Establishment of a Novel Species- and Tissue-specific Metastasis Model of Human Prostate Cancer in Humanized Non-Obese Diabetic/Severe Combined Immunodeficient Mice Engrafted with Human Adult Lung and Bone

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ABSTRACT

Bone is the most common site of metastasis in prostate cancer (PC), and to generate an animal model to investigate the basis of the unique organ tropism of PC cells for bone, we engrafted humanized non-obese diabetic/severe combined immunodeficient (NOD/SCID-hu) mice with human adult bone (HAB) and lung (HAL). Human PC cell lines LNCaP (1 × 10^6) and PC-3 (5 × 10^5) were injected into male NOD/SCID-hu mice via the lateral tail vein at 3–4 weeks after implantation. At 8 weeks after the injection, LNCaP and PC-3 cells had metastasized specifically to HAB in 35 and 65%, respectively, of the mice. The tumors formed by LNCaP appeared to be the osteoblastic type, whereas the PC-3 tumors consisted of osteolytic lesions without any surrounding osteogenic response. A feature of experimental metastasis of PC in NOD/SCID-hu mice was its specificity for HAB tissue. Human PC cells had no or very low metastatic potential in regard to implanted HAL, mouse bone, or native mouse bone. These findings indicate that metastasis of PC cells to HAB is both species and tissue specific. The availability of this small animal model could provide a useful tool for identifying and analyzing important features of the human PC metastatic process that cannot be addressed in conventional metastasis models.

INTRODUCTION

Bone has long been recognized as the most common target organ of PC metabolism (1). More than 80% of PC patients develop bone metastases, predominantly in the spine, and are generally associated with a poor prognosis (2). The basis of the unique organ tropism of PC cells for bone is unclear, in part, because of the lack of good animal models. The several models that have been reported either lack the histological features of clinical PC and/or have a low incidence of metastasis to bone. Apparently, some human tumors require a specific human tissue microenvironment to reproduce their clinical growth and invasion patterns in experimental in vivo models (3–5). In all xenograft models described to date (6–13), transplanted human tumor cells have needed to metastasize to and grow in mouse organs. We hypothesized that the lack of metastasis or lack of tissue-specific metastasis of malignant human cells in immunodeficient rodents is, at least in part, attributable to the mechanisms governing the establishment of tissue-specific metastases also being species specific. The ideal in vivo model for studying human cancer should allow interaction between tumor cells and a human organ environment (14). Previous reports have described the use of the SCID-hu system (15–17) to study the behavior of metastatic human tumor cells; however, this model involves surgical transplantation of human fetal organs into SCID mice.

A new mouse strain (NOD/SCID) was developed recently by crossing SCID mice with NOD mice (18). In NOD/SCID mice, the DNA repair gene defect of SCID mice that severely impairs B- and T-cell development is combined with the reduced natural killer cell activity, absence of complement activity, and defect in macrophage function of NOD mice. The unique feature of this model is that the human cells proliferate, differentiate, and function in implanted human tissues that maintain their normal anatomical architecture. To create a better model of human PC metastasis, we engrafted humanized NOD/SCID mice with HAB and HAL.

In this study, we show that human PC cells introduced into NOD/SCID-hu mice preferentially metastasize to the engrafted HAB and not to implanted HAL or implanted or native mouse bone. This paper reports the first model system for studying bone metastasis by human PC cell lines in HAB.

MATERIALS AND METHODS

Animals. Male NOD/SCID mice, 5 weeks of age, were purchased from CLEA Japan, Inc. (Tokyo, Japan) and maintained at the National Cancer Center Research Institute East (Chiba, Japan) under specific-pathogen-free, temperature-controlled-air conditions throughout this study, according to the Institutional Guidelines. Cages, bedding, and drinking water were autoclaved. Food was sterilized by irradiation. The mice used in all experiments were 6–8 weeks of age. All animal manipulations were performed in a laminar flow hood with sterile techniques under ether inhalation anesthesia, unless noted otherwise.

Protocol for Implantation of Human and Mouse Tissue Fragments into NOD/SCID Mice. After obtaining informed consent from the patients, adult human tissues were obtained from lung cancer patients (age range, 57–79 years) who underwent pulmonary lobectomy in the Division of Thoracic Oncology, National Cancer Center Hospital East. Known or suspected infec-

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3 The abbreviations used are: PC, prostate cancer; NOD/SCID-hu, non-obese diabetic/severe combined immunodeficient-human; HAB, human adult bone; HAL, human adult lung; PSA, prostate-specific antigen; TRAPase, tartrate-resistant acid phosphatase; ALPase, alkaline phosphatase.
Implantation of HAL fragments (NOD/SCID-HAL) was performed as described (15). Briefly, HAL was finely minced into 2×2×2 mm fragments and implanted into the right lower flank. All of the mice received at least two different tissue implants: HAB and HAL; or else HAB, HAL, and mouse bone. We did not observe any signs of inflammation or granulation in the bone or lung grafts or in the surrounding murine tissues.

