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REVIEW

Rat models of bone metastases

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Abstract Bone metastases occur frequently in patients with advanced breast or prostate cancer. Bone metastases can be predominantly osteolytic, osteoblastic or mixed. Studies with animal models allow advances in understanding the molecular basis for bone metastases and provide new targets for therapy. Several animal models have been developed in rat with different pathophysiologies; they required injection or implantation of neoplastic cells into orthotopic locations, bones or the left ventricle of the heart. Several specific strains of rat have an increased incidence of spontaneous tumors. Carcinomas can be induced by either chemicals or physical agents. However, the most used and convenient way to induce bone metastases is a syngeneic transmission. MAT-Ly-Lu cells have been used in several models using Copenhagen rats to induce osteoblastic bone lesions. PA-III cells derived from Pollard tumors can also produce a combination of osteolytic and osteoblastic reactions at the site of transplantation. Osteolytic bone lesions can be obtained with an injection of Walker cells. The use of 13762 or c-SST2 cells allows also leads to osteolysis. Human xenografts can only be used in nude animals. It is essential to validate and correctly interpret the lesions in several models of bone metastasis. No animal model is sufficient by itself to represent the clinical findings observed in humans. The use of models developed in different species should be more predictive and bring a beam of arguments for a better knowledge of pathophysiological and therapeutic mechanisms.

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Introduction

Bone metastases are debilitating diseases with an increased morbidity (pain, impaired mobility, hypercalcemia, fractures, spinal cord or nerve root compression and anemia due to bone marrow infiltration). They occur in approximately 70% of patients with an advanced breast or prostate cancer and 15–30% of patients with a carcinoma of the lung, colon, stomach, bladder, uterus, rectum, thyroid, or kidney [1]. Furthermore, once tumors have metastasized to bone, they correspond to an advanced stage of malignancies and are usually incurable: only 20% of patients with breast cancer are still alive 5 years after the discovery of bone metastasis [2]. Breast and prostate cancer are the two tumor types that most commonly metastasize to bone. This selective homing of tumor cells in bone has been found to be due to (1) a specific chemotaxis of the bone environment for these particular types of cancer (stromal cell-derived factor-1, epidermal growth factor, low glycosylated osteonectin...) (2) a selective adhesion of metastatic cells on the endothelial surface of marrow capillaries (due to a variety of integrins such as $\alpha 4\beta 1$, $\alpha 5\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$) (3) an appropriate growth factors and extracellular matrix proteins present in the marrow microenvironment (parathyroid hormone related protein (PTHrP), tumor growth factor beta (TGFβ), vascular endothelium growth factor (VEGF)...) [3–6]. These 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 [6]).

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 more often found in prostate cancer. Osteolytic lesions are due to a marked increase in osteoclast number with a reduced osteoblastic activity; PTHrP is a major mediator of the osteolytic process [7]. On the contrary, osteoblastic metastases are characterized by a dramatic increase in osteoformation but still possess a resorption component [1]; interactions between prostate cancer cells and osteoblast have been reviewed by Logothetis and Lin [8].

Although cancer is a major cause of death and morbidity in Western countries, one major hindrance in the study of the biology of cancer has been the limited number of laboratory animal models. Animal models provide important knowledge on the pathophysiological conditions that lead to a given disease and allow the preclinical evaluation of new effective treatments. Research using animal models acts as the bridge between in vitro studies and human clinical trials. Mouse has become popular in skeletal research because of the ease with which its genome can be manipulated and investigated. Rat is also a very used model, due to its large availability, low cost, easy handling, easy housing and resistance.

Rat plays a very important role in the bone field especially in the evaluation of metabolic bone diseases. It has been used to study osteoporosis due to ovariectomy or orchidectomy, bone circulation, biocompatibility of prosthetic materials... In this review, we focused on rat as a model for bone metastases: the size of the rat bone allows a better handling and appear interesting to evaluate biomaterials or therapeutic molecules targeting cancer in order to reduce fractures.

