Bone 97 (2017) 83–93

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Full Length Article

Disruption of collagen/apatite alignment impairs bone mechanical function in osteoblastic metastasis induced by prostate cancer

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ARTICLE INFO

Article history:
Received 24 September 2016
Revised 5 January 2017
Accepted 5 January 2017
Available online 06 January 2017

Keywords:
Cancer bone metastasis
Collagen fibrils
Biological apatite
Osteocytes
Preferential alignment
Bone mechanical function

ABSTRACT

Prostate cancer (PCa) frequently metastasizes to the bone, generally inducing osteoblastic alterations that increase bone brittleness. Although there is growing interest in the management of the physical capability of patients with bone metastasis, the mechanism underlying the impairment of bone mechanical function remains unclear. The alignment of both collagen fibrils and biological apatite (BAp) *c*-axis, together with bone mineral density, is one of the strongest contributors to bone mechanical function. In this study, we analyzed the bone microstructure of the mouse femurs with and without PCa cell inoculation. Histological assessment revealed that the bone-forming pattern in the PCa-bearing bone was non-directional, resulting in a spongious structure, whereas that in the control bone was unidirectional and layer-by-layer, resulting in a compact lamellar structure. The degree of preferential alignment of collagen fibrils and BAp, which was evaluated by quantitative polarized microscopy and microbeam X-ray diffraction, respectively, were significantly lower in the PCa-bearing bone than in the control bone. Material parameters including Young's modulus and toughness, measured by the three-point bending test, were simultaneously decreased in the PCa-bearing bone. Specifically, there was a significant positive correlation between the degree of BAp *c*-axis orientation and Young's modulus. In conclusion, the impairment of mechanical function in the PCa-bearing bone is attributable to disruption of the anisotropic microstructure of bone in multiple phases. This is the first report demonstrating that cancer bone metastasis induces disruption of the collagen/BAp alignment in long bones, thereby impairing their mechanical function.

1. Introduction

Prostate cancer (PCa) is a common cancer with high morbidity among men worldwide [1]. It preferentially metastasizes to bone [2]. Although PCa sometimes accompanies osteolysis, the overall character of PCa appears to be osteoblastic with increased bone volume (BV) and clinical evidence of excessive mineral deposition [2,3]. Patients with PCa bone metastasis suffer from bone pain and are at increased risk of pathological fracture due to the deterioration of bone mechanical function [2]. Because these skeletal complications inevitably affect patient survival and quality of life, preventing the impairment of bone mechanical function is important for cancer management. However, the mechanisms through which metastatic bone loses its mechanical function are not well understood and no common treatment for reducing bone fracture risk in patients with osteoblastic metastasis is available.

Bone is a hierarchical composite composed mainly of collagen fibrils and biological apatite (BAp), with an architectural scale ranging from the nanometer (BAp crystallite) to the millimeter (trabeculae) [4]. It is widely accepted that the risk of bone fracture depends on both bone quantity and bone quality [5]. Bone quantity is clinically determined based on bone mineral density (BMD) or BV, both of which are measured by dual-energy X-ray absorptiometry (DXA) or computed tomography (CT). Bone quality includes a wide range of factors from geometry to composition. Geometric factors, such as density or size of osteon, a major remodeling unit in cortical bone, are associated with bone fracture toughness [6]. With respect to compositional factors, some studies revealed that the mineral-to-collagen ratio and mineral crystallinity are associated with bone modulus and strength [7,8], whereas collagen content, maturity, and collagen cross-linking are related to bone toughness or post-yield properties [9–11].

Moreover, the importance of the microstructure created by BAp and collagen in the mechanical properties of bone has been clarified [12–17]. Indeed, both BAp and collagen fibrils show strong longitudinal alignment in long bones under biological conditions [12,14]. BAp crystallization is strongly dependent on collagen structure: BAp crystallites nucleate either within or between fibrils of collagen and subsequently grow parallel to the fibrils. For this reason, BAp and collagen orientations are closely related [18,19]. Nakano et al. demonstrated that the degree of preferential alignment of BAp varies according to the site of

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http://dx.doi.org/10.1016/j.bone.2017.01.004

bone, suggesting that this index reflects the stress distribution or mechanical function of bone under biological conditions [12,13]. Furthermore, the degree of preferential alignment of BAp is strongly correlated to bone Young's modulus in regenerated bone [15], whereas collagen orientation is associated with bone fracture toughness [16,17]. It was also reported that the loss of bone mechanical function is attributable to the alteration in matrix alignment in some pathologies such as osteoporosis [20] and chronic kidney disease [21], both of which are accompanied by osteopenia. Therefore, BAp and collagen alignment strongly contributes to bone mechanical function in both healthy and osteopenic bone.

However, the effects of osteoblastic cancer metastasis on BAp/collagen orientation and its relationship with bone mechanical function have not been examined. In this study, we investigated how the preferential alignment of collagen and BAp is altered by osteoblastic bone metastasis and how this alteration influences bone mechanical function in a mouse model. Quantitative polarized microscopy and microbeam X-ray diffraction were applied to evaluate the degree of orientation of collagen fibrils and BAp, respectively, whereas a three-point bending test was conducted to assess the mechanical properties of whole bone both with and without prostate cancer metastasis.

Additionally, we investigated the alignment of osteocytes, skeletal cells embedded in the bone matrix. Osteocytes show longitudinal alignment synchronously with matrix alignment and are thought to be closely related to bone quality [22]. Because osteocytes have been shown to play a role in bone metastasis [23], insights into the structural features of osteocytes and their contribution to the mechanical properties of bone will make it possible to use the osteocyte as a target for assessing bone metastasis.

