., 1999)] have not replaced histological diagnosis, which remains the essential tool for chondrosarcoma grading.

Different cell lines, such as Swarm rat chondrosarcoma (SRC) cells, CS-OKB and HCS-2/8 (Maibenco et al., 1967; Takigawa et al., 1989; Chano et al., 1998), have been used to study this tumour. SRC, first described as an osteogenic tumour arising spontaneously in a female Sprague-Dawley rat (Maibenco et al., 1967), can be maintained for several years by serial subcutaneous transplantation. SRC is originally composed of cartilage and bone elements, but the latter disappear after repeated transplantations. As collagen II has been identified as the major collagen component of SRC, representing 50% of the total protein synthesised (Smith et al., 1975), SRC has been used to study its biosynthesis (Breitkreutz et al., 1979). SRC has also become a model system for biochemical studies of the structure of cartilage proteoglycans owing to the large amount of tissue available and the ease with which proteoglycans can be extracted. In fact, SRC is a useful source of chondrocytes for the study of proteoglycan

biosynthesis and aggregation (Kimura et al., 1979 ; Stevens et al., 1981). Moreover, numerous SRC cell cultures have been used to test the effects of different factors such as insulin-like growth factor I, insulin, and interleukin-6 (Harper and Harper, 1987; Guerne and Lotz, 1991; Seong et al., 1994). Some culture models (e.g. agarose or alginate) have been developed to maintain the original phenotype of SRC cells (Sun et al., 1986 ; Saito et al., 1988). So, SRC is an appropriate model to study chondrosarcoma as it has been mentioned by Kenan and Steiner (1991). Moreover, the effects of these cells on bone cells have never been studied, despite the fact that chondrosarcoma is a cartilaginous tumour that interacts with bone tissue like many bone metastases. Indeed, solid cancers metastasize to bone by a multistep process that involves interactions between tumor cells and normal host cells. For example, breast cancer grow avidly in bone which is a storehouse of a variety of cytokines and growth factors and thus provide an extremely fertile environment for growing cells (Yoneda et al., 1994). The final step in bone metastasis from breast cancer, namely bone destruction is mediated by osteoclasts that are stimulated by local production of PTH-rP (parathyroid hormone related peptide). These production is enhanced by growth factors produced as a consequence of normal bone remodelling, particularly activated transforming growth factor beta. Thus, a vicious cycle exist in bone between production of soluble mediators by the tumor cells and subsequent production by bone cells of growth factors (Mundy, 1997). The study of interactions between SRC cells and bone cells represents an important step to have a better understanding of chondrosarcoma development into bone.

The purpose of the present study was to develop an intraosseous SRC model simulating the progression of human chondrosarcoma and elucidating its behaviour and biology. Different chondrosarcoma transplantations were performed to investigate interactions and modifications between bone and tumour. A comparison of tumour grading was carried out according to transplantation site. The effects of chondroid cells (SRC cells) on the mineralisation capacities of osteoblasts and on osteoclast differentiation were studied in relation to modifications observed *in vivo* at the cellular level.

Materials and methods

Materials

Swarm rat chondrosarcoma was a generous gift from Dr P. A. Guerne (Geneva, Switzerland). Male Sprague-Dawley rats were purchased from the "Centre d'élevage Janvier" (Le Genest Saint Isle, France). Alpha minimum essential medium (α-MEM), Dulbecco's modified Eagle's medium (DMEM), antibiotics, L-glutamine, and trypsin were obtained from Gibco (Eragny, France); fœtal bovine serum (FBS) from Dutscher SA (Brumath, France); ascorbic acid, dexamethasone, sodium ß-glycerophosphate, and the TRAP detection kit from Sigma (Saint Quentin Fallavier, France); cell culture inserts from AES (Combourg, France); alizarin red-S from Merck (Darmstadt, Germany); collagenase II from Boerhinger Mannheim (Mannheim, Germany); nesdonal 0.5 gr from Specia-Rhône-Poulenc-Rorer (Montrouge, France); and Imalgene 500 from Rhône Mérieux (Lyon, France).

Tumour growth

For SRC propagation, tissue fragments were implanted subcutaneously into the flank of male Sprague-Dawley rats (5 weeks old). After 5 weeks, the tumour was explanted and dissected into fragments that were frozen until cell isolation.