The most used technique for the detection of bone metastases is X-ray examination. However, others techniques have been reported to characterize bone metastases in animal models: scintigraphy [9], micro-computed tomography-X [10]. Others new analytical tools are in development and adaptable to rats: the PET (positron emission tomography) can be performed with 2-[18F] fluoro-2-deoxy-D-glucose (FDG) or 99mTc-bisphosphonate to detect metastases [11]. Fluoroscopy is also an emerging method that can be used to visualize cancer at any stage, including the growth of the primary tumor, tumor cell motility and invasion, interactions between the tumor and its microenvironment [12] or to evaluate the cytotoxicity of therapeutics [13].

The development of bone metastases from spontaneous tumors is rare

Each rat strain and gender appears to have a specific spontaneous tumor profile. For example, the highest susceptibility to mammary fibroadenomas and adenocarcinomas was observed in female Sprague-Dawley rats compared with HanWistar, BASF Wistar and F344 rats [14].

Mammary carcinoma

Rat strains vary in lifetime incidence of spontaneous mammary carcinomas from zero (e.g. Copenhagen 2331) to intermediate (e.g. Sprague-Dawley, Wistar) to high (e.g. Fischer 344, Brown Norway) [15]. Unfortunately, these primary tumors may not be good models for human disease. Most spontaneous mammary carcinomas do not metastasize and are only associated with a mild local tissue invasion. There is a low incidence of spontaneous metastasis to regional lymph nodes and lungs; furthermore, bone metastasis is very rare. In addition, most adenocarcinomas in rodents rapidly loose their estrogen responsiveness and are not good models of estrogen-responsive neoplasms.

Prostatic carcinoma

Spontaneous prostate carcinoma is rare in rodents and the incidence appears very low when compared with humans. However, some rat strains have an increased incidence of prostate neoplasms, including the Lobund Wistar and ACI/Seg rats. Up to 30% of aged Lobund Wistar rats (older than 20 months) develop prostate carcinoma in the anterior prostate/seminal vesicle complex. Lobund Wistar rats have high circulating concentrations of testosterone, which may predispose them to the development of this androgen-dependent adenocarcinoma. In their initial stage, tumors are testosterone-dependent, but when they progress, they become testosterone-independent. The ACI/Seg rats develop a high incidence (80%) of microscopic prostate neoplasia in the ventral lobes and a moderate incidence (16%) of macroscopically evident prostate carcinoma at 36 months of age. However, these tumors do not metastasize to bone [16].

The development of bone metastases in chemical carcinogenesis models is rare

Mammary carcinoma

Mammary carcinomas have been induced by both chemical xenobiotics and physical agents in rats. Chemically induced models of rat mammary cancer are more prevalent than radiation-induced models [17]. The most widely used chemical models include the polycyclic aromatic hydrocarbon DMBA (dimethylbenzanthracene) or the directly acting alkylating agents EMU (*N*-ethyl-*N*-nitrosourea) and MNU (1-methyl-1-nitrosourea). After a single dose of DMBA or MNU, adenocarcinomas develop within 20 weeks in young rats (50–60 days old). These cancers are dysplastic, and sometimes invade the surrounding tissues but they rarely metastasize to distant sites [15]. Mammary adenocarcinomas induced by ENU in Sprague-Dawley rats may metastasize to the lungs. The rats often develop a mild hypercalcemia but bone metastases do not occur spontaneously. The PTHrP production was not analyzed [16]. In contrast to chemically induced carcinomas, radiation-induced cancers have both longer latencies and lower frequencies of occurrence; radiations also induce frequently benign fibroadenomas.

Prostatic carcinoma

Prostate and seminal vesicle adenocarcinomas can be induced in Noble rats with testosterone/estradiol or MNU/testosterone combinations [18]. Administration of MNU and testosterone in Lobund Wistar rats increases the incidence of this tumor and lowers the age of occurrence: approximately 90% of rats will develop prostate carcinoma by 12 months of age [19]. These tumors metastasize uncommonly to the lymph nodes and lungs and do not metastasize to bone.

