Interactions between microenvironment and cancer cells in two animal models of bone metastasis.

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Running title: Bone microenvironment and cancer cells

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ABSTRACT

The preferential proliferation of cancer cells in the bone microenvironment is poorly characterized. Expression pattern of bone marrow and other organs microenvironment in contact with osteolytic (Walker W256) and osteoblastic (MatLyLu MLL) metastases were investigated.

Fisher and Copenhagen rats received respectively W256 and MLL cells injection. Bone and soft tissues were analyzed by immunochemistry for DKK1, cathepsin K, RANKL, MCSF, or IL6 expression. TRAcP+ cells were detected by a histoenzymatic technique.

In bone, expressions of MCSF and DKK1 were shown in stromal cells of the bone marrow, in contact with metastatic foci of both tumors. Many stromal cells were found RANKL+ in the vicinity of the tumors. Cells expressing cathepsin K and multinucleated TRAcP+ cells were found in direct contact with trabeculae but also in bone marrow spaces near metastatic cells.

In extraosseous tumors, cells in contact with malignant cells did not expressed DKK1, MCSF, cathepsin K, IL6. Some RANKL+ cells were found in the periphery of subcutaneous tumors but may represent Langerhans cells. Abnormal presence of TRAcP+ cells was never observed in the vicinity of malignant cells.

Interaction between stromal and cancer cells induces the expression on the formers of characteristics leading to osteoclastogenesis only in the bone microenvironment.

Key words: Bone metastasis; osteolysis; osteoclastogenesis; osteoclast; microenvironment.

INTRODUCTION

Bone is a preferential site for metastasis in different types of cancer. Bone metastases induce an increased morbidity (pain, fractures, nerve compression, hypercalcemia…) and compromise the long-term survival. They occur in approximately 65-75 % of patients with an advanced breast or prostate cancer and 15 to 40 % of patients with a carcinoma of the lung, bladder, skin or kidney. The median survival after first recurrence of breast cancer in bone is 20 months (Coleman, 2001). Once a tumor has metastasized to bone, it is virtually incurable and only palliative therapies can be proposed (Mundy, 1997).

Interactions between the bone marrow environment and cancer cells were first advocated by Paget who described them as 'the seed and the soil' theory (cited by (Guise & Mundy, 1998)): bone provides the 'fertile soil' in which certain cancer cell ('seeds') find favourable conditions for growth. Mineralized bone matrix is known to represent a rich storehouse of growth factors which are mobilized by osteoclastic resorption and become active in the local microenvironment. The release of these growth factors constitutes one of the key mechanisms in the bone remodeling allowing the physiological coupling between osteoblasts (the bone forming cells) and the osteoclasts (bone resorbing cells) (Mundy et al., 2001). When localized in the marrow spaces, tumor cells, in turn, secrete additional factors that interfere with bone remodeling leading to tissular changes that characterize the osteolytic or osteoblastic expression of the metastases. Thus, local interactions between tumor and bone cells form a vicious cycle which underlies the development of skeletal metastases. During the last decade, it has become evident that interactions between tumor and bone cells may change the phenotype of both populations.

Breast and prostate cancer are the two tumor types that most commonly metastasize to bone. On one hand, bone has characteristics that allow the selective homing of tumor cells: 1) a release of attractive molecules for these particular types of cancer (stromal cell-derived factor-1, epidermal growth factor, collagen degradation products, low glycosylated osteonectin…) inducing specific chemotaxis (Cooper et al., 2003) 2) the expression of adhesion molecule on the endothelial surface of bone marrow capillaries (due to a variety of integrins such as $\alpha4\beta1$, $\alpha5\beta1$, $\alpha v\beta3$, $\alpha v\beta5$)(Roodman, 2003) 3) appropriate growth factors and extracellular matrix proteins present in the bone marrow microenvironment (parathyroid hormone related protein (PTHrP), tumor growth factor beta (TGF-β), vascular endothelium growth factor (VEGF)…) (Guise & Mundy, 1998; Keller et al., 2001; Woodhouse et al., 1997). On the other hand, these peculiar tumor cells express proteins which favor propensity to migrate and anchor in bone: this includes chemotaxis (CXCR4) (Muller et al., 2001), extravasation and bone marrow

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homing (integrins, E-cadherin) (Woodhouse et al., 1997), pericellular proteolysis and invasion (MMPs, ADAMs) (Woodhouse et al., 1997; Zigrino et al., 2005), angiogenesis (Woodhouse et al., 1997), osteoclastogenesis (Roodman, 2004), growth factor regulation (follistatin) (Reis et al., 2004; Risbridger et al., 2001), and extracellular matrix alteration (proteoglycan-1) (Timar et al., 2002). A multigenic study of breast cancer metastasis to bone has provided evidences for a causal role of IL-11, connective tissue-derived growth factor (CTGF), and CXCR4, along with osteopontin in osteolytic metastases. The combined overexpression of these genes give to transfected cells a metastatic activity close to that of the highly aggressive cell populations endogenously expressing the entire bone metastasis gene set (Kang et al., 2003).

