ABSTRACT

Commonly used in vivo models of prostate cancer metastasis include syngeneic rodent cancers and xenografts of human cancer in immunodeficient mice. However, the occurrence of osseous metastases in these models is rare, and in xenograft models, species-specific factors may limit the ability of human cells to metastasize to rodent bone. We have modified the severe combined immunodeficient (SCID)-human model to test the ability of circulating human prostate cancer cells to home to macroscopic fragments of human bone and other organs previously implanted into SCID mice. We have also compared the growth of human prostate cancer cells in various human and mouse tissue microenvironments in vivo. Macroscopic fragments of human fetal bone, lung, or intestine (16–22 weeks gestation) or mouse bone were implanted subcutaneously into male CB.17 SCID mice. Four weeks later, human prostate cancer cells were injected either i.v. via the tail vein (circularizing cell colonization assay) or directly into the implanted tissue fragments (endothelial growth assay). Tumor growth was followed for 6 weeks by palpation and magnetic resonance imaging. After 6 weeks, tumors were enumerated in implanted human and mouse organ fragments and native mouse tissue. Tumors were characterized by histology, immunohistochemistry, and chromosomal analysis. After i.v. injection, circulating PC3 cells successfully colonized implanted human bone fragments in 5 of 19 mice. Tumors were easily followed by palpation and imaging and had an average volume of 258 mm³ at autopsy. Histological examination revealed osteolysis and a strong desmoplastic stromal response, which indicated intense stromal-epithelial interaction. Bone tumors were subcultured, and chromosomal analysis demonstrated that the tumors were derived from the parental prostate cancer cell line. Microscopic tumor colonies were also found in a few mouse lungs after i.v. injection of PC3, DU145, and LNCaP cells, however the volume of the lung nodules was less than 1 mm³ in all of the cases. No colonization of human lung or intestine implants, the mouse skeleton, or other mouse organs was detected, demonstrating a species- and tissue-specific colonization of human bone by PC3 cells. Direct injection of 10⁴ prostate cancer cells into human bone implants resulted in large tumors in 75–100% of mice. PC3 and DU145 bone tumors were primarily osteolytic, whereas LNCaP bone tumors were both osteoblastic and osteolytic. PC3 and LNCaP bone tumors showed a desmoplastic stromal response, which indicated intense stromal-epithelial interaction. All three of the cell lines formed tumors in implanted human lung tissue; however, the tumors were all ≤10 mm³ in volume and showed minimal stromal involvement. No tumors formed after either s.c. injection or injection of cells into implanted mouse bone demonstrating both species- and tissue-specific enhancement of growth of human prostate cancer cells by human bone. The severe combined immunodeficient-human model provides a useful system to study species-specific mechanisms involved in the homing of human prostate cancer cells to human bone and the growth of human prostate cancer cells in human bone.

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INTRODUCTION

Bone has long been recognized to be the most common target organ of prostate cancer metastasis (1), and the phenomenon of osseous metastasis signals the final, incurable stage of disease. The reasons underlying the proclivity of prostate cancer to metastasize to bone remain unclear; however, the competing hypotheses can be divided into two categories: anatomical/hemodynamic factors (Batson’s plexus) and “seed and soil.” In 1940, Batson (2) described a venous portal system between the plexus of veins draining the prostate and the plexus of veins surrounding the lumbar spine. In animal experiments and in human cadavers, Batson demonstrated that dye injected into the deep dorsal vein of the penis passed through the prostatic plexus and continued through longitudinal, valveless veins to the sinusoidal venous structures of the lumbar spine (2, 3). These experiments suggested that passive hemodynamic patterns may contribute to the tendency of prostate cancer to metastasize to the lumbar spine. The alternative hypothesis has been attributed to Paget, who surmised that the metastatic pattern of a given type of carcinoma is dependent on the interaction of individual tumor cells with the microenvironment of the target tissue (4). Thus the high frequency of osseous metastases in prostate cancer is not due to passive hemodynamic patterns but to a molecular affinity between prostate cancer cells and the bone marrow microenvironment.

The phenomenon of prostate cancer metastasis to bone has been difficult to mimic in animal models. In syngeneic animal prostate cancer models such as the Dunning rat model (5) and in the transgenic mouse model TRAMP (6), lymph node and pulmonary metastases are common, but the occurrence of bone metastasis from a primary tumor site is rare. It has also been difficult to mimic clinical patterns of osseous metastasis in immunodeficient murine xenograft models of human prostate cancer. For example, i.v. injection of up to 1 × 10⁶ PC3 or LNCaP human prostate cancer cells does not typically result in the development of bone metastases (7, 8). Increased osseous metastasis of prostate cancer cell lines has been achieved by various experimental manipulations, including coinjection of tumor cells with bone stromal cells (9), orthotopic injection of prostate tumor cells (10), or brief occlusion of the vena cava during i.v. injection of cells (11). Nonetheless, these xenograft models all share the fact that human cells must metastasize to and grow in mouse organs. It is possible that many of the molecules involved in the metastatic process such as proteases, adhesion molecules, chemotactic factors, and growth factors and their receptors, are species-specific, which may contribute to the difficulty in achieving bone metastases in xenograft models of prostate cancer.