Syngeneic models of bone metastasis

A syngeneic model consists in injecting tumor cells in a host animal; these cells being derived from another animal of the same species (also called allograft). Many syngeneic models (Table 1) have been used to obtain bone metastases with different protocols for injecting the tumor cells (Fig. 1).

Osteoblastic metastasis

The MAT-Ly-Lu androgen-insensitive subline of the rat Dunning prostate carcinoma (R3327)

The parental tumor from which all the following in vivo sublines were derived is the original R3327 tumor, initially discovered in 1961 by W. F. Dunning in a 22-month-old inbred Copenhagen (Cop) male rat. Following serial in vivo passages of the original R3327 tumor, sublines with differing biological characteristics were obtained and characterized. Since one of these sublines produced metastases to both the lymph nodes

Table 1 Syngeneic rat models of bone metastasis

Cell line	Type of metastasis	Mode of injection	Time frame and observations
Mat-Ly-Lu	Osteoblastic	Intravenously (tail vein) after surgical clamping of the lower caval vein (Gedolf-Rao model) of Copenhagen rats	Tumor foci visible in the 5th and 6th lumbar vertebrae within 4 days after inoculation/ Hindlimb paresis and paralysis within 14 days
		Intraarterial injection in Copenhagen rats	Hindlimb paralysis and death within 2 weeks (mainly osteolytic lesion)
		Intraosseous inoculation in Copenhagen rats	Euthanasia within 1 month to avoid expansion of tumor. Significant osteoblastic lesions visible
PA-III	Osteoblastic	Deposit over the calvarium or the scapula of L-W rats (disruption of the local periosteum by the inoculation needle)	Combination of osteolytic and osteoblastic reaction at the site of implantation few days after surgery
Walker 256	Osteolytic	Intraarterial injection	Hindlimb paralysis and death within 2 weeks
		Intraosseous inoculation	Euthanasia within 1 month to avoid expansion of the tumor
13762	Osteolytic	Into the left ventricle of Fisher 344 female rats	Tumor foci visible in the tibiae, femur and fibulae 4 weeks post inoculation
		Intramedullar implantation in the proximal tibia of female Fisher 344 rat	Osteolysis visible 7 days after implantation on radiographs; trabecular BMD significantly decreased 21 days after implantation
cSST2	Osteolytic	Into the thoracic aorta by catheter insertion in the left common carotid artery of SHR rat	90% of animals developed bone metastases after 4 weeks, mainly in the spine