Bone metastases are commonly characterized as osteolytic or osteoblastic from a radiological point of view. This represents two extremes of a continuum in which dysregulation of the normal bone remodeling process occurs and alters bone mass and bone microarchitecture. Breast cancer is most often associated with osteolytic metastases while osteoblastic metastases are common in prostate cancer. Osteolytic lesions are due to a marked increase in osteoclast number with a reduced osteoblastic activity. PTHrP is a major mediator between tumor and bone cells and induces the osteolytic process through the RANKL pathway (Guise, 2000). On the contrary, osteoblastic metastases are characterized by a dramatic increase in osteoformation but always possess a resorption component (Logothetis & Lin, 2005; Mundy, 2002).

Most researches have focused on the interactions between tumor cells and osteoclasts or osteoblasts. However interactions with surroundings cells have been poorly investigated. Because osteoblast precursors are stromal cells (present in the marrow spaces) that interact with hematopoietic osteoclast precursors via the RANK/RANKL/osteoprotegerin system (Thomas et al., 1999), the potential influence of stromal cells on metastatic cells needs to be investigated.

The aim of this study was to induce metastases in the rat to investigate the expression pattern of these key molecules in the bone microenvironment and also in the stroma of other organs invaded by a metastasis. Two cell lines known to induce either osteolytic or osteoblastic metastases were used.

MATERIALS AND METHODS

Cell lines and culture conditions

Two malignant cell lines capable of inducing bone metastases were used in the present study:
Walker 256/B cells. The Walker 256/B (W256) cells (issued of a rat mammary carcinoma) are
associated with osteolytic metastases (Blouin et al., 2005; Rizzoli & Fleisch, 1987; Wingen et
al., 1986). W256 cells, (kindly provided by Pr. R. Rizzoli, Geneva, Switzerland), were grown
in Dulbecco's modified Eagle's medium (DMEM - Eurobio, Les Ulis, France) containing 10%
heat inactivated fetal calf serum (FCS-Seromed Biochrom, Berlin, Deutschland), 100 UI/ml
of penicillin (Eurobio), 100µg/ml of streptomycin sulfate (Eurobio), 1% of Non Essential
Amino Acids (NEAA – Cambrex, Walkersville, MD, USA), and 1 mM of sodium pyruvate
(Eurobio). Cells were cultured at 37°C in a humidified incubator with 5% CO_2. In order to
obtain cells with a bone trophicity, 10^7 cells were serially passaged intraperitoneally at 7 days
intervals in Fisher rats to obtain malignant ascite.

MatLyLu cells. The MatLyLu cell line is an androgen independent, highly metastatic, and
anaplastic tumor of rat origin, which spreads to the lymph nodes and invariably to the lung.
metastases are reported to be osteoblastic in the vertebrae (Vieweg et al., 1994). The
MatLyLu tumor cell line was obtained from ECCAC (European Collection of Animal Cell
Cultures, Salisbury, Wiltshire, UK). MatLyLu cells were grown in Roswell Park Memorial
Institute 1640 medium (RPMI 1640 - Eurobio) containing 10% heat inactivated fetal calf
serum (FCS-Seromed Biochrom, Berlin, Deutschland), 100 UI/ml of penicillin (Eurobio),
100µg/ml of streptomycin sulfate (Eurobio) and 250nM of dexamethasone (Sigma, Saint
Quentin Fallavier, France). Cells were cultured at 37°C in a humidified incubator with 5%
CO_2.

Animals

The Animal Care and Use committee at the University of Angers approved all procedures.
Nine female Fisher F344/NHsd rats (Harlan, Gannat, France), 3 weeks old were maintained 6
months to the local vivarium conditions (24°C and a 12-h/12-h light/dark cycle). Nine female
Copenhagen born from COP/NCrl rats (Charles River, Sulzfeld, Germany) and bred in our
University animal facilities were maintained 6 months to the same conditions. Rats were
given a standard laboratory food (UAR, Villemoison sur Orge, France) and water *ad libitum*.
F344 rats were anesthetized with isoflurane (AErrane®, Baxter S.A, Belgium) and received
an intracardiac injection of 10^7 W256 cells in 0.25 ml saline. They also received

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intramuscular (quadriceps femoris) and subcutaneous (base of the neck) injection with 10^7 W256 cells.

