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ARTICLE

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The effect of an osteolytic tumor on the three-dimensional trabecular bone morphology in an animal model

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Abstract *Objective*. To investigate the application of micro-computed tomography (µCT) for the assessment of density differences and deterioration of three-dimensional architecture of trabecular bone in an experimental rat model for tumor-induced osteolytic defects.
Design and materials. Walker carcinosarcoma 256 malignant breast cancer cells (W256) were surgically implanted into the medullary canal of the left femur of 15 4-month-old rats. Twenty-eight days after surgery all animals were killed and both femora from each rat were harvested. A total of 30 specimens (left and right femur) were scanned in a desktop µCT imaging system (µCT 20, Scanco Medical) to assess densitometric and architectural parameters. For each specimen a total of 200 micro-tomographic slices with a resolution of 30 µm in the distal metaphysis was taken. Bone mineral content (BMC) was analyzed for both cortical and trabecular bone (ctBMC), and for trabecular bone only (tBMC). Architectural indices (BV/TV, Tb.N, Tb.Th, Tb.Sp) according to standard definitions used in histomorphometry were calculated for trabecular bone.
Results. The quantitative analysis of density parameters revealed significantly (P<0.001) lower values for ctBMC and tBMC in the tumor-bearing group (T) of 26% and 31%, respectively, compared with the contralateral control group. The quantitative analysis revealed significant (P<0.001) changes in the architectural parameters in the tumor-bearing bones compared with the contralateral control group: BV/TV was 30% lower, Tb.N and BS/TV decreased by 24% and 21%, respectively, Tb.Th. decreased by 10% and Tb.Sp. increased by 94%.
Conclusions. This study demonstrates that µCT is able to provide three-dimensional parameters of bone mass and trabecular structure in an animal model for tumor-induced bone loss. Recent advances in therapeutic approaches for skeletal diseases such as osteoporosis and metastatic bone disease rely on an understanding of the effects of the agents on the mechanical properties of bone. In order to quantify the structural changes of the affected bones the application of a non-destructive method is mandatory. The use of µCT seems to be a great advantage, since biomechanical tests and further histologic analysis can be done for the same specimens.

Keywords µCT · Trabecular bone structure · Bone density · Tumor osteolysis · Animal model

Introduction

Non-osseous primary cancer spreading to bone is an important cause of morbidity, which is often associated with local osteolysis and the subsequent development of pain, hypercalcemia and pathologic fractures [1, 2, 3]. Bone metastases mediate an increase in bone resorption which results in rapid loss of bone mass and alteration of the architectural integrity, and subsequently the load-bearing capacity, of the affected bone [1, 4]. Most research efforts have been directed toward understanding the biology and pathogenesis of metastases, from the patterns of invasion to the changes in bone metabolism. As with most structures, the strength of normal and pathologically altered bone is a function of geometric variables, material properties and functional demands [5]. Since bone strength and fracture risk are considered to be closely related to bone mineral density [6], bone mineral density has been studied extensively in experimental and clinical studies in osteoporosis [7]. However, there is evidence that it is not the only variable for trabecular bone strength and that the three-dimensional (3D) microstructure of trabecular bone is another key factor influencing the mechanical behavior of cancellous bone [8, 9]. Traditionally, the quantification of structural parameters in vitro, such as trabecular thickness and trabecular separation, was based on histomorphometry of two-dimensional (2D) bone sections [10, 11]. Nevertheless, 2D sections are limited in describing cancellous bone architecture in normal and pathologic bone [12].

Non-invasive methods of bone densitometry have been widely used to quantify changes in bone mineral content and bone mineral density in experimental animal studies mimicking metabolic bone disease [4, 13, 14, 15]. Most recently new imaging techniques for the determination of the alteration of the 3D architecture of trabecular bone in osteoporosis have been introduced [16, 17]. As with osteoporosis, tumor-induced osteolysis is characterized by reduced bone mass and a deterioration in bone structure which results in an increased fracture risk [12]. Hence, for tumor-associated osteolytic bone lesions, there is a similar need to quantify the bone loss by densitometry and 3D structure analysis. Micro-computed tomography (μCT) is a new and emerging technique for the non-destructive assessment and analysis of 3D bone density and architecture. Feldkamp et al. [18] established an X-ray-based micro-tomography system to create a 3D object with a resolution of 50 μm. While the early implementations of 3D micro-tomography focused on the technical and methodologic aspects of these systems more recent studies have emphasized the practical aspects of this technology [19]. Several studies using human bone biopsies have been published, including the recent demonstration of excellent correlation between 3D μCT morphometric parameters and standard 2D histomorphometry [20].