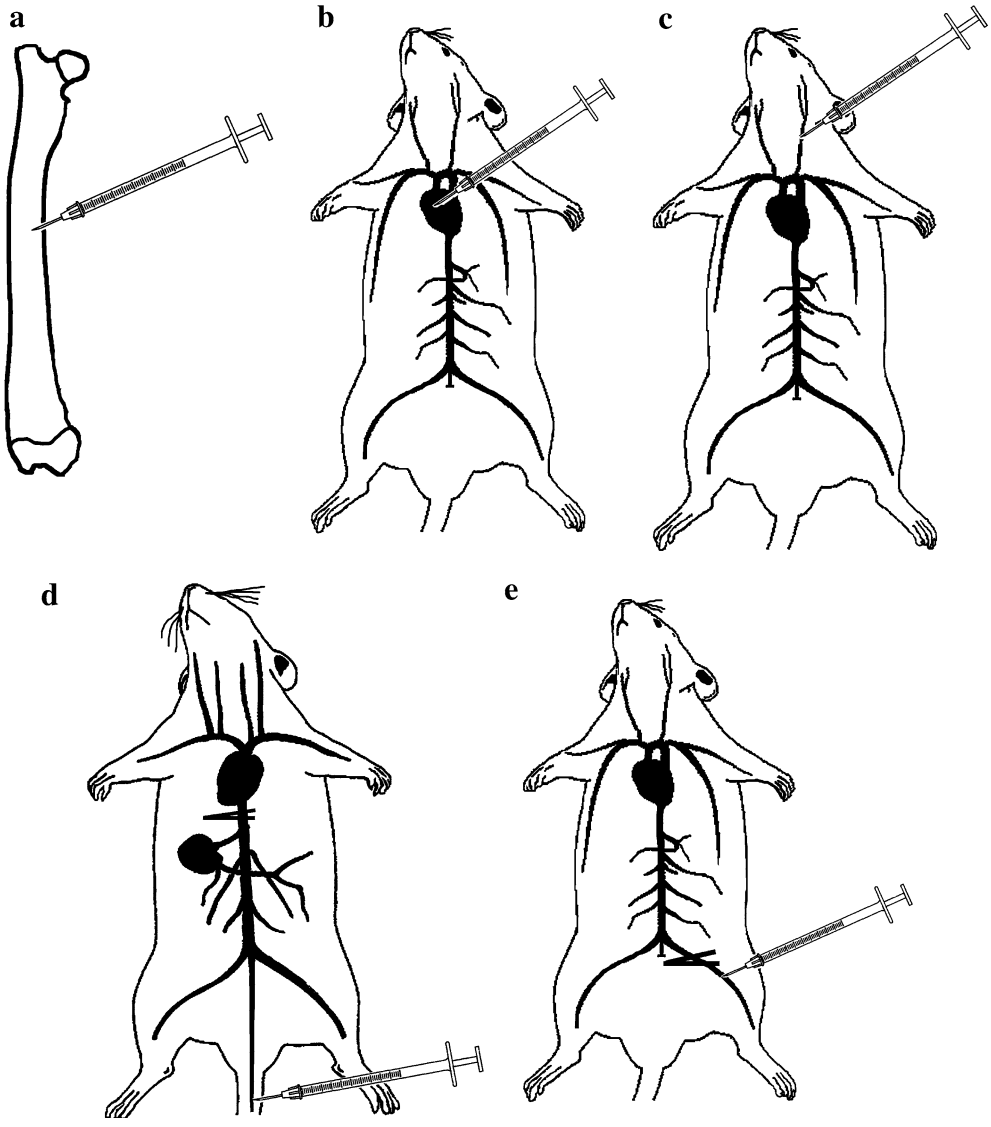

Fig. 1 Syngeneic models of rat bone metastasis can be performed by injecting tumor cells directly into bones such as the femur (**a**), into the left ventricle of the heart (**b**), into the carotid artery (**c**), into the tail vein after a transient surgical clamping of the lower caval vein (**d**) or into the femoral artery after a transient surgical clamping (**e**)

and lung; it was termed the MAT-Ly-Lu subline for: Metastatic Anaplastic Tumor metastasizing to Lymph node and Lungs. The MAT-Ly-Lu can be used in different experimental ways:

The Gedolf-Rao model [20–24] The R3327-MAT-Ly-Lu tumor cells were injected intravenously via the tail vein in male Copenhagen rats that had transient surgical clamping of the lower caval vein. This procedure reproducibly resulted in metastatic tumor growth in the lumbar region of the vertebral column. Microscopically, tumor growth became visible in the fifth and sixth lumbar vertebrae within 4 days after inoculation. Clinical signs of nerve function disablement (hindleg paresis and paralysis) followed within 14 days of such procedure. From a pathological point of view, a clear response of concomitant osteoclastic and osteoblastic activities was observed in the lumbar spine. In the serum, a transient phase of hypercalcemia could be evidenced. The development of skeletal metastases in these animals was not reflected by significant alteration in serum levels of acid phosphatase, prostatic-specific antigen, or osteocalcin. Treatment with a bisphosphonate (dichloro methylene bisphosphonic acid—Cl_2MDP) suppressed the metastatic potential and delayed the development of the hindleg paralysis.