Recent reports have described the use of the SCID-hu system to study the behavior of metastatic human tumor cells. It was found that small cell lung carcinoma cells that did not grow in murine organs after i.v. injection specifically colonized human fetal lung and bone marrow tissues implanted in SCID mice (12). Application of the SCID-hu system to the study of human colon carcinoma has also led...
to the identification of adhesion molecules that may be important in colon cancer metastasis (13). These studies clearly illustrate the importance of species- and tissue-specific interactions in the design of in vivo metastasis models. Using the SCID-hu system, we have found that circulating human prostate cancer cell lines preferentially colonized implanted human bone tissue. In addition, growth of human prostate cancer cells was specifically enhanced by direct interaction with the human bone marrow microenvironment.

**MATERIALS AND METHODS**

**Cell Lines and Cell Culture.** DU145, LNCaP, and PC3 human prostate cancer cell lines were purchased from the American Type Culture Collection. DU145 cells were maintained in culture in DMEM-10% fetal bovine serum, and LNCaP and PC3 cells were maintained in RPMI 1640–10% fetal bovine serum. All of the culture reagents were purchased from Life Technologies, Inc. (Gaithersburg, MD). Cell lines were used at low passage number in all of the experiments.

**Animal Care and Human Tissue Implantation.** Male homozygous C.B-17 scid/scid mice, aged 5 weeks, were purchased from Taconic Farms (Germantown, NY). Mice were maintained according to the NIH standards established in the “Guidelines for the Care and Use of Experimental Animals,” and all of the experimental protocols were approved by the Animal Investigation Committee of Wayne State University. Human male fetal tissues were obtained with informed consent according to regulations issued by each state involved and the federal government. Methoxyflurane anesthesia was used during all of the surgical procedures. Implantation of 6-week-old mice with human fetal lung fragments (SCID-hu-L) or human fetal intestine fragments (SCID-hu-I) was performed as described by Shrivelman (12). Briefly, human fetal lungs or intestines of 16–22 weeks of gestation were cut into 2 × 2 × 2 mm fragments and implanted s.c. in the ventral surface of the mouse via a small skin incision. Implantation of mice with human fetal human bone fragments (SCID-hu-B) was performed as described previously (14). Briefly, human fetal femurs and humeri of 16–22 weeks of gestation were divided in half longitudinally and then again in half transversely into four fragments approximately 1 cm long and 3 or 4 mm in diameter. These bone fragments were implanted s.c. in the flank through a small skin incision with the opened marrow cavity against the mouse muscle. As a control for species-specific growth of prostate cells in human bone, femurs were harvested from normal SCID mice, and bone fragments were implanted in the flank of recipient mice in an identical fashion (SCID-n-B). All of the mice received at least two different tissue implants; human bone in the flank and human lung or intestine in the abdomen or human bone and mouse bone in opposite flanks.

**Circulating Cell Colonization Assay.** Cultured prostate cancer cell lines were harvested by incubation with trypsin/EDTA and washed twice in Hank’s balanced salt solution. Cell number and viability were measured by trypan blue. For in vivo circulating cell colonization experiments, 1 × 10⁶ cells in a volume of 200 µl were injected into SCID-hu mice via lateral tail vein using a 27-g needle. In all of the experiments, cancer cells were injected 4 weeks after tissue implantation because this time point was found to be optimal for colonization of human tissues by carcinoma cells in previous reports (12).

**End Organ Tumor Growth Assay.** For experiments measuring growth of human prostate cancer cells in human metastatic organ microenvironments in vivo, 1 × 10⁶ cells in a volume of 20 µl were injected directly into implanted human or mouse tissue fragments through the skin using a 27-g needle. After injection, injections were performed 4 weeks after tissue implantation. For the bone fragments, the opened marrow surface was targeted with the needle. Some control mice also received the same number of cells injected s.c. in the shoulder region as a further control for tumor growth. For the LNCaP cell line, a second group of mice received a larger inoculum of 5 × 10⁶ cells (see “Results”).