**Cell Lines and Cell Culture.** Human PC cell lines, LNCaP (20) and PC-3 (21), were purchased from the American Type Culture Collection (Rockville, MD). LNCaP, an androgen-responsive human PC cell line originally derived from a supravacular lymph node with PC metastasis, was cultured in RPMI 1640 supplemented with 10% fetal bovine serum. PC-3, an androgen-independent human PC cell line derived from a bone metastasis specimen, was cultured in Ham’s F-12 with 10% fetal bovine serum. The medium was changed every week. Tumor volume was estimated by the formula \( \frac{4}{3} \pi a^3 b^2 \), where \( a \) is the longest dimension and \( b \) is the width.

**Histological Examination.** Mice were killed by cervical dislocation under anesthesia 8 weeks after injection of PC cells. Human tissues and certain mouse tissues (lumbar vertebrae, ribs, visible lymph nodes, liver, kidneys, adrenal glands, and lungs) were examined grossly and histologically for the presence of tumors. All of the other organs and the skeleton were inspected carefully. Adrenal glands, and lungs) were examined grossly and histologically for the presence of tumors. All of the other organs and the skeleton were inspected carefully.

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To determine whether the active cells and surrounding matrix derived from the implanted HAB and HAL were of human or mouse origin, a monoclonal antibody specific for human HLA-DP, -DQ, -DR class II, which recognizes human cells or associated matrix but does not cross-react with mouse cells or matrix, was used to stain sections from the 16-week postimplant grafts. Bone marrow elements were of human origin, showing specific staining for human HLA class II (Fig. 1H).

To monitor implanted bone function throughout the experiment, human IgG in the peripheral blood of NOD/SCID mice was quantitatively determined by ELISA methods. Fig. 2 shows the course of human IgG levels during the experiment in the NOD/SCID-hu mice. The human IgG levels were low 2 weeks after implantation, increased rapidly between 2 and 4 weeks, and reached a plateau thereafter. Constant levels of IgG were maintained for as long as 16 weeks (Fig. 2).

Metastasis of Human PC Cells to HAB and HAL in NOD/SCID-hu Mice. The ability of PC cells to colonize human tissues in vivo was tested by injection of LNCaP or PC-3 cells into NOD/SCID-hu mice via the lateral tail vein.

To determine the optimal experimental conditions for formation of metastases, we initially i.v. injected various numbers of LNCaP and PC-3 cells into NOD/SCID mice at various times after implantation of human and mouse tissue. Bolus i.v. injections of $4 \times 10^7$ LNCaP or more or $2 \times 10^7$ PC-3 cells or more resulted in 75% mouse mortality within 10 min of the injection. More frequent PC cell metastasis to HAB was observed in mice implanted with HAB 3–4 weeks prior to injection than implanted 6–8 weeks before injection (data not shown). Consequently, in subsequent experiments $1 \times 10^7$ LNCaP or $5 \times 10^6$ PC-3 cells were inoculated into NOD/SCID-hu mice at 3–4 weeks after implantation, and there were no complications. At 8 weeks after the injection, the presence of PC cells was analyzed by morphologically. Histological evaluation of the incision lesions did not reveal any evidence of PC cell colonization. In the control group, one of the five LNCaP cell-injected mice had distant metastasis (retroperitoneal lymph node), whereas two of the five PC-3-cell-injected mice developed distant metastases, including to the lung (one of five) and bone (one of five; data not shown).

Table 1 summarizes the incidence of metastasis to implanted HAB and HAL. The LNCaP cell line, originally isolated from a lymph node metastasis, metastasized to 35% of the implanted HAB, and PC-3...
cells, originally isolated from a bone metastasis, metastasized to 65% of the implanted HAB. Four weeks after the injection of PC cells, the NOD/SCID-hu mice developed grossly palpable and measurable tumors in the implanted HAB. The difference in the size of bone tumors was evident as early as 6 weeks after the i.v. injection (average sizes, 174.68 ± 31.3 mm³ for PC-3 tumors and 84.34 ± 26.0 mm³ for LNCaP tumors). Eight weeks after the injection of PC cells, the average size of the PC-3 metastatic bone tumors (228.45 ± 50.2 mm³) was significantly larger than that of LNCaP tumors (116.72 ± 35.4 mm³; P < 0.05; Fig. 3).