Transplantation of SRC into bone

Male Sprague-Dawley rats (4 to 5 weeks old) were anaesthetised by an intraperitoneal injection of thiopental sodium (Nesdonal 25 mg/ml), followed by intramuscular injection of ketamine (Imalgene 40 to 80 mg/ml). Four groups of 3 rats each were used for the different transplantations performed:

Group A (intraosseous model)

Using a lateral approach facing the right femoral condyle, a 3-mm diameter hole was drilled to give access to the medullary cavity where an SRC fragment was implanted with a curet. The incision was sutured with 3-mm non-resorbable yarn (Flexocrin®, Melsungen, Germany). For the contralateral limb (control), a 3-mm diameter hole was drilled, but no tumour fragment was implanted.

Group B (intraosseous model)

The same surgical procedure as above was used for the right femoral condyle. The left femoral condyle was incised and drilled, and a fragment of rat articular cartilage was taken extemporaneously from another specimen and implanted into the medullary cavity.

Group C (extraosseous model)

Using a right femoral approach, the cortical surface of the diaphysis was scarified laterally on 10 mm. An SRC fragment was placed contiguous to the scarified surface, and the cutaneous and muscular wound was sutured. The same procedure was performed for the left femoral condyle, but no tumour fragment was implanted.

Group D (extraosseous model)

The right femoral condyle was operated on as described above. Only the periosteum of the diaphysis

was opened and resected along a length of 10 mm, and underlying bone was intact. A chondrosarcoma fragment was then placed in contiguous to the exposed bone surface without periosteum. For the left femur, a chondrosarcoma fragment was implanted without cutting into the periosteum.

Two weeks after transplantation, each rat was radiographed to observe tumour development. Five weeks after transplantation, radiographs were taken again, and the rats were sacrificed. The femora and tumour of each rat were removed and fixed at 4 °C for 48 h in formol buffer (pH 7.4). The femora were cut longitudinally or transversally. After decalcification, femoral fragments were embedded in paraffin and cut into 5-μm sections. Subcutaneous tumour explants were treated in the same way as femoral tumour explants.

SRC cell isolation

Single cells were isolated from fresh or frozen subcutaneous tumour fragments that had been washed in DMEM, minced and then digested in 1 mg/ml trypsin solution for 10 min at 37 °C with constant shaking. The fragments were then transferred into a digestion buffer containing DMEM and 1 mg/ml collagenase II solution for 2.5 h at 37 °C with constant shaking. After washes, the cells were cultured in DMEM containing 5% FBS, 2 mM L-glutamine and 1% antibiotic mixture (100 U/ml penicillin; 100 μg/ml streptomycin).

Co-cultures of tumour cells with bone marrow

Isolation of bone-marrow cells (Bohic et al., 1998)

Bone-marrow cells were isolated from adult male Sprague-Dawley rats (3 months old). Briefly, both femora and tibias were dissected aseptically and cleaned of soft tissues. The epiphyses were removed, and bone-marrow cells were flushed out with a syringe fitted with a 21-gauge needle containing α-MEM supplemented with antibiotics and 2 mM L-glutamine. A single cell suspension was prepared by repeated pipetting. Unfractioned bone-marrow cells were cultured in α-MEM containing 15% FBS, 2 mM L-glutamine, 1% antibiotics, 10^{-8} M dexamethasone, and 50 μg/ml of freshly prepared ascorbic acid.

Co-cultures

SRC cells were seeded at various densities (50, 250, 500, 750, 1,000, 5,000, and 10,000 cells/well) into 6-multiwell plates in α-MEM supplemented with 5% FBS, 2 mM L-glutamine, and 1 % antibiotics. After adhesion of tumour cells to plastic, the culture medium was removed and bone-marrow cells were added at 10^7 cells/well (4 ml). After one week of culture, Na α-glycerophosphate 10 mM was added to the medium. The cell cultures were then placed at 37 °C in a 5% CO_2 humidified atmosphere and maintained for three weeks, with total renewal of the culture medium every two days. Similar experiments were done using cell culture inserts (pore diameter: 0.4 μm). Bone-marrow cells were plated at 10^7 cells/well, and SRC cells were seeded at various densities (50, 500, 1,000, and 5,000 cells) into cell culture inserts. Culture conditions were as above.

Alizarin red-S staining

Alizarin red-S staining was used to detect the mineralisation nodules formed in vitro. After 3 weeks of culture, adherent cells were washed twice with phosphate-buffered saline (PBS). The cells were then fixed with ice-cold ethanol 70% for 1 h. The ethanol was then removed, and the fixed cells were washed with distilled water and incubated with alizarin red-S (40 mM, pH 7.4) for 10 min at room temperature. Finally, the cells were washed with distilled water to eliminate alizarin red-S. Mineralisation nodules were observed by light microscopy.

