New Model for the Induction of Osteoblastic Bone Metastases in Rat

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Abstract. Background: We have investigated different models for osteoblastic lesions. Currently, there are two models using MatLyLu R-3327 prostate cancer cells: tumor cell application in the left heart ventricle and intravenous application with concomitant transient surgical clamping of the lower caval vein. Materials and Methods: Thirty male Copenhagen rats (age 9±2 months, mean weight 323±21 g) were each injected with 200,000 R-3327 prostate cancer cells. In 10 rats the left ventricle route was used (group 1), in another 10 rats the intravenous route (group 2), while in the third group of 10 rats a new model of a direct intra-osseous route was applied (group 3). Additionally, a control group of 5 rats underwent the same procedure as in group 3, but only saline without tumor cells was administered. A 99mTc-HMDP bone scan and histological examination of bone and lung were performed for follow-up. Results: In the bone scan, bone lesions could not be visualized in groups 1 and 2, but in group 3 osteoblastic lesions were observed in both femora in 9 out of 10 rats. Upon histological examination, there were lung metastases in animals from groups 1 and 2, but not in group 3. Clinical signs for bone metastases in the lumbar spine (motor disablement of the hind legs) were found in groups 1 and 2. Conclusion: The intra-osseous administration of MatLyLu R-3327 prostate cancer cells represents a useful and effective model for osteoblastic bone lesion, and allows further autoradiographic evaluation of bone uptake using bone-seeking radiopharmaceuticals.

In men, prostate adenocarcinoma is the most frequently diagnosed cancer and the second leading cause of cancer death in the US and Europe (1, 2). The incidence of bone metastases in advanced prostate cancer patients is 80 to 100% (3, 4). Common sites of bone metastases in prostate cancer derived from autopsy studies are the spine, pelvis and rib cage (5). Osseous spread in prostate cancer is often accompanied by severe bone pain and other complications, which necessitate palliative treatment.

Usually, bone metastasis is divided into osteolytic and osteoblastic lesions. From this traditional viewpoint, osteolytic metastases are believed to be caused by osteoclast activating factors induced by tumor cells in the bone microenvironment. The most important factor might be parathyroid-hormone-related peptide (PTHrP). In contrast, osteoblastic metastases are believed to be caused by the cancer cell production of factors that stimulate osteoblast proliferation, differentiation and bone formation. Here endothelin-1 and the transforming growth factor-β family might be most important. It is now realized that osteolytic and osteoblastic lesions are two extremes; morphological analysis has revealed that most cases of bone metastases have osteolytic as well as osteoblastic elements (6).

Bone metastases formation is a multifactorial process. In the first step, primary tumor cells invade the surrounding tissue by producing proteolytic enzymes. In this process, tumor cells traverse the walls of small blood vessels or of those induced by the tumor and enter the circulation (7). Most of the tumor cells do not survive the protective host-surveillance mechanism in this initial stage (8, 9). The surviving cells enter the bone marrow cavity (6) and are believed to adhere to the endothelium (10). The bone provides chemotactic factors, especially in prostate cancer, to attract tumor cells to enter the bone and become bone metastases (11). This view is further supported by the observation that bone undergoing active resorption facilitated adhesion (12). The subsequent bone destruction results from direct and also indirect factors released by tumor cells into the vascular system. Some factors result in necrosis (13), while others consist of tumor products (14), osteoclast-activating factors (15), alpha transforming growth factor (16), parathyroid hormone-like substances (17),...
tumor necrosis factor (18), and perhaps of other as yet unidentified agents (10, 19).

In the case of prostate carcinoma, it appears that the induction of osteoblastic-mediated mineralization outweighs the increase in osteoclast resorption, resulting in an overall formation of osteoblastic lesions (11). The balance between resorption and mineralization of normal bone is impaired, but the resorption by osteoclasts is not completely lost (10). Clinical evidence demonstrated increased systemic markers of bone production and resorption in prostate carcinoma patients (20, 21).

Unfortunately, the biology of skeletal metastasis is incompletely understood. In this study, animal models were investigated and further developed in an attempt to mimic human prostate carcinoma skeletal metastasis and to improve the therapeutic options, e.g. palliative treatment with bone-seeking radiopharmaceuticals. For studies to investigate the uptake of these radiopharmaceuticals, animal models with osteoblastic bone metastases are required. The red marrow radiation-absorbed dose limits the applied activity, but the actual models of calculation are based on incorrect assumptions, e.g. the percent uptake in cortical and trabecular bone (22). Also, the effect of high beta emitters, e.g. rhenium-188-HEDP, and low beta emitters, e.g. samarium-153-EDTMP, on the red marrow radiation-absorbed dose is insufficiently known. Further data are necessary to improve treatment with these pharmaceuticals: there is a paucity of good preclinical models to study osteoblastic lesions.