PC cells had no or a very low metastatic potential in regard to implanted and native mouse bone, suggesting that they specifically metastasize to human tissue. PC-3 metastatic lesions in mouse vertebral and implanted mouse bone marrow were limited, and they remained confined to the marrow space. Despite the injection of a high number of cells (5 × 10⁶ or 1 × 10⁷), histological evaluation of implanted HAL did not reveal any evidence of colonization. In a small number of mice, PC cells formed colonies visible in the mouse lung; however, these colonies were always <1 mm³ in volume. PC-3-cell-injected mice had distant metastases, including to the lung (5 of 20), liver (1 of 20), and mediastinal lymph nodes (2 of 20), whereas LNCaP-cell-injected mice developed distant metastases, including to the lung (2 of 20) and retroperitoneal lymph nodes (3 of 20). All mice with PC tumors in mouse tissues had an HAB tumor.

Histological Appearance of Metastases of Human PC Cells to HAB. Histological examination of the bone tumors formed by each of the cell lines revealed large, poorly differentiated tumors with a variable degree of stromal-epithelial interaction. The tumors formed by PC-3 showed a desmoplastic stromal response with large areas of necrosis and also consisted of osteolytic lesions without any surrounding osteogenic response (Fig. 4, A and C). Viable tumor was also present among the implanted HAB, but no invasion of surrounding muscle was detected. The distribution of tumor cells was demonstrated by immunohistochemical staining for cytokeratin (Fig. 4E).

PC-3 tumor cells remained unreactive with the antibody against PSA (not shown). Expression of human CD68 is a valuable marker for identification of cells of the human monocyte-macrophage lineage, and multinucleated cells on bone were uniformly CD68 positive, consistent with their identity as osteoclasts. Numerous multinucleated cells on the bone surface in areas of osteoclastic bone resorption were CD68 positive (Fig. 4G). CD68-positive cells were also localized in bone marrow. The number of osteoclasts was abnormally high compared with nonmetastatic regions. Expression of TRAPase activity is a characteristic phenotypic marker of osteoclasts and osteoclast precursors, and it is expressed in osteoclasts resorbing bone. The TRAPase-positive multinucleated cells were mainly located on or close to the surface of resorption lacunae of bone with a TRAPase-positive cement line (Fig. 4I). By contrast, TRAPase-positive mononuclear cells were located a distance from the bone surface. There was no osteogenic response around the tumor. Only a few ALPase-positive cells were seen, and they stained very weakly (data not shown).

The tumors formed by LNCaP cells appeared to be of the osteoblastic type (Fig. 4B), and regions of bone deposition of new bone matrix could be seen (Fig. 4D). The surface of the bones with metastatic tumor involvement rarely showed active resorption. The layer of stromal cells between the tumor cells and the bone surface was reduced more extensively in the LNCaP tumors than in the PC-3 tumors, and only a few osteoclasts were seen. Numerous osteoblastic cells lined the surface of newly formed bone. Immunohistochemical staining of the tumors in this group of mice was positive for PSA (Fig. 4F). LNCaP cells retained the ability to produce PSA while growing in a HAB microenvironment in vivo. Only a few multinucleated cells on the bone surface around the tumor were CD68 positive (Fig. 4H), and they were weakly stained with TRAPase (data not shown). The ALPase-positive osteoblasts, on the other hand, were located on the bone surface with an ALPase-positive cement line (Fig. 4J).

**DISCUSSION**

We established a NOD/SCID-hu model that can be used to study metastasis of human PC in a species- and tissue-specific manner. The metastatic process is determined not only by the characteristics of the tumor cell itself but by its surrounding microenvironment. Previous xenograft models (6–13) all required human cells to metastasize to and grow in mouse organs. Although recent reports (15–17) have described the use of the SCID-hu system to study the behavior of metastatic human tumor cells, the models have been generated by surgical transplantation of human fetal organs into SCID mice. Embryonic and adult hematopoietic and osteogenic progenitors differ with respect to cycling rates (28), response profiles to hematopoietic factors (29), and differentiation capacities (30), and to improve these
human PC metastatic models, we engrafted NOD/SCID-hu mice with HAB and HAL. Immunohistochemical characterization of the HAB demonstrated that the osteoblastic, osteoclastic, and endothelial cells were of human origin (specific staining for human HLA class II, CD68, and CD34). These cells, including the human osteoblastic, osteoclastic cells, and human bone marrow stromal cells, survived and were functional up to at least 16 weeks. These morphological findings, together with the results of kinetic studies of IgG expression showing that constant levels of IgG were maintained in vivo for as long as 16 weeks after implantation, indicate that transplantation of a human bone fragment can provide a stromal microenvironment suitable for human hematopoietic and osteogenic progenitor cells and maintain it for a long time. Thus, this model allows metastatic spread of human tumor cells to human tissues.