Nodule microanalysis

After 3 weeks of culture, cells were washed 3 times with PBS and fixed with ice-cold ethanol for 24 h at 4 °C. Samples were then dehydrated through a graded alcohol series and conserved in 100% ethanol until analysis before being coated with carbon (Jeol JEE 4B, Tokyo, Japan). The nodules were then analysed using a scanning electron microscope (Jeol JSM-630, Tokyo, Japan) coupled to an elementary analysis system in dispersive energy (Link EXLII, Oxford, Great Britain).

Co-cultures of SRC cells with total rabbit bone cells

Isolation of total rabbit bone cells

Neonatal rabbit bone cells were isolated using a previously reported method (Guicheux et al., 1998). Briefly, 11-day-old rabbits were killed by cervical dislocation, and each leg was cut at the hip and the astragalo-calcalean joint. The bones were freed aseptically from soft tissue, minced with scissors, and vortexed in 20 ml α-MEM medium for 30 sec. After sedimentation for 2 min, the cell suspension was harvested (repeated twice). The cells were then washed in α-MEM and resuspended in α-MEM supplemented with 10% FBS and 1% antibiotics.

Co-cultures

SRC cells were plated at various densities (500; 1,000; 2,500; 5,000; 10,000 and 20,000 cells/well) on glass coverslips in 24-multiwell plates in DMEM supplemented with 5% FBS, 2 mM L-glutamine, and 1% antibiotics. Once tumour cells adhered to glass coverslips, culture medium was removed and total rabbit bone cells were added to each well. Total rabbit bone cells were seeded in glass coverslips in 24-multiwell

plates at a density of 10^7 cells/well. The cell cultures were maintained for 4 days in a humidified 95% air, 5% CO_2 atmosphere at 37 °C.

Characterisation of the multinucleate cells formed

To confirm the presence of osteoclasts and the formation of resorption pits in this model, the total rabbit bone cell preparation was seeded on dentin slices in 24-multiwell plates at a density of 10^7 cells/well. After 4 days of culture, dentin slices were sonicated for 2 min in distilled water and gold palladium-coated for scanning electron microscopy (SEM) studies and semiautomatic image analysis.

Further confirmation was obtained using tartrate-resistant acid phosphatase (TRAP) staining as an enzyme marker for osteoclasts. After 4 days of culture, SRC cells cultured with total rabbit bone cells on glass coverslips were washed with PBS, fixed and stained to detect the presence of TRAP. The number, surface area, and diameter of osteoclasts and the number of nuclei per cell were scored using an image analyser (Q500MW, Leica, Cambridge, UK).

Statistical analysis

All experiments were performed in triplicate. Results are expressed as the mean ± SD. Comparative study of means was done using the ANOVA statistical test. Results were considered significantly different when $p < 0.01$.

Results

SRC transplantation induces extensive bone remodelling

Bone remodelling induced by SRC transplantation was detected radiographically two weeks after intraosseous transplantation. At 5 weeks, radiographic changes were observed for the right femora of groups A and B, which had been implanted with SRC tumour. Osteolytic areas with calcified septa were observed as well as cortical thinning and breaches and exuberant periosteal accretion. These modifications occurred well beyond the area of intraosseous grafting (Fig. 1a,b). However, no modifications were observed for the left femora without SRC grafting. Right femoral scarifications (group C) or periosteal lesions (group D) with tumour accretion showed similar modifications to those of groups A and B, but with a prevalence of periosteal reactions (Fig. 1c,d). No radiographically discernible modifications were noted for left femora.

Bone environment modulates SRC malignancy

After subcutaneous development, SRC was whitish (translucent to opaque), well delimited, poorly vascularised, lobular in organisation, and soft (unlike normal cartilage). Central tumour necrosis was generally observed 5 weeks after subcutaneous transplantation. Histological examination revealed chondroid tumour proliferation consisting of lobules of variable size containing chondroid stroma and delimited by fine fibrous septa (Fig. 2a). Hypercellularity was quite notable, especially at the periphery of lobules. SRC cells had large ovoid or occasionally multilobular nuclei with densified chromatin and often a small nucleolus. Many mitoses could be seen (Fig. 2b). In subcutaneous sites, SRC displayed the histological features of grade II chondrosarcoma.