COP rats were anesthetized with isoflurane and received an intracardiac injection of 3.25×10^4 MLL cells in 0.25 ml saline. They also received intramuscular, intraliver, intraspleen and subcutaneous injections with 3.25×10^4 MLL cells in the same locations than above. For both types of rats, the cell number to be injected was determined as the most appropriate in preliminary experiments. Data were compared with results obtained on control rats.

Rats were sacrificed by asphyxiation with CO_2, 9 days (F344) or 15 days (COP) after the intracardiac injection of tumor cells. These durations have been determined by preliminary studies and were found sufficient to obtain bone metastases before animals die from other tumor localization. The hindlimbs were carefully dissected; bones and other tissue samples (liver, spleen, brain, subcutaneous and intramuscular nodules) were placed in a formalin-alcohol based fixative at +4°C during 24 h.

Histological staining methods on paraffin sections

Paraffin sections (4μm) were deparaffined and stained by Hematoxylin Phloxine Saffron (HPS) as a standard tissue stain. Immunohistochemistry was performed on femur and organs of 6 F344 and 6 COP rats. Bone decalcification was done using a ready-to-use decalcifying fluid according to the manufacturer's recommendations (Surgipath®, Labonord, Templemars, France). Bone and other tissues were then dehydrated, embedded in paraffin and sectioned at 4 μm. Antigen retrieval was performed by bain-marie heating in a citrate buffer (pH 6). Immunochemistry was performed using DAKOCYTOMATION® procedure with the Dako autostainer automat (Dako, Glostrup, Denmark). The following proteins were traced using specific antibodies:

- Dickkopf-1 (Dkk-1) is an antagonist of the canonical Wnt signaling by binding directly to the LDL receptor-related protein 5/6 (LRP5/6). The interaction between DKK1 ant LRP5/6 induces an inhibition of bone formation (Johnson et al., 2004). The reagent was a purified goat polyclonal antibody (Tebu-bio, sc-14949) used at a dilution of 1:50. Positivity is characterized by a cell membrane labeling.

- Interleukin 6 (IL-6) is a pleitropic cytokine. Its production by bone marrow stromal cells enhances osteoclastogenesis (with subsequent bone resorption) and tumor cell growth (Roodman, 2003). We used a purified goat polyclonal antibody (Tebu-bio, sc-1265) at a dilution of 1:50. Positivity is characterized by a cytoplasmic labeling.

- RANKL is produced by osteoblasts and stromal cells and induces osteoclastogenesis by interacting with its receptor at the osteoclastic surface (Roodman, 2003). We used a purified goat polyclonal antibody (Tebu-bio, sc-7628) at a dilution of 1:200. Positivity is characterized by a cell membrane labeling.

- Cathepsin K is a cysteine protease highly expressed by osteoclasts and is implicated in the bone matrix collagen breakdown (Yasuda et al., 2005). The purified goat polyclonal antibody (Tebu-bio, sc-6507) was used at a dilution of 1:200. Positivity is characterized by a cytoplasmic labeling.

- Macrophage-colony stimulating factor (M-CSF) is expressed by stromal cells and osteoblasts. It is required together with RANKL to induce the development of mature osteoclasts (Boyle et al., 2003). The purified goat polyclonal antibody (Tebu-bio, sc-1324) was used at a dilution of 1:200. Positivity is characterized by a cytoplasmic labeling.

Control positive tissues were obtained for each antibody. Sections were counterstained with hematoxylin.

TRAcP cells detection

Tartrate Resistant Acid Phosphatase (TRAcP) positive cells were detected by a histoenzymatic technique in 3 F344 and 3 COP rats. TRAcP is a key enzyme found in high level in osteoclasts; it is involved in the intracellular degradation of collagen and non-collagenous proteins of the bone matrix (Vaaraniemi et al., 2004). Tissues were embedded in methylmethacrylate at 4°C to maintain enzyme activity. Bone samples were embedded undecalcified. Sections (7μm thick) were cut dry on a heavy-duty microtome equipped with 50° tungsten carbide knives (Leica Polycut S, Rueil-Malmaison, France). TRAcP was revealed by a simultaneous coupling reaction using 1-naphtyl phosphate (Sigma-Aldrich Chemical, Illkirsh, France) as substrate and Fast Violet B as the diazonium salt. Sections were counterstained with phosphomolybdic aniline blue (Chappard et al., 1983). TRAcP positive cells appeared with a brownish tint. Collagen fibers densely packed in the form of bone matrix or in the tissue stroma appear with a blue tint.