The intracardiac injection. When inoculated into the left heart ventricle, Copenhagen rats reliably developed a hindlimb paralysis, a clinical marker of bone metastasis within 2–3 weeks. Paralysis is due to the spinal cord compression by tumor cells extending from the vertebral body. The MAT-Ly-Lu cells were found adhering preferably to bone marrow stromal and endothelial cells rather than to other bone-derived cells, fibroblasts and hepatic endothelial cells. These

results suggest that preferential adhesion of prostate cancer cells to sinusoid capillaries of the bone marrow plays a significant role in the incidence of bone metastases [25]. Animals grafted with cells overexpressing urokinase, developed hindlimb paralysis significantly earlier and had a more widespread appearance of skeletal metastases [26]. When cells overexpressing PTHrP were inoculated, this resulted in an increase in osteolytic skeletal metastases [27].

The intraosseous injection. Recently, Liepe et al. developed a new model using an intraosseous injection of MAT-Ly-Lu cells after drilling a hole in the femoral shaft. They observed osteoblastic bone lesions by scintigraphic evaluation and histological study [9]. Our team has also developed this model by injecting 10^3 cells in 10 µl intraosseously after drilling a hole in the median part of the femoral shaft. After injection, the cortical hole is filled up by bone wax. The development of an osteoblastic tumor is clearly identified 1 month after injection, in the femoral shaft on radiographs (Figs. 2 and 3). However, it represents the local development of a malignant tumor rather than a true metastasis localized in bone after vascular embolization. Metaplastic trabeculae made of woven bone can be identified by histology under the spongiosa. A massive periostic reaction is often found with metaplastic bone invading the soft tissues.

Fig. 3 Femur from Copenhagen rats (**a**) sham and (**b**) injected intraosseously with 5×10^3 MAT-Ly-Lu cells. Animals were euthanized 1 month after injection. Note the considerable osteocondensation in the diaphysis. Radiographs obtained on a Faxitron machine (Faxitron, Edimex, France)

The Pollard model [28–32] Pollard tumors are prostate adenocarcinomas (PA) developing spontaneously among 10% of aged Lobund-Wistar (L-W) rats. The incidence of such prostate tumors increases significantly following treatments by *N*-Nitro-*N*-Methylurea, depotestosterone and with a high fat diet. Four PA cell lines derived from four L-W rats (PA-I, -II, -III and -IV) that manifested spontaneous prostate cancer have demonstrated a metastatic capacity. After SC injection in the flank region, the spread pattern of tumors PA-III and -IV was found involving only ipsilateral lymphatic channels to related lymph nodes leading eventually to the lungs [31]. Based on blood assay, no tumor cell (PA-I, -III and -IV) was detected in the blood stream of grafted animals. The spread of PA-II cells initially involved ipsilateral lymphatic channels to related lymph nodes and then through blood, they invade bilateral lymph nodes, lungs, liver and less frequently, kidneys on both sides. The rate and extent of the tumor spread in PA tumor-bearing rats was found accelerated by oral administration of sodium barbiturate and retarded by indomethacin and aspirin [28]. When deposited over