Observations were similar after SRC intraosseous development. Histological examination showed extensive invasion of bone and surrounding tissues by grade II to III chondrosarcoma. SRC developed in the medullary space, pushing back bone marrow to the cortex, which was thinned and perforated, facilitating the entry of bone-marrow and tumour cells into the extraosseous site (Fig. 2c). During infiltration of surrounding tissues, a periosteal reaction was observed, corresponding to high cellular activity. Marked hypercellularity occurred locally, and nuclei were larger than in the subcutaneous site and also vesicular and highly nucleolated. After intraosseous transplantation, histological examination revealed that the SRC was grade II with foci of grade III (Fig. 2d). The histological examination of extraosseous chondrosarcoma was identical to the intraosseous model except the presence of foci of grade III (data not shown).

For left femurs of each group, histological examination has revealed any modification compared to right femur.

SRC cells reduce the number of mineralised nodules formed by bone-marrow cells in vitro without influence of osteoclastic differentiation

After three weeks of co-culture of rat bone-marrow cells with SRC cells, nodular structures expressing alizarin-red staining were observed (Fig. 3). SRC cells decreased the capacity of bone marrow to form mineralised nodules. Thus, the mean number of nodules after three weeks of culture was 65±20/well for 10^7 bone-marrow cells (Fig. 4a). This indicated that 500 and 750 SRC cells reduced the number of nodules significantly (46%; $p<0.01$) compared to bone-marrow cells alone. The decrease was 66% with 1,000 SRC cells ($p < 0.001$), and no nodules were observed with 5,000 and 10,000 SRC cells. Nodule surface area also decreased significantly in the presence of SRC cells (Fig. 4b).

As this decrease in bone nodule formation could have been due to soluble or membrane factors, co-cultures were performed with inserts to determine the nature of these factors. No detectable modifications in nodule number or surface area were observed in the presence of 50 SRC (Fig. 4c), whereas a decrease of 40.7% and 96.6% occurred respectively with 500 and 1,000 SRC cells, and inhibition was total with 5,000 SRC cells. SRC cells also decreased nodule surface

areas (data not shown). SEM analysis performed to determine the elements composing mineralised nodules showed that their inner layer was composed of globular calcified accretions closely associated with the organic matrix. EDX analysis of mineral phases revealed the presence of prominent peaks for calcium and phosphorus and to a lesser extent for magnesium and sodium. No qualitative modification of nodules was observed in the presence of SRC cells (data not shown).

Co-cultures of total rabbit bone cells with SRC cells were performed to detect the effects of SRC cells on osteoclastic differentiation. Positive TRAP staining of these co-cultures revealed the presence of multinucleate cells (data not shown). The presence of lacunae on dentin slices indicated that these multinucleate cells had resorbance capacity. No change in the number of osteoclasts was observed in the presence of SRC cells as compared to total bone cells (data not shown). Similarly, osteoclast surface area and diameter were not altered by addition of SRC cells. The number of nuclei per cell was not modified, respectively 6.5±0.35 per osteoclast without and 6.3±0.4 with SRC cells.