In the present study, we investigated the application of μCT for the assessment of density differences and deterioration of 3D architecture of trabecular bone in an experimental rat model for tumor-induced osteolytic defects. For this purpose we asked (1) whether the presence of an osteolytic tumor has an effect on the micro-structure of trabecular bone and (2) whether the negative effect on bone mass can be quantified by μCT in a small animal model.

Methods and materials

Animal model and surgical protocol

We employed a previously described animal model for the evaluation of densitometric and biomechanical properties in tumor osteolysis [4]. Fifteen 4-month-old virgin Sprague-Dawley rats were obtained from Taconic Co. Laboratories (Germantown, NY, USA). All animals were maintained in accordance with federal regulations and the study was conducted with approval of the Institutional Animal Care and Use Committee. The widely used Walker carcinosarcoma 256 malignant breast cancer cells (W256) were cultured at a concentration of 2×10^6 in 0.6% agarose gel solution. After anesthesia by an intraperitoneal injection of ketamine (75 mg/kg) and xylazine (5 mg/kg) the tumor cells in agarose gel were surgically implanted into the medullary canal of the left femur via a drill hole in the intercondylar notch. The drill hole was sealed with bone wax after tumor implantation, the patella was then relocated and the extensor mechanism was reconstructed. The soft tissues and the skin were closed with 4.0 vicryl sutures. The limb was checked for normal post-operative motion of the knee joint. The rats were allowed unrestricted ambulating in their cages after recovery and were observed daily for activity and weight-bearing of the operated limb.

There were no complications with the surgical procedure. Twenty-eight days after surgery all animals were killed and both femora from each rat were harvested and cleaned of soft tissue for image analysis.

Before scanning, all femora were radiographed in an anterior/posterior plane using a Faxitron machine (Hewlett-Packard, McMinnville).

Image processing

For the first time, we employed a desk-top μCT imaging system (μCT 20, Scanco Medical, Zurich, Switzerland) to assess densitometric and architectural parameters in small animal whole bones. The details of the μCT system have been previously described [19, 20]. The system is equipped with a microfocal X-ray tube providing a 10 μm focal spot. The source produces a fan beam that is detected by a charge coupled device (CCD) array with 1024 elements. The specimen is loaded on a turntable that can be shifted automatically in the axial direction. After a data acquisition time set at 200 ms the turntable with the specimen is rotated by 1° and a new data acquisition process is performed. After the entire plan has been covered the specimen is moved upward by 30 μm and a new series of data acquisition begins. A standard convolution backprojection procedure with a Shepp and Logan filter is used to reconstruct the CT images in 1024×1024 pixel matrices. The spatial resolution is typically defined by the 10% contrast level in the modulation transfer function, resulting in a spatial resolution of 28 μm in plane [19].

A total of 30 specimens (left and right femur) were scanned. For each specimen a total of 200 micro-tomographic slices with a

resolution of 30 µm was taken starting from a reproducible anatomic landmark immediately behind the boundary of the condyles covering a region of 6 mm of the distal femur. From the 2D slices obtained a 3D reconstruction was automatically performed by using a triangulation algorithm.

Two regions were considered for the quantitative analysis. Bone mineral content (BMC) based on the measured bone volume (BV) and the assumption of a constant tissue density was analyzed for a volume of interest (VOI) in the metaphysis consisting of both cortical and trabecular bone (ctBMC), and for a sphere consisting of trabecular bone only (tBMC). Architectural indices were calculated for the spherical volume of interest only according to standard definitions used in histomorphometry [11]. Bone volume density (BV/TV) and trabecular plate number (Tb.N) were determined directly from the binarized 3D images [19]. From these primary indices, bone surface density (BS/TV), trabecular thickness (Tb.Th) and trabecular separation (Tb.Sp) were derived using a parallel plate model. All 30 specimens were analyzed using both a visual assessment and quantitative morphometry. A Wilks-Shapiro test for normality was conducted on all parameters. A paired *t*-test was used to test the null hypothesis that the tumor-bearing bones have a lower density and that differences in morphometric parameters as compared with the contralateral controls can be found.

Results

We found in all tumor-bearing animals soft tissue tumors around the affected bone which indicated the viability of the implanted tumor cells. Radiologic examination revealed that all tumor-bearing bones had evidence of intramedullary lesions which extended from the distal metaphysis to the mid-diaphyseal area. We found a severe compromise of metaphyseal bone but no evidence of gross pathologic fractures. The radiographs of the contralateral controls showed no apparent evidence of bone compromise caused by spread of the tumor cells.