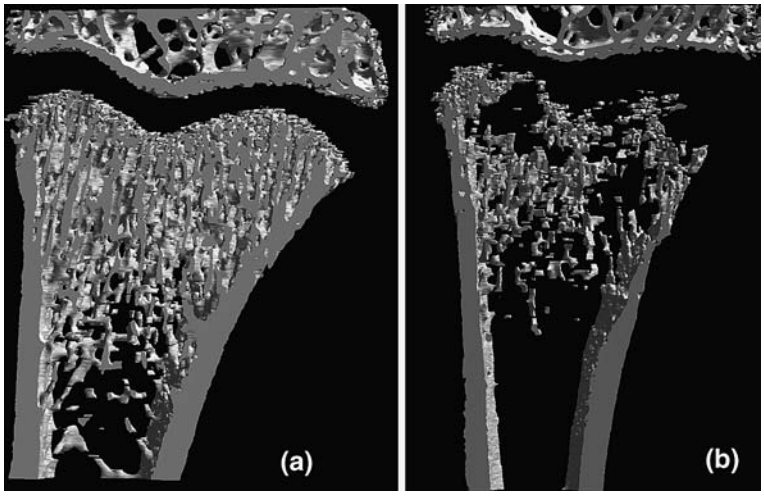

Fig. 2 Femur from Copenhagen rats: (**a**) sham and (**b**) injected with 5×10^3 MAT-Ly-Lu cells into the left heart ventricle. Animals were euthanized 15 days after injection. Note the focal osteolysis induced by MAT-Ly-Lu cells. 3D-models obtained with a Skyscan 1072 X-ray computed microtomograph (Skyscan, Aartselaar, Belgium)

the calvarium or the scapula of L-W rats, PA-III cells demonstrated the capacity to produce: (a) a local development of tumor with a combination of osteolytic and osteoblastic reaction at the site of transplantation; (b) metastasis to the lungs and production of secondary tumors. The development on bone required the disruption of the local periosteum by the inoculation needle. Periosteum appeared to act as a barrier for the establishment of PA-III cell tumors and the osteoblastic reaction. Subcutaneous administration of the bisphosphonate Cl_2MDP blocked the osteolytic process; the osteoblastic process was suppressed by X-rays delivered at the tumor site [29]. Castration did not affect either growth or metastatic capacity of PA-III cells in vivo since these cells are lacking androgen receptors. PA-III cells possess only the type-I IGF receptor and insulin; IGF-I and IGF-II increased cell proliferation in a dose-dependent manner. The local production of IGF-I by osteoblasts and the IGFs contents of the bone matrix could play an important role in the establishment of PA-III cells [32]. The bioavailability of IGF-I was regulated by urokinase which was produced by PA-III cells and acted as a paracrine factor [30]. These cells possess also glucocorticoid receptors and the administration of dexamethasone resulted in a remarkable inhibition of their proliferation due to the inhibition of urokinase expression [30].

Osteolytic metastasis

Walker 256

The Walker 256 rat tumor was first observed by Walker in 1928 at the John Hopkins University School of Medicine; it arose spontaneously in the region of the mammary gland of a pregnant albino rat, about 10 months of age [33]. The tumor was considered as a ''carcinosarcoma'' since it exhibited 2–3 cell types with different shapes. On microscopic examination, the patterns of a carcinoma and sarcoma were evidenced as independent entities, or could be intermingled. The hypotheses were that the tumor may come from a mixed neoplasia or from a multipotent stem cell from which all cell types arise (Stewart cited by [33]).

W256 cells can be maintained and propagated in vitro or in vivo by repetitive SC [34, 35], IM [36] or ascitic passages [37–47]. After an in vitro passage, W256 cells are susceptible to develop subcutaneously, intramuscularly or in ascite but they cannot induce bone metastases after an IA injection. Two passages in vivo are required before cells can induce bone metastases in the recipient animals after IA injection (Fig. 4). The W256 cells are the original line and are lacking estrogen receptors. They produce PTHrP allowing them to be a model of hypercalcemia [36, 48–50]. They were also used for the induction of bone metastases. Different ways of inoculation were used including IA injection [37, 42–44, 47] and bone inoculation [10, 51, 52]. These animal models, using W256 cells, were described to test the effect of bisphosphonates which are powerful anti-osteolytic agents against bone tumors.