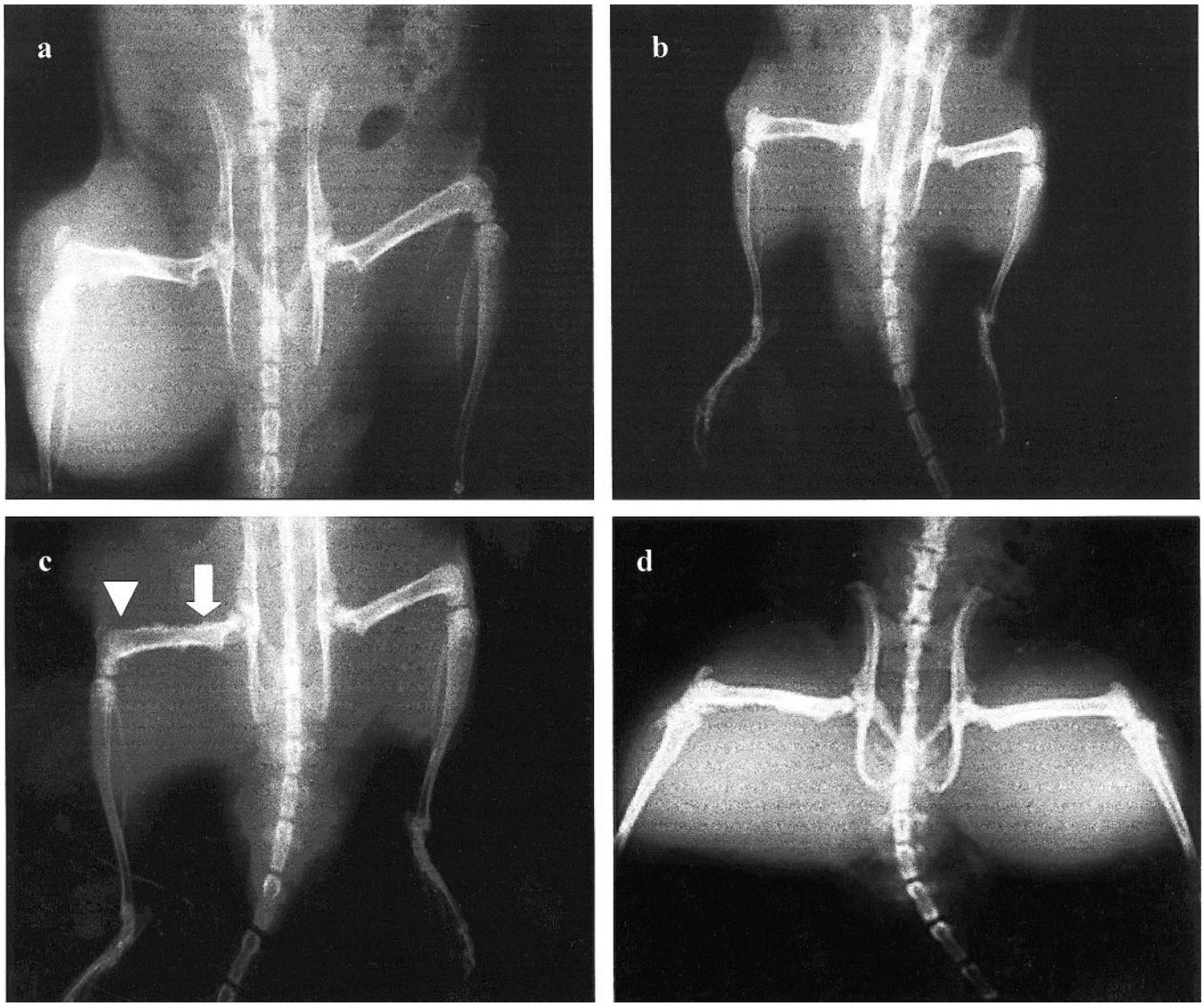

Fig. 1. Radiographs of rat femora 5 weeks after implantation of Swarm rat chondrosarcoma. **a.** Group A. The right femur after intramedullary implantation of an SRC fragment displays extensive osteolysis, cortical thinning and discrete periosteal apposition. The left femur (without transplantation - control) shows normal radiographic features. **b.** Group B. The right femur (SRC implantation) displays cortical effraction with considerable periosteal reaction. The left femur (cartilage transplantation - control) shows normal radiographic features. **c.** Group C. The right femur (scarifications and SRC apposition) displays numerous periosteal appositions and cortical irregularities. The left femur (scarifications without tumour) shows normal radiographic features. arrow: periosteal apposition, arrowhead: cortical irregularity. **d.** Group D. The right femur (periosteal lesions and SRC apposition) displays cortical irregularities and periosteal appositions. The left femur (SRC apposition) shows normal radiographic features.