RESULTS

Tumor growth in animals

F344 rats developed tumors at subcutaneous and intramuscular site of injection. They also developed pulmonary, liver, brain and bone tumors secondary to the intracardiac injection of W256 cells. In the femur, osteolytic foci were preferentially localized below the growth cartilage and formed a large and irregular band filled with malignant cells.

COP rats similarly developed tumors at subcutaneous, intramuscular, liver and spleen sites of injection. They also developed pulmonary and bone tumors after the intracardiac injection. In the femur, focal areas of osteolysis were disseminated all over the metaphysis. No osteosclerotic lesions were found.

Histological and immunohistochemical characteristics of bone tumors

Tumor cells appeared most often as closely packed nodules ill-demarcated from the hematopoeitic marrow. Some tumoral cells were also found isolated in bone marrow. For both line cells, tumor cells were large with a high nuclear/cytoplasmic ratio, they exhibited proeminent nucleoli and a basophilic cytoplasm without vacuoles. Numerous mitoses were observed. An increased osteoclastogenesis was assessed in the immediate vicinity of the tumor foci, leading to a dramatic increased of active erosion surfaces and the resorption of complete bone trabeculae (Fig. 1). Siderophages (macrophages containing brownish cytoplasmic particles) were frequently found, they reflect the necrotic and remodelling steps occurring in the hematopoietic bone marrow secondary to tumor invasion (Weinberg, 1983). These cells were positive with the Perls reaction for iron. They are readily distinguishable from immunolabeled cells under the microscope, although they can appear with a similar intensity on black and white microphotographs.

Both models exhibited the same immunohistochemical labeling pattern: tumor cells never exhibited significant labeling for M-CSF, Dkk-1, RANKL, IL-6 or cathepsin K (Fig. 2). A strong membrane expression of M-CSF was found in most bone marrow stromal cells in contact with the metastatic foci. Dkk-1 was also expressed at high levels in stromal cells and osteoblasts in contact with metastatic foci in both models (Fig.2 A). At distance, there was no significant labeling for Dkk-1 in these cell populations. Many stromal cells showed RANKL positivity in the vicinity of the tumor (Fig. 2C). RANKL labeling was also seen on osteoblasts at distance of the tumor foci. These stromal cells (which were labelled by Dkk1, M-CSF and RANKL) were found at distance from trabeculae: they were mononucleated with often a large granular cytoplasm; some of them appeared spindle-shaped. The IL-6 detection was not conclusive due to a non specific label induced by the prior decalcification.

Two types of cells were found to express a faint and homogenous cytoplasmic positivity for cathepsin K: typical multinucleated osteoclasts at the surface of bone trabeculae and multi- or mono-nucleated cells (either round or with cytoplasmic branches) in the marrow spaces. These cells were mixed with haematopoietic cells and were at distance from bone surfaces (Fig. 2E); they concentrate in the close vicinity of the tumor foci, on the contrary, they appeared scantily distributed at distance.

Two types of cells exhibited TRAcP positivity (TRAcP+): cells apposed on trabeculae were multinucleated; cells encountered in bone marrow spaces were either multi- or mono-nucleated; they were situated far from trabecular surfaces and in the close vicinity of metastatic cells, their density decreased at distance from tumoral foci (Fig. 3A).

Histological and immunohistochemical characteristics of extraosseous tumors

Tumor nodules of both types of carcinomas were dense and well-delimited by a fibrous capsule. There were composed of a large majority of tumor cells mixed with less numerous stromal cells. Tumor cells showed a different aspect according to the metastatic site: brain metastases were composed of spindle-shaped cells whereas in other sites, they appeared round or polygonal (especially with Walker cells). For both line cells, tumor cells were large with a high nucleocytoplasmic ratio; prominent nucleoli were often present and the cytoplasms were devoid of vacuoles. Numerous mitoses were observed but small foci of necrosis were also noted inside nodules.