The 13762 rat mammary carcinoma cell line

The 13762 rat mammary carcinoma cell line was initially developed by Segaloff (cited by [53]). The line was established in Fisher 344 female rats by administration of 7,12-dimethylbenzanthracene. It is a spontaneously metastatic syngeneic rat tumor, which has been extensively characterized for its growth, both in vitro and in vivo [53–57]. The tumor, when implanted SC into the mammary fat pads of female Fischer 344 rats, metastasizes at low frequency solely to lymph

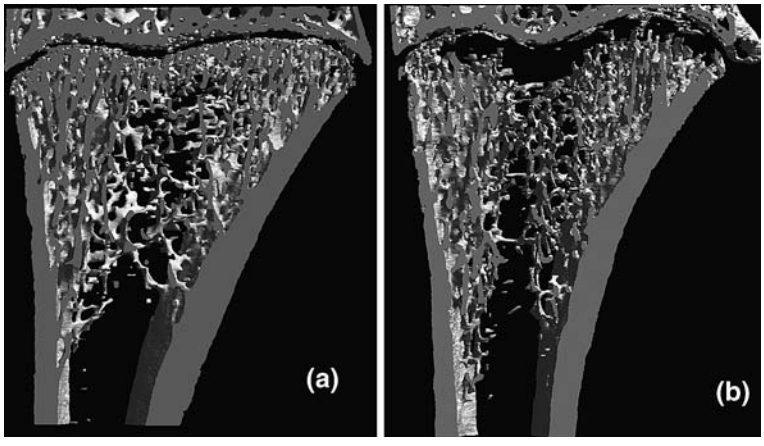

Fig. 4 Femur from Fisher rats (**a**) sham and (**b**) injected into the left heart ventricle with 10^6 W256 cells. Animals were euthanized 15 days after injection. Note the osteolysis of the primary spongiosa induced by W256 cells

nodes and lung. Several individual tumor sublines and lung metastasis-derived clones were compared for their spontaneous metastatic potentials, cell shape in culture, histologic types at primary implant and secondary metastatic sites, and growth characteristics in vivo and in vitro [58, 59]. The 13762 carcinoma was used as a model of tumor-induced osteolysis. Alvarez et al. evaluated the tumor progression by using radiographs and microcomputed tomography (microCT) after intramedullar implantation in the proximal tibia of female Fisher 344 rats. Osteolysis developed 7 days after implantation and was evidenced on radiographs; trabecular BMD was significantly decreased by 21 days after implantation. Rats treated with a bisphosphonate (alendronate, pamidronate or risedronate) showed a decreased osteolysis. Using immunohistochemical techniques, they also demonstrated that these cells express both PTHrP and TGF-β1 when grown as sc, intratibial or pulmonary nodules [53].

The 13762 carcinoma tumors metastasize to bone after injection into the left ventricle of Fisher 344 female rats. Synthetic organic compounds of a library were selected using high-throughput screening to identify small molecules that enhance the activity of the promoter of the human OPG gene of the levels seen with TGF-β1. The most potent of these compounds was found to inhibit osteoclast formation and parathyroid hormone-induced resorption of calvaria. It was found to reduce the ability of the 13762 cells to metastasize to bone [57].

The c-SST2 rat mammary carcinoma cell line.

The c-SST-2 cells are breast cancer cells which spontaneously occurred in SHR (spontaneously hypertensive rats). Neither estrogen nor progesterone receptors are expressed in this cell line. Subcutaneous inoculation of this cell line produces high rates of metastatic lesions in lung, kidney, heart, pericardium and lymph nodes. The inoculation of c-SST-2 cells into the thoracic aorta (by catheter insertion in the left common carotid artery) induces bone metastases in SHR strain rats. Two studies have used this model to examine the relationship between the progression of bone metastases and the therapeutic effects of pamidronate using bone markers like pyridinoline and deoxypyridinoline. Pamidronate inhibited the progression of bone resorption, caused by osteoclastogenesis due to the bone metastasis. Bone markers could be useful in the follow-up of tumoral progression and treatment because they were significantly correlated with volume of the bone metastases [60, 61]. Pamidronate also prevented the graft of tumor cells inside bone and the subsequent osteolysis [62].