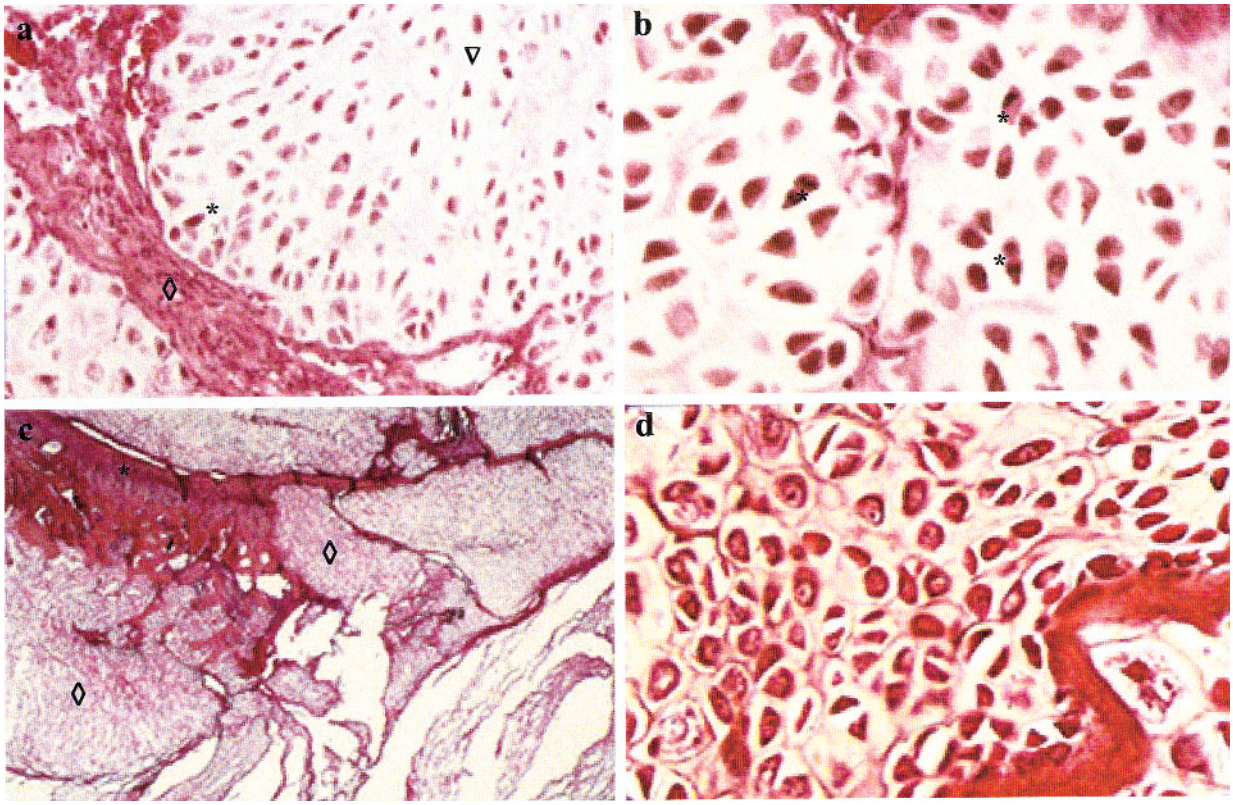

Fig. 2. Swarm rat chondrosarcoma in different implantation sites. Hematoxylin-eosin-safran (HES) staining. **a.** Swarm rat chondrosarcoma in a subcutaneous site. Tumour organisation is lobular, showing considerable chondrocytic hypercellularity (chondroid cells), especially at the lobule periphery. rhomb: fibrous septum, triangle: lobule periphery; *: lobule centre. **b.** Swarm rat chondrosarcoma in a subcutaneous site. Grade II. Tumour cells with large nuclei are observed as well as some mitoses (triangle). **c.** Features of Swarm rat chondrosarcoma in an intraosseous site 5 weeks after implantation. Massive cortical resorption has been replaced by tumour proliferation. rhomb: Swarm rat chondrosarcoma; *: cortex. **d.** Swarm rat chondrosarcoma in an intraosseous site. Grade III. Considerable hypercellularity and large, irregular vesicular nuclei are observed. a, x 25; b, x 400; c, x 16; d, x 400

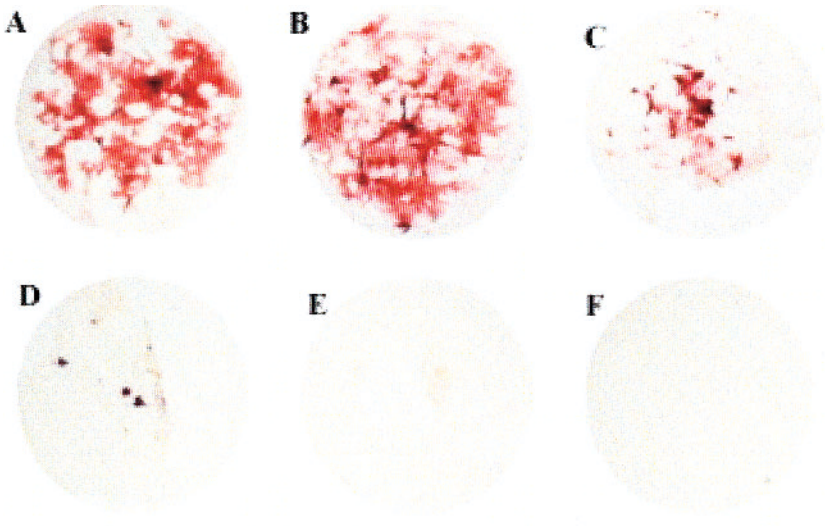

Fig. 3. Mineralisation nodules obtained after 21 days of bone-marrow culture (Alizarin red-S staining). 10^7 rat bone-marrow cells have been cultured for 21 days in α-MEM supplemented with FBS (15%), L-ascorbic acid (50 μg/ml) and dexamethasone (10^{-8} M) in presence or absence of SRC cells. After 7 days of culture, 10 mM Na ß-glycerophosphate is added to the culture medium. **A:** bone marrow; **B:** bone marrow + 250 SRC cells; **C:** bone marrow + 500 SRC cells; **D:** bone marrow + 1,000 SRC cells; **E:** bone marrow + 5,000 SRC cells; **F:** 1,000 SRC cells.