Both tumor models exhibited the same immunohistochemical labeling pattern: tumor cells never exhibited any significant labeling for M-CSF, Dkk-1, RANKL, IL-6, cathepsin K. Stromal cells in contact with malignant cells did not express Dkk-1, M-CSF, cathepsin K. At distance from tumor foci, they also failed to express these markers. IL-6 expression was found homogeneous in the liver parenchyma with diffused granular cytoplasmic expression in the hepatocytes. The staining was found in all the liver parenchyma and not restricted to the areas surrounding the tumor. Few stromal cells were located around the tumoral foci and did not exhibit IL-6 expression (Fig. 3C). Some RANKL positive (RANKL+) stromal cells were found at the periphery of subcutaneous tumors. These mononucleated cells were round or stellate with an abundant cytoplasm. No RANKL+ cells were found in any other extraosseous site.

TRAcP+ cells were found interspersed in the spleen parenchyma whether tumor cells were present or not. These mononucleated TRAcP+ cells appeared round with a large granular

cytoplasm; the nucleus was ovoid or reniform (Fig. 3B). TRAcP+ cells were never observed in the vicinity of malignant cells in any other extraosseous metastases.

DISCUSSION

It is an oversimplification to consider only osteoblasts and osteoclasts as the interacting partners of tumors cells in the vicious cycle of cancer bone metastases. In addition to a variety of matrix components, the tumor stroma contains a rich cell population, which includes fibroblasts, smooth muscle cells, endothelial cells, dendritic cells, macrophages and other inflammatory cells (Zigrino et al., 2005). This forms a network with a complex crosstalk influencing cellular activities. For example, various actors of the tumor microenvironment such as fibroblasts, immune cells and the extracellular matrix influence the ability of TGF-β to promote or suppress carcinoma progression and metastasis (Bierie & Moses, 2006). In a previous work, we have shown an influence of the microenvironment on tumor cell in the 5T2 multiple myeloma murine model since a high bone remodeling (induced by ovariectomy) dramatically increased tumor growth (Libouban et al., 2003). This altered microenvironment was able to select a highly aggressive myeloma cell line (Libouban et al., 2004). Others have also reported that parathyroid hormone injection in athymic mice induced a preferential localization of PC-3 prostate cancer cells in bones (Schneider et al., 2005).

Interactions of malignant cells with endothelial cells in bone metastases have been previously investigated. Van der Pluijm *et al.* observed significantly elevated mRNA levels for VEGF-A and -B in bone metastases compared to soft tissue metastases induced by the human breast cancer cells MDA-MB-231 (van der Pluijm et al., 2001). Di Raimondo *et al.* similarly detected higher concentrations of the angiogenic peptides (VEGF, bFGF (basic fibroblast growth factor) and HGF (hepatocyte growth factor)) in bone marrow than in the peripheral circulation of patients with multiple myeloma. This suggests the specific induction within the bone microenvironment of angiogenic factors that allows the development of a neovascular network which favours the tumor growth (Di Raimondo et al., 2000).

Interaction between tumor cells and other stromal components also plays a major role in cancer progression. Cancer cells can alter the surrounding connective tissues and modulate the metabolism of fibroblasts thus resulting in the production of a collagenous stroma that supports the tumoral process. Fibroblast are a key component of tumors because they acquire a modified phenotype similar to those associated with wound healing. Such "activated" fibroblasts has been coined "carcinoma-associated fibroblasts" (CAFs) (Kalluri & Zeisberg, 2006). CAFs are implicated in extracellular matrix production which provides a lattice that facilitates cancer progression; they are one of the main sources of VEGF, thus providing more

nutriment to the metastatic cells (Kalluri & Zeisberg, 2006). Shimamura *et al.* have investigated the microenvironment of osteolytic metastases caused by MDA-MB 231 breast carcinoma cells in nude mice. Two weeks after an intracardiac injection with MDA-MB 231 cells, foci of tumor cells were associated with osteoclastogenesis together with angiogensis related to intense the expression of VEGF (Shimamura et al., 2005). In our study, we have also found specific modifications in the bone microenvironment around the tumor foci with expression of M-CSF, Dkk-1, and RANKL in non-osteoblastic cells. Moreover, mononucleated TRAcP+ cells were also encountered in bone marrow spaces far from trabecular surfaces. This denotes an increased induction of osteoclast precursors since mononuclear TRAcP+ cells in the bone marrow are a source for mature multinucleated osteoclasts (Baron et al., 1986). These mononucleated TRAcP+ cells expressed cathepsin K and were found in medullar spaces at distance from bone surfaces. All these cells were found with a density decreasing as the distance from tumor cells increased. It is probable that a gradient of cytokines released from malignant cells can explain this finding. Tumor cells produce a variety of soluble factors (PTHrP, TGF-β, IGF, Endothelin-1…) which are employed as a paracrine communication system with surrounding cells or as an autocrine regulation system (Guise & Mundy, 1998; Guise et al., 2003; Lee et al., 2003). W256 and MatLyLu cells are unable to resorb bone by themselves and have been reported to produce high levels of PTHrP which promotes osteoclastogenesis (Rabbani et al., 1999; Rizzoli & Fleisch, 1987).