2. Materials and methods

2.1. Cell culture

The androgen-independent human metastatic prostate cancer cell line MDA PCa 2b (PCa-2b) [24] was obtained from ATCC (Manassas, VA) and maintained in Ham's F-12K (Wako Pure Chemical, Osaka, Japan) supplemented with 20% fetal bovine serum (Gibco, Waltham, MA), 25 ng/mL cholera toxin, 10 ng/mL mouse epidermal growth factor, 5 μM phosphoethanolamine, 100 pg/mL hydrocortisone, 45 nM selenious acid, and 5 μg/mL bovine insulin, at 37 °C in 5% CO_2. Growth factors were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Intrabone injections

Five-week old male severe combined immunodeficiency disease mice (n = 9) were obtained from Charles River Laboratories (Wilmington, MA), and housed in a facility with constant humidity and temperature and a 12-h light-dark cycle. Mice had *ad libitum* access to standard mouse feed and water. All animal experimentation was approved by the Animal Care and Use Committee of Osaka University. PCa cell inoculation was conducted on seven mice as previously described [25] with few modifications. Briefly, aliquots of 1.0×10^6 of PCa-2b cells were diluted in 5 μL PBS and then injected into the right femurs of each mouse using a Hamilton syringe. The contralateral femurs were injected with PBS and used as internal controls. Two mice were kept intact to be used as the external control. Mice were then monitored twice weekly for tumor bulk and sacrificed at six months post-operation by overdose administration of sodium pentobarbital. The femurs were excised from five mice immediately after sacrifice without fixation and subjected to micro-CT analysis. Two cancer-bearing mice as well as two external-control mice were perfused with 4% paraformaldehyde through the cardiac left ventricle, and the femurs were immersed in 4% paraformaldehyde at 4 °C for 7 days for histochemical analysis.

2.3. Histology

Two cancer-bearing mice were used for histological assessment; one mouse was used for the detection of both homing of cancer cells and osteoclastic activity, and the other mouse was used for the detection of the osteoid. In each histochemical assessment, the cancer-inoculated femur was compared with the contralateral femur of the same mouse.

A pair of femurs was processed to prepare conventional decalcified paraffin sections (4 μm-thick) and was stained for prostate specific antigen (PSA) and tartrate-resistant acid phosphatase (TRAP) to confirm homing of the cancer cells to the inoculated femur and to evaluate the osteoclastic activity, respectively. For the detection of PSA, antigen retrieval was performed using 10 mM citrate (pH 6.0) at 25 °C for 2 h on the deparaffinized sections followed by the blocking of endogenous peroxidase using 3% hydrogen peroxide in methanol. The samples were incubated in 1% (w/v) bovine serum albumin for 1 h to block nonspecific binding and then incubated with an anti-PSA antibody (Abcam, ab140337, 1:100) at 4 °C overnight. The samples were incubated with a peroxidase-conjugated secondary antibody according to the manufacturer's instructions (Histofine Mouse Stain Kit, Nichirei Bioscience). The reaction was visualized with 3,3-diaminobenzidine tetrahydrochloride. Counterstaining was performed using methyl green. TRAP staining was carried out with the TRAP Stain Kit (Wako).

A pair of femurs was treated with cyanunic chloride solution for osteoid staining as described by Yoshiki et al. [26]. Briefly, tissues were dehydrated through an ascending series of ethanol and immersed in 0.5% (w/v) cyanunic chloride (Wako) in anhydrous methanol containing 1% *N*-methylmorpholine (Wako) for two days at 25 °C. After decalcification with a 0.5 M EDTA-2Na solution (pH 7.4) for 7 days at 4 °C, specimens were dehydrated through a graded series of ethanol, embedded into paraffin, and then cut into 4-μm-thick sections. Deparaffinized sections were stained with Mayer's hematoxylin (Wako) and 1% aqueous eosin Y (Sigma-Aldrich). In this method, the osteoid matrix is strongly stained with eosin and distinguishable from poorly stained mineralized matrix [26].

2.4. Micro-CT analysis

Unfixed femurs were scanned using micro-CT (Shimadzu, Kyoto, Japan) at X-ray energy settings of 80 kV and 36 μA, with a 1.0 mm aluminum filter and a nominal resolution of 10 μm. Bone was morphometrically analyzed using TRI/3D-bon imaging software (Ratoc, Tokyo, Japan) to obtain BV and bone volume fraction. Femurs were wetted with saline solution during scanning, readily frozen in saline-soaked gauze after the scan and stored at −80 °C until use in DXA, X-ray diffraction and a three-point bending test. Based on micro-CT analysis, a reference point for the following assessments was chosen as the region where osteogenesis most readily occurred in the PCa-bearing femur. The corresponding region was always used for the analysis of the control femur.

Immediately before a series of assessments including DXA, X-ray diffraction and a three-point bending test mentioned below, the femurs were thawed and transaxially cut into 10-mm-long cylinders using a diamond saw (South Bay Technology, San Clemente, CA) to designate the 6-mm-long cylinders as the reference regions with 2-mm extras at both ends for each.

2.5. DXA analysis

The bone mineral content (BMC) and BMD of the femurs were measured by DXA using a densitometer equipped with the dedicated analysis software (DCS-600-EX-IIIR, Hitachi Aloka Medical, Tokyo, Japan). Dual-energy X-rays were generated with tube voltage of 35/65 keV and tube current of 0.8 mA. Femurs were transaxially scanned from the anterior side toward the posterior side with 1-mm pitch. The reference regions of the 6-mm-long cylinders were analyzed for the