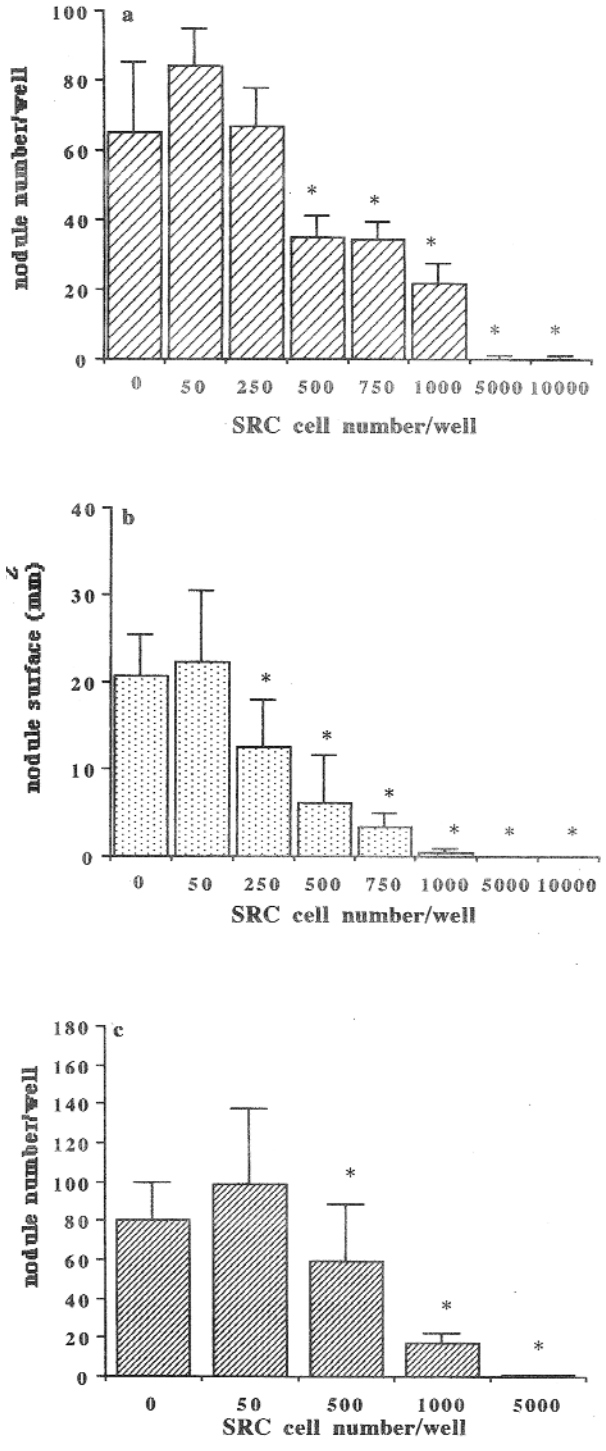

Fig. 4. Effects of SRC cells on bone nodule formation. 10^7 rat bone-marrow cells have been cultured for 21 days in α-MEM supplemented with FBS (15%), L-ascorbic acid (50 μg/ml) and dexamethasone (10^{-8} M) in presence or absence of SRC cells (50 to 10,000 cells/well). After 7 days of culture, 10 mM Na ß-glycerophosphate is added to the culture medium. **a:** nodule number in the presence of SRC cells in direct contact. **b:** nodule surface area in mm^2 in the presence of SRC cells in direct contact. **c:** nodule number in the presence of SRC cells using cell culture inserts. *: $p<0.05$ compared to bone-marrow cells alone (0).

Discussion

Interactions between bone and tumour were noted for the different types of SRC transplantation performed in this study. Intramedullary transplantation of SRC induced extensive bone remodelling, with trabecular bone rarefaction, periosteal apposition and cortical thinness. Femoral scarifications of the diaphysis or periosteal lesions, followed by tumour apposition, induced the same modifications. Histological examination confirmed these results, revealing SRC development in the medullary space that pushed bone marrow back to the cortex, inducing destruction of trabecular as well as cortical bone. All of these results are coherent and confirm that SRC induces extensive bone remodelling, as do most human central chondrosarcomas (O'Neal and Ackerman, 1952; Dahlin and Henderson, 1956; Barnes and Catto, 1966).