In the literature, two models using prostate cancer cells in Copenhagen rats have been described (23-26). Intravenous injection of neoplastic cells into the tail vein with concomitant occlusion of the inferior vena cava results in colonization of lumbar vertebrae. Using this procedure, 94% of the injected rats showed histological evidence of bone metastases in the lumbar spine and 94% of rats showed clinical sign of bone metastases (hind leg paralysis) (25). The left cardiac ventricular route leads to widespread metastasis to bone (24, 26). These models result in a combination of both lytic and blastic metastases.

Here, we compared these different models for induction of osteoblastic bone metastasis and the development of a new animal model.

Materials and Methods

Animals and tumor cells. The Dunning (R-3327) rat prostate adenocarcinoma model of prostate cancer was developed from a spontaneously occurring adenocarcinoma found in a male Copenhagen rat (27). Several sublines with varying characteristics have been developed from the primary tumor (28). The MatLyLu subline is a rapidly growing, highly metastatic, poorly-differentiated adenocarcinoma cell line, and androgen/estrogen receptor-negative (29). Copenhagen rats, originally obtained from Charles River Laboratories (Sulzfeld, Germany), were bred and housed in our animal facilities according to the institutional animal welfare regulation. In the study, 9±2-month-old male rats with a weight of 233±21 g were used. These were housed under constant humidity and temperature, with a 12-hour light and dark cycle. They were allowed free access to water and standard rat feed. All experimental procedures received approval from the Institutional Laboratory Animal Care and Use Committee of the University of Dresden, Germany, as well as from the local government authority. The animals were anesthetized with ketamine 90 mg/kg and xylazine 10 mg/kg intraperitoneally. The animals were monitored daily for signs of posterior paralysis (index of spinal cord compression due to lumbar vertebrae metastases), pain (change in posture, immobilization of extremities, limiting of movement) or local swelling (index of local metastases in extremities). Sacrifice of the animals was performed 14 to 20 days after administration of the tumor cells.

The original history and tumor characteristics of the Dunning (R-3327) prostate cancer cells have been reported in detail previously (28, 29). The establishment of the cell culture has been described by Geldof et al. (30).

Induction of skeletal metastases. We used three different procedures for the induction of skeletal metastases and injected each animal with 200,000 R3327 Mat LyLu cells in 0.2 ml Hank’s solution in groups 1 and 2, and each animal with 100,000 R3327 Mat LyLu cells in 0.1 ml Hank’s solution in both femoral shafts in group 3. There were 10 animals per group.

- Intra-cardiac route 1 (group 1), (26): The left ventricle was injected with the tumor cells percutaneously, using a 25-gauge needle attached to a syringe. Visualization of bright red blood entering the hub of the needle in a pulsatile fashion indicated the correct position of the needle in the left ventricle. Sacrifice of the animals was performed within 15 days of administration of tumor cells because of signs of change in posture and limitation of movement.
- Intravenous route (group 2), (25): Following a midline abdominal incision, the abdominal cavity was exposed, and the inferior vena cava isolated and prepared. A small surgical clamp was used to interrupt blood flow through the vena cava for one minute. Immediately thereafter, the tumor cells were injected intravenously through the tail veins. After the procedure, the abdominal cavity was closed. The surgical procedure was undertaken under aseptic conditions. Sacrifice of the animals was performed within 15 days of administration of tumor cells because of signs of change in posture and limitation of movement.
- Intra-osseous route (group 3): In accordance with experience in immunodeficiency disease mice and MDA PCa 2b tumor cells (31), the prostate tumor cells were instilled into the bone marrow of the right and left femur after a muscle-splitting incision by drilling a hole in the femoral shaft, using pediatric bone biopsy instruments. After this procedure, the prostate tumor suspensions were applied directly into the bone marrow with a syringe. Sacrifice of the animals was performed within 20 days of administration of tumor cells because of scintigraphic signs for osteoblastic bone metastases or change in posture.

- Control group: In 5 rats the same procedure as in group 3 was performed. After drilling a hole in the femoral shaft, 0.1 ml saline was instilled into the bone marrow of the right and left femur.
Figure 1. Bone scintigraphy $^{99m}$Tc-HMDP of a rat 14 days after intravenous administration of MatLyLu tumor cells (group 2): no signs of osteoblastic lesions, especially in the lumbar spine.