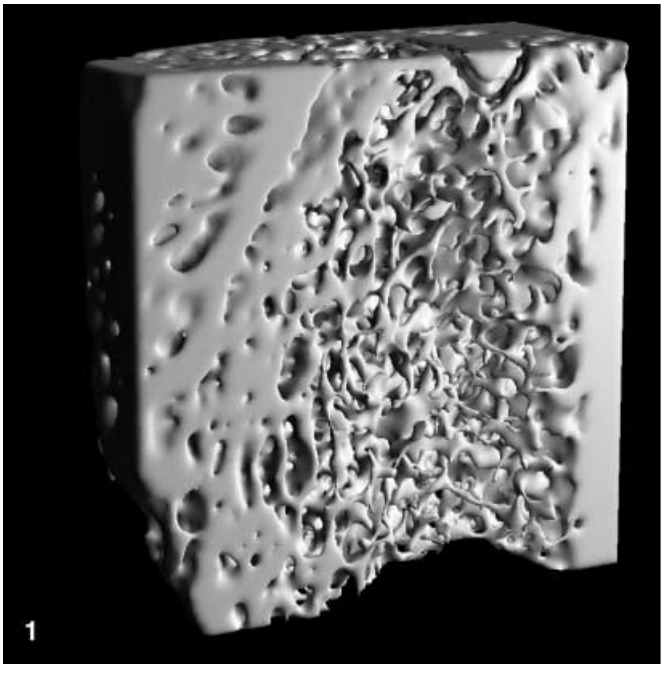
Descriptive results for all densitometric and morphometric properties are given in Table 1. Representative three-dimensionally reconstructed µCT images of the distal femur (left and right) are shown in Fig. 1 and Fig. 2. The loss of trabecular bone and deterioration of the trabecular architecture following the implantation and growth of tumor are clearly visualized. The quantitative analysis of density parameters by µCT revealed significantly ($P<0.001$) lower values for ctBMC and tBMC in the tumor-bearing group (T) of 26% and 31%, respec-

Table 1 Summary of data, as mean (SD), for the tumor-bearing and control groups (*ctBMC* bone mineral content for cortical and trabecular bone, *tBMC* bone mineral content for trabecular bone, *BV/TV* bone volume density, *Tb.N* trabecular plate number, *BS/TV* bone surface density, *Tb.Th* trabecular thickness, *Tb.Sp* trabecular separation)

	Tumor group (n=15)	Control group (n=15)
ctBMC (mg)	52.2 (12.1)	70.2 (9.1)
tBMC (mg)	24.6 (7.9)	35.4 (5.8)
BV/TV (%)	33.78 (11.30)	48.23 (5.95)
BS/TV (mm^2/mm^3)	6.66 (1.72)	8.41 (0.643)
Tb.N (1/mm)	2.79 (1.03)	3.69 (0.51)
Tb.Th (mm)	0.09 (0.01)	0.10 (0.02)
Tb.Sp (mm)	0.33 (0.03)	0.17 (0.05)

Fig. 1 Three-dimensional micro-tomographic (µCT) visualization of a representative normal distal femur (6 mm) of a rat (control group)

Fig. 2 Three-dimensional micro-tomographic (µCT) visualization of a tumor-bearing distal femur (6 mm) of a rat (tumor group). The loss of trabecular bone and deterioration of the trabecular architecture following the implantation and growth of tumor are apparent



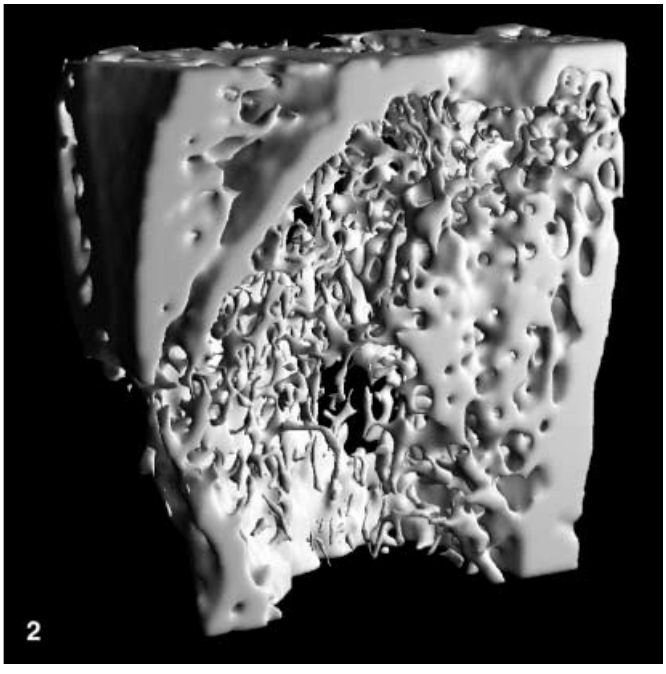


Table 2 Group differences, in percent, for all densitometric and morphometric parameters ($P<0.001$)

	Tumor versus control
ctBMC	–26%
tBMC	–31%
BV/TV	–30%
BS/TV	–21%
Tb.N	–24%
Tb.Th	–10%
Tb.Sp	+94%

tively, compared with the contralateral control group. The quantitative analysis in the trabecular sphere revealed significant ($P<0.001$) changes of the architectural parameters in the tumor-bearing bones compared with the contralateral control group: BV/TV was 30% lower, Tb.N and BS/TV decreased by 24% and 21%, respectively, Tb.Th. decreased by 10% and Tb.Sp. increased by 94%. (Table 2).