Interactions between the bone environment and tumour were studied using an original model (intraosseous SRC transplantation) to simulate the conditions of human chondrosarcoma development. SRC was chosen because of its capacity for rapid growth after subcutaneous transplantation. This model has been well characterised histologically, biochemically, and structurally (Breitkeutz et al., 1979; Kimura et al., 1979; Hascall, 1980; Hascall and Kimura, 1981). Kenan and Steiner (1991) showed that SRC is a well-differentiated malignant tumour, histologically similar to well-differentiated human chondrosarcoma, which produces metastases and ultimately death in the rat. Our study suggested that bone destruction and remodelling with this model were due to biological changes rather than mechanical factors. Transplantation of articular cartilage into bone did not induce modifications of bone structure. Bone remodelling and destruction were obtained after femoral scarification and SRC apposition (extraosseous model) without intramedullary mechanical stress. However, no remodelling, but only tumour growth similar to that of the contralateral control, occurred when SRC was implanted contiguous to femoral diaphysis without periosteal lesion. Thus, our experiment suggests that biological interactions occur between bone and SRC. However, as SRC induced bone remodelling and destruction only when bone lesions existed, the factors involved in periosteal and cortical repair could also have played a role. In fact, the complex interaction of many local and systemic factors is involved in periosteal and/or cortical healing. Multifactor environment involving growth factors such as bone morphogenetic proteins and transforming growth factor beta may play an important role (Barnes et al., 1999). These results tend to confirm that the mechanism of normal bone repair is accelerated by chondrosarcoma, which would lead to overexpression of local factors inducing bone remodelling, disturbance of physiological repair phenomena, and further tumour development.

SRC induced bone modifications, but the bone environment also had an effect on chondrosarcoma

behaviour. According to standard chondrosarcoma classification (O'Neal and Ackerman, 1952), the SRC was grade II in the subcutaneous site and after intraosseous implantation, it was grade II with foci of grade III, but no modification of grade was observed in extraosseous model. Grading is important in the diagnosis of chondrosarcoma, and several studies have confirmed its usefulness in predicting biological behaviour and prognosis (Evans et al., 1977; Sanerkin, 1980; Gitelis et al., 1981). As noted by Unni and Dahlin (1984), grade II indicates moderate malignancy and grade III high malignancy. Cartilage tumours frequently show some variations in grading within the same tumour. Marcove et al. (1972) studied 152 chondrosarcomas (primary and recurrent) and noted five changes of grade among 36 patients who had undergone more than one operation. In two cases, the lesion changed from grade I to II, and in three from grade II to III. In our study, the variations in SRC grade suggest that bone environment was partly responsible for increasing chondrosarcoma malignancy. As no grading modication was observed in extraosseous model, the importance of confined bone environment, and level of soluble factors released may have a part in modification of chondrosarcoma grade.

The effects of SRC cells on bone cells were studied *in vitro* to elucidate the nature of interactions between SRC and bone. SRC cells decreased mineralised bone nodule formation by producing soluble factors, as indicated by co-cultures with cell culture inserts. In relation to the modifications observed in vivo, these studies suggested that SRC, after being activated by elements in the bone environment such as periosteal healing factors, decreased the capacities of bone to regenerate, particularly by acting on osteoblastic cells. SRC can produce soluble factors capable of acting on mature osteoblasts by decreasing their capacities to form bone or by decreasing osteoblastic differentiation.

As no modifications in osteoclast number, surface area, and diameter were observed in the presence of SRC, the tumour appeared to have no action on osteoclast differentiation. This may have been due to a limitation implicit in the model itself through the use of two different species for co-cultures. As large osteolytic areas were observed in vitro, SRC cells may also have had an indirect effect on osteoclasts. One possibility is that SRC cells affected bone directly through a resorption mechanism acting independently of bone cells. In this respect, Galasko (1976) noted that tumour cells are frequently found adjacent to resorbed bone margins in the apparent absence of resorbing osteoclasts. Our histological studies in which SRC was sometimes observed close to resorbed bone tend to support this hypothesis. However, the indirect action of SRC cells on osteoclast differentiation is a more likely mechanism. As in the vicious circle described for bone metastasis (Yoneda et al., 1994), SRC cells could stimulate osteoclast resorption indirectly. SRC cells may produce soluble factors that act on osteoblasts, which in turn produce factors stimulating osteoclast differentiation.

This study elucidates certain interactions between bone environment and tumour behaviour. Our experimental model, involving conditions with or without interaction with adjacent tumour, provides a terrain for the study of bone environment relative to tumour progression and malignancy and for investigation of the role of tumour in physiological bone remodelling and healing.

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