The stromal expression of important cytokines involved in the bone remodeling was found altered only when metastatic cells were developing in a bony microenvironment. No expression of these cytokines could be observed in the tumor stroma when metastatic cells had developed in other organs. The few RANKL+ cells found at the periphery of subcutaneous tumors were probably Langerhans cells. TRAcP+ cells encountered both in invaded and non-invaded spleens probably corresponded to activated macrophages, a condition previously noted by others (Hayman et al., 2000). TRAcP+ cells were previously observed in the stroma of bone tumors (Yam et al., 1987). TRAcP+ osteoclast-like cells are also observed in giant cells tumors which can develop in bone (and cause osteolytic lesions) or in soft tissues (Teot et al., 1996). In this tumor, the malignant component is of fibroblastic origin and express RANKL which provokes osteoclastogenesis (Murata et al., 2005). In our models, stromal cells were able to express RANKL and to induce osteoclastogenesis only in the bone compartment.

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Tumor cells do not have the same cytokine pattern of expression according to their location and can modulate it depending on the surrounding stromal cells. For example, breast cancer cells have an increased expression of PTHrP in bone metastases when compared to other sites (Mundy, 2002). As a consequence, the microenvironment can also influence the tumor cells: Walker cells acquired a spindle-shape only when located in brain metastases, whereas they appeared round or polygonal in other sites. The difference may also be supported by soft tissue stromal cells which can have different surface receptors than bone stromal cells, making them unable to response to tumor cell stimulation.

In conclusion, interaction between stromal and cancer cells induces the expression on the formers of characteristics leading to osteoclastogenesis in the bone microenvironment. These modifications allow the growth of the metastases in a convenient microenvironment. It thus explains the preferential localization observed in our bone metastasis models. The major role of osteoclasts and osteoblasts in bone metastasis formation has led to underestimate the critical function of microenvironment in metastasis growth.

Acknowledgments

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FIGURES

Fig.1. Increased osteoclast number (arrows) and resorption lacunae on trabeculae near metastatic foci of tumor cell (T) in bone (HPS staining). Original magnification: x400.

Fig.2. A) Dkk-1 expression in cells at distance of tumoral foci (T) in bone with osteoblasts (arrow) and stromal cells (arrow head) exhibiting membrane and cytoplasmic labelling. Other cells appearing in black at the top of the image are siderophages (S).

B) the negative control section stained with the same concentration of control antibody, note that a group of osteoblasts (arrows) is negative.

C) RANKL expression near tumor foci in bone. Osteoblasts (arrow) and stromal cells (arrow head) are RANKL positive. Tumor foci are RANKL negative.

D) the negative control section stained with the same concentration of control antibody, note that a group of osteoblasts (arrow) is negative.

E) Cathepsin K expression near tumoral foci (T) in bone. Cathepsin K+ multi- and mononucleated cells (arrow head) have a diffuse and faint cytoplasmic labelling. Both can be found in marrow spaces at distance from bone surfaces. A siderophage (iron-loaded macrophage) is also evidenced (S).

F) the negative control section stained with the same concentration of control antibody, note that an osteoclasts (arrow) is negative.

For all images, the original magnificationis x400.

Fig. 3. A) Multinucleated TRAcP+ cells in bone are apposed on trabeculae. In bone marrow spaces, multi- (arrow) and mononucleated (arrow head) TRAcP+ cells are also encountered far from trabecular surfaces and near tumor cells (T) (undecalcified plastic section). Original magnification: x200.

B) TRAcP+ cells are found interspersed in the spleen parenchyma in the presence or not of tumor cells. These mononucleated TRAcP+ cells are round with a large cytoplasm in the red pulp, note that the white pulp is negative (M= Malpighi corpuscle). (Plastic section). Original magnification: x50.

C) IL-6 expression in the liver. Note that tumoral foci (T) are negative whereas hepatocytes show a homogeneous granular cytoplasmic expression. Original magnification: x50.