**MRI.** MR images were acquired on a Bruker AVANCE spectrometer equipped with 4.7-T horizontal-bore magnet and actively shielded gradients. During MR observation, the mouse was anesthetized (1.5%/v/v halothane) and immobilized on a bed that was heated by circulating temperature-controlled water. A whole-body RF water-cooled receiver coil (70-mm diameter) was used to transmit homogeneous RF excitation and hold the animal bed. A RF surface transceiver coil (30-mm diameter) was placed on the back of the mouse to receive MR signals because it produced images with increased signal:noise ratios compared with the whole-body coil. The surface coil and whole-body coil were actively decoupled to avoid signal interference. High-resolution T₂-weighted spin echo images (TE = 15 ms, TR = 545 ms, flip angle = 90°, 8.0 cm FOV, and 256 × 256 matrix size) were acquired in coronal sections to serve as the detailed anatomical reference for tumor position. However, tumor cannot be easily distinguished from surrounding normal tissue in these T₂-weighted images. To enhance contrast between tumor and normal tissue, a multishlice T₂-weighted spin echo sequence (TE = 60 ms, TR = 1225 ms, flip angle = 90°, 2 averages) with fat saturation was used. T₂-weighted images of 10 contiguous slices covering the whole tumor were obtained in two sections (axial and coronal), yielding in-plane resolution <0.1 mm² with a slice thickness of 1.0 mm (6.4-cm or 8.0-cm FOV, 256 × 256 matrix size).

**Data Analysis and Histology.** Mice were killed 6 weeks after injection of tumor cells by cervical dislocation while under methoxyflurane anesthesia. Human tissues and certain mouse tissues (lumbar vertebrae, ribs, visible lymph nodes, and lungs) were dissected, photographed, and measured with calipers. Tumor volume was estimated by the formula $a \times b^2/2$, where $a$ is the longest dimension and $b$ is the width. All of the other organs and the mouse skeleton were visually inspected. Mouse lungs were stained in Bouin’s fluid to reveal tumor nodules. Portions of each tissue were fixed in 10% buffered formalin, decalcified (bone specimens), embedded in paraffin, sectioned, and stained with H&E for routine histological examination. Immunohistochemical staining was also performed to confirm the presence of tumor cells in bone tissues. Briefly, sections were deparaffinized and rehydrated through graded alcohols. For antigen retrieval (15), slides were placed in 10 mM citrate buffer and boiled by microwave heating for 5 min. Nonspecific sites were blocked by incubation with Superblock (ScyTek, Logan, UT). Sections were incubated with either monoclonal anti-cytokeratin antibody (C-25, Sigma, St. Louis, MO) or nonspecific mouse immunoglobulins at 1:200 dilution. Positive immunoreactive sites were detected using a Vectastain alkaline phosphatase kit (Vector, Burlingame, Ca) and Sigma Fast Red substrate, and nuclei were briefly counterstained with hematoxylin.

Mean tumor volumes were calculated for each cell line test group and tumor site, and statistical comparisons of mean tumor volumes between groups were performed using Student’s $t$ test (one-tailed analysis).

**Subculture of Tumors and Genetic Analysis.** Tumors arising in implanted human bone tissues after i.v. injection of tumor cells were analyzed to confirm the cell of origin. Small portions were minced under sterile conditions and cultured in Keratinocyte-SFM (Life Technologies, Inc.) containing EGF, bovine pituitary extract, and 10% fetal bovine serum until a confluent monolayer of cells was established. This medium was chosen to retard the growth of fibroblastic cells. After the first and second passages, cultures were stained for cytokeratin as described above (“Histology”) to assess the purity of the tumor cell populations. By the second passage, the cultured tumor cells were nearly 100% cytokeratin-positive, suggesting minimal stromal cell survival in culture. At this point, cells were analyzed by CGH.

CGH analysis was performed essentially as described previously (16, 17). DNA was prepared separately from bone tumor subcultures and parental cell lines by proteinase K digestion and phenol/chloroform extraction. One µg of tumor DNA or reference normal DNA was nick-translated in the presence of FITC-12-dUTP (green fluorescence) or Texas Red-5-dUTP (red fluorescence, DuPont NEN, Boston, MA), respectively. Labeled tumor and normal DNA were hybridized together with 30 µg of Cot-1 DNA (Life Technologies, Inc.) onto normal male metaphase spreads (Vysis, Downers Grove, IL) for 2–3 days. After washing, slides were counterstained with 0.1 mM DAPI. Ten to 15 metaphase images were acquired for each tumor-normal hybridization, of which the best images were chosen for quantitative analysis. Quantitative image analysis was performed as described previously using the QUIPS CGH system (Vysis Inc., Downers Grove, IL; Ref. 18).