Figure 2. Bone lesions in the right and left femur in both sides 17 days after intra-osseous administration of MatLyLu tumor cells (group 3) in bone scintigraphy: administration of tumor cells in the bone marrow of the right and left femur by drilling a hole in the femoral shaft and following application of suspension directly into the bone marrow using a syringe.

Figure 3. Bone scintigraphy in the control group with application of saline without tumor cells and the same procedure as in group 3: only a small increase in both femoral shafts after drilling.
Figure 4. Example of an osteoblastic bone lesion in the right femur 18 days after intrasosseous administration of MatLyLu tumor cells (group 3).

Figure 5. Example of a lung metastasis 15 days after intravenous administration of MatLyLu tumor cells and clamping of the lower caval vein (group 2).
Scintigraphic evaluation of skeletal metastases. Bone scans using 99mTc-HMDP in anesthetized rats were performed daily from 10 days after application of tumor cells for evaluation of osteoblastic bone metastases in all animals. Geldof et al. (25) described histological evidence of bone metastases in 74% of rats 10 days after intravenous inoculation of tumor cells. Whole body images were obtained using a dual-headed, large field-of-view gamma camera (Genesys, ADAC Laboratories, Milpitas, CA, USA) at 3 hours after injection of 99mTc-HMDP.

Histology. Skeletal tissue with signs of osteoblastic bone metastases, visualized in a 99mTc-HMDP bone scan, were fixed in phosphate-buffered formalin (4%), decalcified in Kristensen’s solution (foric acid), and processed routinely for paraffin embedding. Sections (4 μm) were stained with hematoxylin/eosin (25). This procedure was performed in 5 rats in group 3 with lesions visible on the bone scan. In groups 1 and 2, no skeletal tissue sampling was obtained because of lack of lesions visualized in the 99mTc-HMDP bone scan. In addition, in 3 animals per group, lung tissue was sampled.

Results

No treatment-induced changes in either posture, conduct, or general condition could be observed in the rats that recovered completely from the surgery within the first 7 days. However, from day 12 in group 3 a local swelling and signs of motor disablement of the hind legs were observed. In groups 1 and 2, there were also signs of motor disablement of the hind legs from the tenth days after administration, but not complete paralysis. Three animals died within 14 days after the surgical procedure and were not evaluated.

Scintigraphic results. In groups 1 and 2, no bone scan abnormalities were visualized in the 99mTc-HMDP bone scan within 15 days of administration (Figure 1). A longer observation period was not sustainable because of the poor general condition of the rats and ethical aspects, and so no statements can be made about bone lesions at a later time. The poor general conditions were probably caused by pulmonary metastases. In group 3, we found lesions in the femora on both sides within 20 days of administration in 9 out of 10 rats (Figure 2). The rats in the control group showed only a slightly higher bone uptake of 99mTc-HMDP in the femoral shafts in comparison to the normal bone (Figure 3).

Histological finding. In all 5 rats with skeletal tissue sampling in group 3, osteoblastic bone metastases were confirmed in the histological investigations, in the same location as indicated in the bone scan (Figure 4). Skeletal tissue sampling in groups 1 and 2 was not performed, because of lack of evidence of bone metastases in the bone scan. In the histology of the lung, metastases were found in all examined samples in groups 1 and 2 (Figure 5), but not in group 3.

Discussion

Only localized prostate cancer can be effectively cured by surgery or radiotherapy, with 10-year survival rates of approximately 90% (32). Advanced prostate cancer will become hormone-refractory and, after developing metastatic disease to the bone, patients have a median survival of 3 years. Metastatic bone disease caused by advanced prostate cancer is an important clinical problem as it often leads to severe pain and causes secondary complications, such as pathologic fractures or spinal cord compression, requiring additional interventional treatment.

In recent years, metabolic bone disease associated with metastatic prostate cancer has been better understood (33). Metastases secondary to prostate cancer are mixed lesions with a predominant osteoblastic component, but increased osteoclast-mediated osteolysis (34-36). In addition to direct metastatic bone destruction, patients with prostate cancer seem to have pre-existent low bone mineral density, although the reasons for this are poorly understood (37). Androgen-deprivation therapy, which is highly effective and the main treatment modality for hormone-therapy-naïve patients with advanced disease, can lead to severe additional reductions in bone mineral density (38, 39).

Pain and secondary complications of bone metastases require treatment by androgen deprivation and in hormone-refractory prostate cancer by bisphosphonates, radiotherapy, bone-seeking radiopharmaceuticals and/or analgesic medication (40). Although palliative in nature, treatment of metastatic bone disease in prostate cancer can effectively improve the quality of life and prevent secondary complications. With the high prevalence of prostate cancer, the treatment of metastatic disease poses a significant burden on the medical system.