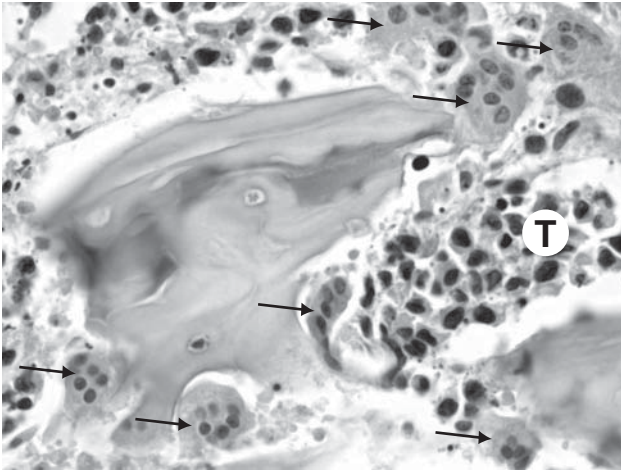D) the negative control section stained with the same concentration of control antibody, note that an osteoclasts (arrow) is negative. Original magnification: x50.
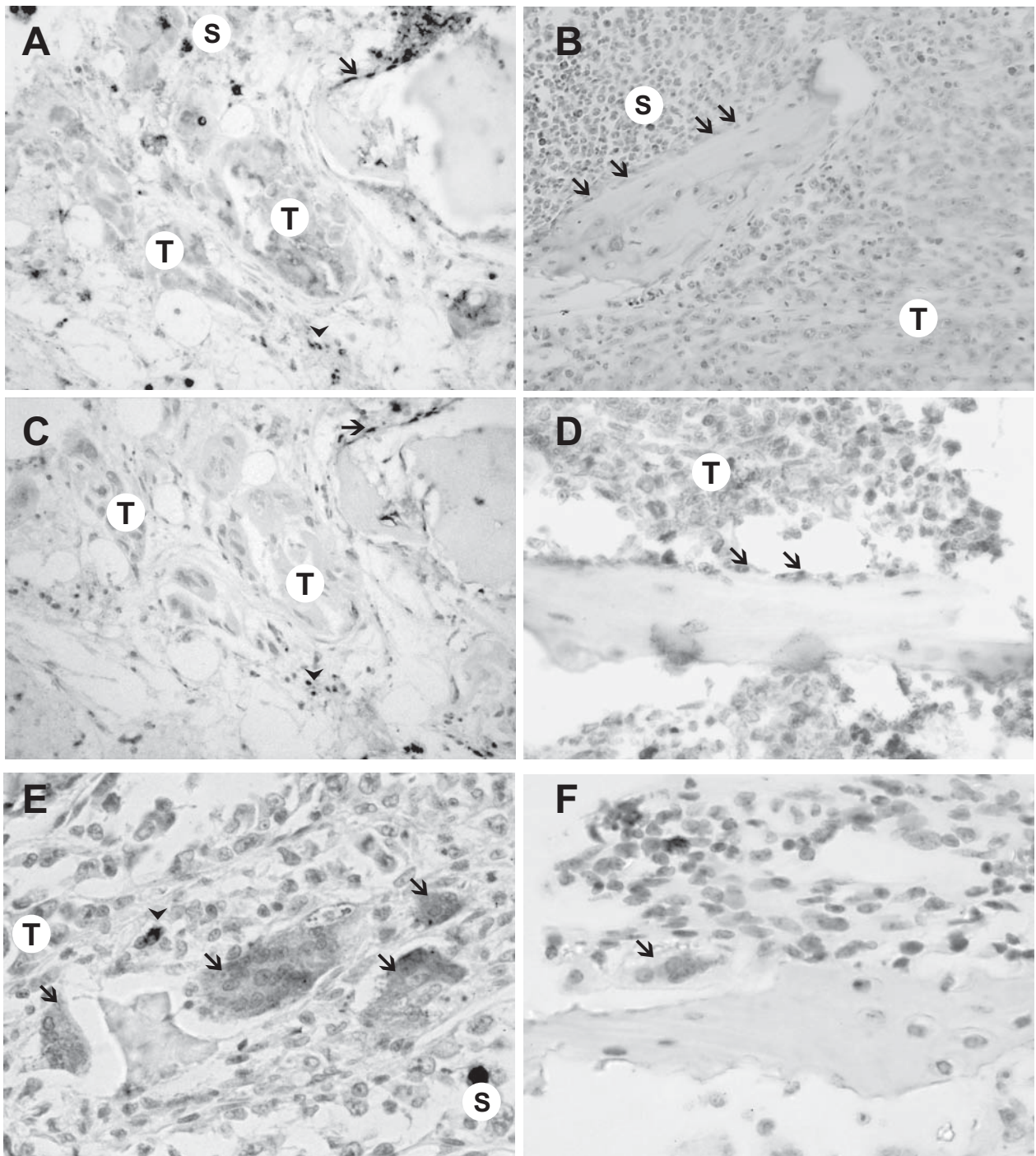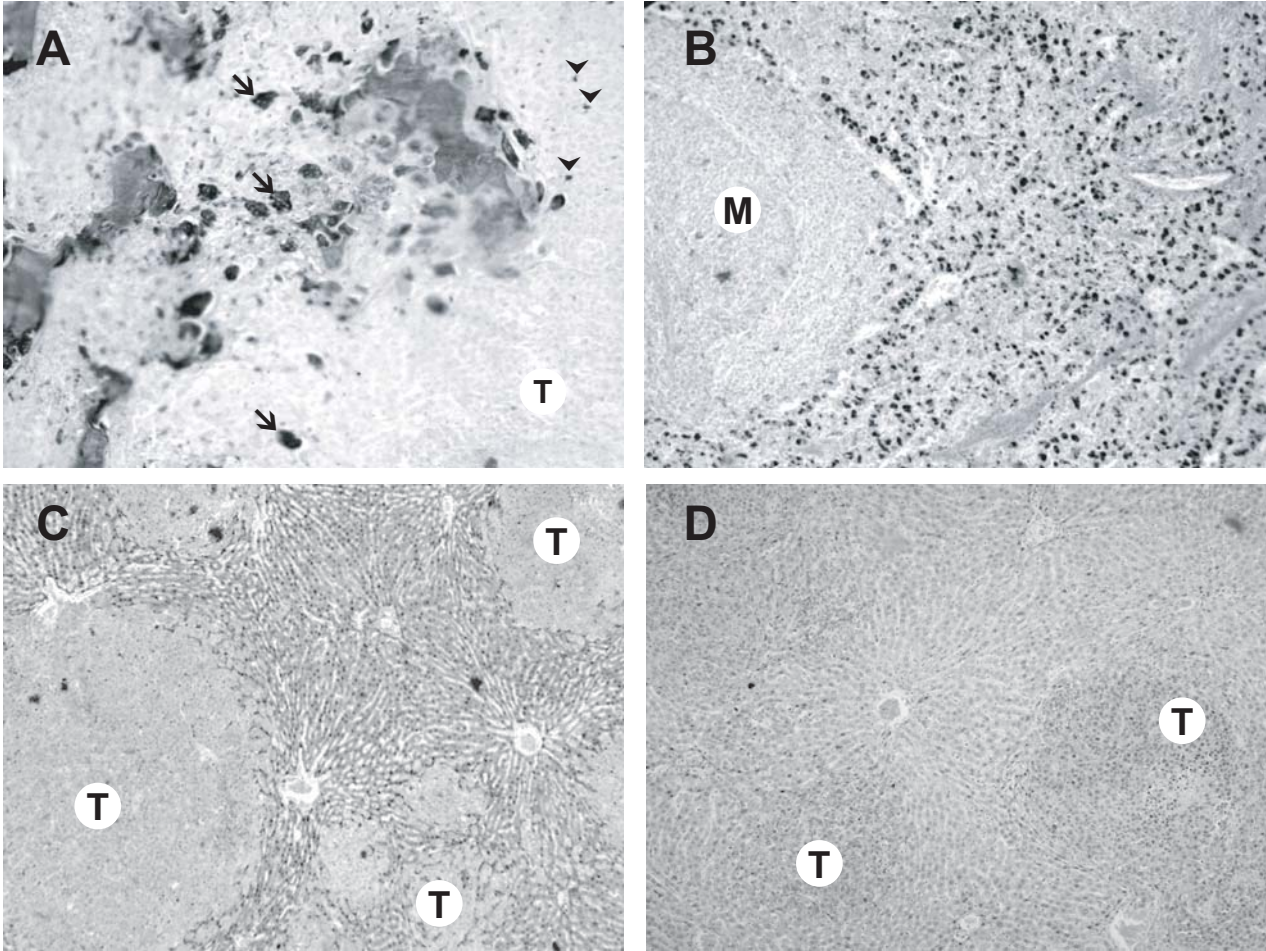
Figure 1

Figure 2

Figure 3