Discussion

This study has demonstrated that the µCT is able to provide 3D parameters of bone mass and trabecular structure in an animal model for tumor-induced bone loss. This new technology offers a non-destructive approach to the study trabecular bone architecture in preclinical animal models of metabolic and neoplastic bone disease. We found significant differences in 3D morphometric parameters between the tumor-bearing bones and the contralateral controls. A decrease in trabecular number, bone volume and trabecular thickness by 24%, 30% and 10%, respectively, as well as an increase in trabecular separation by 94% demonstrates clearly the destruction of the normal bone structure by the tumor-induced process. An interesting finding is the small effect on trabecular thickness of the remaining trabeculae in the tumor-bearing bones compared with the control bones and the large increase in trabecular separation. The biologic effect of the tumor on bone seems to be an elimination of entire bone elements and not thinning of the trabeculae.

The destruction of bone architecture can best be seen in additional 3D visualizations as illustrated in Fig. 1 and Fig. 2.

In an osteoporotic animal model, a 3D X-ray tomographic microscope was used to image the trabecular bone architecture in the proximal tibia of rats. The ovariectomized rats lost approximately 50% of their trabecular bone volume over a period of 50 days after ovariectomy. The connectivity and the number of trabeculae was significantly reduced, but no thinning of trabeculae was found [16]. In another study using µCT, investigating the first lumbar vertebra of rats that had undergone ovariectomy, a difference of 31% for BV/TV at 3 months post-ovariectomy and 36% at 19 months post-ovariectomy was found. The trabecular number was decreased by 14% and 4% at 3 and 19 months post-ovariectomy, respectively. For trabecular thickness decreases of 19% and 34%, respectively, were found [21]. The authors stated that their results are in accordance with those found by several authors using conventional histomorphometry in the same animal model [21]. A third study using µCT technology in an ovariectomy model revealed a decrease of 62% in bone volume, 46% in trabecular number, 35% in trabecular thickness and an increase of 131% in trabecular separation compared with the sham ovariectomy group [17]. The discrepancy between the different studies using 3D analysis in osteoporotic animals might be due to different study designs and endpoints. The results from the present study are similar to the findings of Kinney et al. [16] and Kapadia et al. [17], with a remarkable decrease in bone volume and trabecular number and an increase in trabecular separation, but less change in trabecular thickness. Since similar pathophysiologic processes in osteoporosis and tumor osteolysis lead to an increased in osteoclast-mediated bone resorption, a similar pattern of alteration of trabecular structure can be expected. Only the time course seems to be different.

Additionally, the generally known loss of bone mass [4, 22] in tumor osteolysis was confirmed by the µCT densitometric analysis. Osteoclast-mediated bone resorption resulted in a decrease in bone mass of 36% for the volume of interest containing cortical and trabecular bone and 45% for trabecular bone alone. In a previous study using dual-energy X-ray absorptiometry (DXA) and peripheral quantitative computed tomography (pQCT) [4], we found a decrease in BMD and BMC (DXA) of 9% and 12%, respectively, compared to the contralateral non-tumor-bearing control bones [4]. The average bone density (pQCT) for the tumor-bearing bones was 18% lower than for the contralateral controls. The differences between the two experimental groups were much larger for µCT in this study than for the DXA- and pQCT-generated parameters in a previous study. We attribute this effect mostly to the superior resolution and positioning capabilities of µCT.

This paper describes the application of µCT technology to research in tumor-induced bone diseases. The evaluation of bone architecture in pathologically altered bone is of great importance for the understanding of the biomechanical behavior of bone [23]. Furthermore, it is of great clinical importance to understand that tumor in bone affects the bone structure. The aim of therapeutic approaches to tumor osteolysis is to restore the structure of bone and not only to increase bone mass.

To our knowledge this is the first time that µCT technology has been applied to an animal model for tumor-induced osteolysis. In preclinical research numerous animal studies are necessary for the development of new

therapeutic agents for metabolic bone diseases. The evaluation of the effect of the new agents on bone biomechanics and bone structure is of special interest. Recent advances in therapeutic approaches for skeletal diseases such as osteoporosis and metastatic bone disease rely on an understanding of the effects of the agents on the mechanical properties of bone [4]. In order to quantify the structural changes in the affected bones [13, 15, 24] application of a non-destructive method is mandatory [14]. The use of μCT seems to be a great advantage, since biomechanical tests and further histologic analysis can be done on the same specimens [23].

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