However, many aspects of the treatment of prostate cancer metastatic to the bone are poorly understood and improvements in treatment efficacy and durability are highly desirable. An animal model, which would allow the reproduction of metastatic bone disease comparable to human prostate cancer bone disease, would enable the effective study of several treatment and combination treatment modalities, such as the use of bone-seeking radionuclides. Therefore, such an animal model would be a significant step towards improving treatment of hormone-refractory metastatic prostate cancer.

In this study, we investigated a new model for osteoblastic bone metastases. This model is necessary for further autoradiographic investigation to evaluate the uptake of bone-seeking radiopharmaceuticals in normal skeleton and bone metastases. For a good quality of autoradiography, we need osteoblastic lesions with a high uptake of radioactive diphosphonates. Unfortunately, although there are a lot of animal models for osteolytic bone metastases, there is a paucity of good models to study osteoblastic lesions (41). The PA-III cell line derived from a spontaneous prostate
adenocarcinoma in a Lobund Wistar rat induced a mixed osteolytic and osteoblastic bone lesion when transplanted adjacent to bone (42). Other models used LNCaP and ARCaP, androgen-dependent and androgen-repressed human prostate carcinoma cell lines, in athymic mice and showed osteoblastic reaction when tumor cells were harbored in the skeleton (43, 44). Yang et al. (31) found osteoblastic reaction with MDA PCA2 and MDA PCA2b prostate cancer cells in immunodeficient mice using intra-bone application.

For MatLyLu R-3327 prostate cancer cells, there are two models in Copenhagen rats: intravenous application with concomitant transient surgical clamping of the lower caval vein (25) and left ventricle administration (26).

Using the left ventricle route (26), skeletal metastases appear in long bones and could be visualized by radiography as osteolytic lesions. Upon histological examination of the vertebrae, more osteolytic bone metastases are revealed. The Geldof model (25) using the intravenous application presented bone lesion especially in the lumber spine after 10 days, with osteoblastic and osteolytic activities. In further autoradiographic examinations using rhenium-188-HEDP, an incorporation of radioactivity mainly in trabecular bone was observed (45).

In our study, we could not detect bone lesions with a high uptake of $^{99m}$Tc-HMDP after application of MatLyLu R-3327 prostate cancer in group 1 (the left ventricle route) or group 2 (clamping of the lower caval vein and intravenous application). These two models are insufficient for further autoradiographic studies with rhenium-188-HEDP to study the uptake in bone metastases. We, therefore, introduced a new model with intraluminal tumor cell application, in the style of mouse studies (31). In 9 out of 10 rats, osteoblastic lesions were shown with a high uptake in the $^{99m}$Tc-HMDP bone scan after this procedure. In contrast, in all 3 rats with lung metastases seen in groups 1 and 2, histological preparation of the lung confirmed the metastases, but none were demonstrated in group 3. In the control group, there was only slightly less increase of bone uptake in the femoral shaft compared with the uptake in group 3. The increase of $^{99m}$Tc-HMDP bone uptake was not simply caused by the procedure of drilling a hole in the femoral shaft.

The standard gold for detection of bone metastases is the X-ray examination, especially for lytic metastases. However, bone metastases which could not be seen on the $^{99m}$Tc-HMDP bone scan were unsuitable for further studies of uptake of bone-seeking radiopharmaceuticals and so X-rays were not performed.

In groups 1 and 2, there were signs of bone lesions in the lumbar spine, illustrated by motor disableness of the hind legs, but these were not visualized on the $^{99m}$Tc-HMDP bone scan. This could be explained by the existence of more osteoblastic bone metastases. Alternatively, the intra-osseous tumor cell inoculation route permits for a longer observation time (20 days) compared to the other routes. This may allow an osteoblastic response to mount and become detectable.

An animal model, such as ours, which reproducibly establishes PSA-producing bone metastases of prostate cancer, would also be interesting for studies looking at other palliative treatment strategies for advanced prostate cancer, such as combination chemotherapy or bisphosphonate treatment. Reliable PSA production in such a model enables highly sensitive monitoring of treatment effects comparable to clinical practice in humans. Thus, the model described here might have numerous, potentially extremely interesting experimental applications.

**Conclusion**

The intra-osseous administration of MatLyLu R-3327 prostate cancer cells represents a useful and effective model for the induction of osteoblastic bone lesions, and allows further autoradiographic evaluation of bone metastasis uptake using bone-seeking radiopharmaceuticals.

**References**

Liepe et al: Osteoblastic Bone Metastases


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