Several animal models of human PC metastasis using both LNCaP and PC-3 cell lines have been established (6–13, 17). The PC-3 cell line was isolated from a PC that metastasized to the vertebral bodies of a patient with hormone-insensitive PC, which is commonly associated with bone metastasis, and use of the PC-3 cell line in NOD/SCID-hu system for studying bone metastasis would therefore be of considerable value. The LNCaP cell line is useful and important because it is the only human PC cell line with functional androgen receptor and PSA expression that has been established. These cell lines possess a few differentiated cell features that are the predominant morphological characteristics of PC; clinical extrapolation can be deduced from the present experimental results. The finding that LNCaP and PC-3 cells preferentially metastasize to implanted HAB, with considerably less preference for grafts of HAL, may reflect the characteristic clinical features of PC, which preferentially metastasizes to the bones of patients rather than their lungs. In addition, the characteristic osteoblastic bone metastases of PC can be reproduced by using HAB. This study reports the first model system for studying bone metastasis of human PC cell lines to HAB. Future studies will be performed to determine the mechanism of the PC bone metastasis under conditions similar to those in the human body (NOD/SCID-hu system) by using more differentiated tumor cells established from clinical specimens of human PC or cell lines isolated from primary tumors.

LNCaP and PC-3 cells injected or implanted orthotopically have been reported to fail to form bony metastases (11, 12), and i.v. injection of up to $1 \times 10^6$ LNCaP or PC-3 human PC cells does not typically result in the development of bone metastases (7, 13). In our in vivo experiments, LNCaP and PC-3 cells produced a high incidence

Fig. 4. Histological analysis of PC-3 (left panels) and LNCaP (right panels) tumors in HAB implanted in NOD/SCID mice. A and C, PC-3 tumors exhibit a desmoplastic stromal response with large areas of necrosis and osteolytic lesions (H&E, ×40 and ×100). E, PC-3 cells stained for cytokeratin (immunostaining for cytokeratin, ×200). G, numerous multinucleated cells on the bone surface stained positive for CD68 (immunostaining for CD68, ×100). I, several TRAPase-positive multinucleated osteoclasts are visible on the bone surface. Lacunae and a TRAPase-positive cement line are present. TRAPase-positive multinuclear cells can be seen among the stromal cells (TRAPase staining, ×200). B and D, LNCaP tumors that have undergone an osteoblastic change. Regions of newly deposited bone matrix are visible (H&E, ×40 and ×100). F, LNCaP cells stained for PSA (immunostaining for PSA, ×200). H, only a few CD68-positive cells on the bone surface around the tumor are visible (immunostaining for CD68, ×100). J, ALPase-positive osteoblasts with an ALPase-positive cement line are present on the bone surface (ALPase staining, ×100).
of metastasis to implanted HAB, although both PC cell lines were capable of infrequent metastasis not only to implanted mouse bone but to native mouse bone as well. These results combined with previous data indicate that experimental metastasis of PC cells to HAB in the NOD/SCID-hu system is exquisitely species specific, as confirmed by the following facts: (a) native mouse bones are vastly superior in size and vascularization compared with HAB implants; and (b) LNCaP and PC-3 cells were barely capable of metastasis to implanted and native mouse bone. The tissue specificity of the metastases in NOD/SCID-hu mice was also demonstrated by the lack of metastases of PC to HAL grafts. These observations further support the notion that the colonization of HAB involves species- and tissue-specific mechanisms and is not attributable to the passive lodging of tumor cells in bone. Vascular access, although required, is insufficient in itself for the establishment of bone metastases.

The preference for bone as the site of PC metastases is thought to be governed by factors that recruit extravasated tumor cells, such as the presence of chemotractants produced by the target organ, preferred stromal elements, and/or angiogenic factors suitable for metastatic cell growth (31–33). Another possible mechanism is that bone marrow endothelial cells serve as a site of specific adhesion for bone-homing PC cells (34–36). Bone marrow-derived endothelial cells express adhesion ligands for PC cells that are not expressed on hepatic endothelial cells or on nonendothelial cells of the bone marrow (8, 37).

Introduction of green fluorescent protein into tumor cells has recently made it possible to visualize tumor metastasis at higher resolution. This method is much easier than the traditional cumbersome pathological examination procedures (38, 39). To elaborate the precise interaction between PC cells and bone marrow stromal cells, future studies using green fluorescent protein expression will be performed to determine whether human metastatic cancer cells attach to human bone marrow endothelial cells in HAB on the microscopic level.

Because the high incidence of metastatic colonies is reproducible, this model may be useful in evaluating the therapeutic efficacy of antimetastatic agents. The model includes osteoblastic (LNCaP) and osteolytic (PC-3) types of bone metastasis of human PC that differ in hormone sensitivity, PSA status, histogenesis, and malignancy. Thus, this small animal model should enable the identification and analysis of important features of the human PC metastatic process that cannot be addressed in conventional models of metastasis.

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