Xenografts in immuno-deficient animals

The establishment of animal models based on human cancer cells (xenografts) has an obvious utility in basic and preclinical research. In this way, the cytotoxic activity of many experimental treatments, evaluated in vitro, can be validated in vivo. However, human tumors can only be tolerated in immuno-compromised animals. Murine mutants with impaired T lymphocyte function, such as the athymic nude mice are commonly used. If more severe immuno-supression is required to increase the chances of engraftment, SCID mice can be used. Athymic and nude rats are also available. The anti-tumor effect of the α-particle-emitting ^{223}Ra has been evaluated in an experimental skeletal model of metastases in nude rats injected with MT-1 human breast cancer cells [63]. The human estrogen-independent breast cancer cell line MDA-MB-231 has also been successfully used in nude rats with an innovative method of inoculation. MDA-MB-231 cells were injected via a polyethylene catheter directly into the femoral artery of both hindlimbs. Following injection of tumor cells, arteries were completely ligated. Osteolytic lesions became visible on radiographs 18 days post injection with a take rate of 95%. Histology showed the predilection area for osteolytic lesions to be the distal femur and proximal tibia, close to the epiphysal growth plates. Metastases were characterized by a marked destruction of trabeculae and replacement of bone marrow by tumor cells [64]. In a refined type of this model, tumor cells were injected in the superficial epigastric artery of nude rats. Osteolytic lesions occurred exclusively in the femur, tibia and fibula with a tumor take rate of 93% [65]. The MRMT-1 rat mammary gland carcinoma cells [66], the OHS osteosarcoma cell line [67] or the CWR22 human prostate cancer cells [68] have also been used in immunodeficient rat. However, nude animals are very inconvenient to use: they are expensive, need to be kept under sterile conditions and have a short life-span. All xenograft models have also the disadvantage of lacking a physiological interaction between the host and the tumor, in part because the impaired immune system of the animals does not force the tumor to display the escape mechanisms that occur in humans. In addition, cancer cells may not respond to the factors produced by the animal microenvironment, and vice versa. Nevertheless, the histological appearance of the

human tumors is reasonably well preserved in the animals.

Advantage of rat models over mice models in bone metastasis research

Mice, like rats, develop rarely bone metastases from spontaneous breast cancer or prostate cancer. It is also easier to induce carcinomas by chemicals xenobiotics in rats than in mice. In addition, mouse mammary carcinoma is often associated with a viral etiology (the so-called Bittner virus) [69]. The rat mammary carcinoma more closely resembles human breast cancer in both etiology and biology: both are heterogeneous in their responses to estrogenic hormonal interventions while only a few murine cell lines are hormone-responsive [70]. Few syngeneic murine models of bone metastases have been described. The most known is the orthotopic or intracardiac administration of the 4T1 mammary tumor subline in syngeneic female mice (Balb/c) [71]. Murine models have advantages over rat models in that many biotechnics have been developed; however, nude or SCID mice are required when using human cancer cell lines like MDA-MB-231, MDA-MB-435, MC-F7, PC-3 or ZR-75-1 by intraarterial or intraosseous injection. It is likely that bone marrow microenvironment and adhesion molecules markedly differ from human. Furthermore, the use of immunodepressed animals differs from clinical conditions.

Mouse models do not allow studies on biomaterials due to the small size of the bones. In the last decades, several bone biomaterials have been proposed for a local delivery of active molecules (e.g. cytostatic agents or bisphosphonates can be embedded for a controlled release) [72, 73]. Orthopedic methods commonly used in tumor surgery, like the medullary nailing of a long bone after resection, cannot be applied in mice [74].

Conclusion

Animal models are indispensable to investigate the pathogenesis of bone metastases in vivo and to conduct preclinical pharmacological trials. However, differences in metabolic pathways, adhesion molecules or cytokine networks may differ from human and should always be taken into account. Rat models offer an interesting approach to the study of bone metastasis since numerous syngeneic cell lines are available. Genomic and proteomic approaches will help to identify key targets to limit bone lesions.

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