**Serum PSA Measurements.** Serum samples were taken from mice bearing LNCaP tumors in implanted human bone fragments at the time the mice were killed. Total PSA levels were measured by routine methods at the Clinical Chemistry Laboratory of the Detroit Medical Center.

**RESULTS**

**Implantation of Human Fetal Tissues.** SCID-hu mice were used for circulating cell colonization and end organ growth experiments in vivo.
3–4 weeks after the implantation of human tissues. Previous studies demonstrated higher rates of colonization of implanted human tissues when cells were injected at early time points, before the recovery of hematopoiesis in the bone marrow compartment (12). To assure viability of implanted human fetal tissues at the time of injection, four SCID-hu mice bearing different human tissue implants were killed 4 weeks after implantation, and the tissues were examined histologically. The marrow compartment of human bone implants contained mainly stromal elements and some residual hematopoietic cells, and evidence of new bone formation could be seen (Fig. 1A). Small blood vessels containing RBCs were clearly visible in the tissue sections. Human lung implants grew in size, and the tissue appeared to become more differentiated with evidence of alveolar structures, columnar epithelium lining bronchioles, and cartilage (Fig. 1B). Likewise, implanted human fetal intestine appeared healthy, with a well-differentiated epithelial lining (Fig. 1C). These results are consistent with previous reports describing the biology of implanted human fetal tissue fragments (12, 14) and suggest that the implanted tissues were healthy and adequately vascularized.

Colonization of Human Tissue Implants by Circulating Tumor Cells. The ability of circulating prostate cancer cells to colonize human tissues in vivo was tested by the injection of DU145, LNCaP, or PC3 cells into SCID-hu mice via the lateral tail vein. Three weeks after the injection of cancer cells, 5 (26%) of 19 mice receiving PC3 cells developed grossly palpable and measurable tumors in implanted human bone fragments (Table 1). Six weeks after injection, tumors were confirmed and measured at autopsy. Histological analysis revealed large solid tumors composed of cohesive nests of tumor cells intermingled with a cellular desmoplastic stromal response (Fig. 2B). Close examination showed areas of bone destruction and resorption, which indicated an osteolytic tumor phenotype (Fig. 2C).

Immunohistochemical staining for cytokeratin confirmed the presence of epithelial tumor cells (not shown). DU145 and LNCaP cells did not form macroscopic palpable tumors in human bone tissues after tail vein injection (Table 1), and this observation was confirmed histologically and by immunohistochemical staining for cytokeratin (not shown). Histological evaluation of implanted human lung and intestine tissues did not reveal any evidence of colonization by any of the cell lines tested (Table 1), which suggested that circulating PC3 cells prefer to colonize human bone as opposed to other human tissues, and the colonization of human bone by PC3 cells was not merely an effect of the implantation procedure.

Because cells injected i.v. via the tail vein must pass through the mouse lung before entering the general circulation, we examined the mouse lungs for signs of colonization. In a small number of mice, the three cell lines formed between 5 and 15 colonies visible on the lung surface (Table 1), however these colonies were always less than 1 mm³ in volume and did not cause any adverse respiratory effects. Of the two mice with PC3 tumors in the mouse lungs, one occurred in a mouse that also had a human bone tumor. A representative lung colony formed by circulating PC3 tumor cells is shown in Fig. 2D.

None of the mice experienced limb paralysis, which was consistent with a lack of spinal metastases, and all of the mouse tissues examined were clear of tumor deposits by gross and histological inspection. The absence of metastasis to mouse bone suggested that species-specific factors favored targeting to the human bone environment.

To confirm the origin of tumors arising in implanted human bone tissues after i.v. injection of PC3 cells, explant cultures of these tumors were established. After the second passage, the cultures were greater than 98% epithelial cells, as judged by cellular morphology and positive staining for cytokeratin (not shown), confirming that the tumors were of epithelial origin. One of the resulting cell populations, termed PC3-HB1, was characterized by CGH analysis. We found gains in the regions of chromosomes 7p, 8q, and 11q, and losses in the regions of chromosomes 4q, 9p, and 10p (not shown). Analysis of the parental PC3 cells revealed the same pattern of chromosomal alterations as PC3-HB1. This pattern of gains and losses was consistent with the recent findings of Nupponen et al. (19). Genetic analysis thus confirmed that the bone tumors that were formed in the circulating cell colonization assay originated from circulating PC3 cells.

The ability of circulating PC3-HB1 cells to form tumors in SCID-hu mice was also tested. Injection of 1 × 10⁶ cells into SCID-hu mice via the lateral tail vein did not result in any detectable tumors in human bone or lung tissues nor in the mouse lungs (Table 1). The reason for this result was unclear.

End Organ Tumor Growth Assay. To measure the effects of various human tissue microenvironments on the in vivo growth of human prostate cancer cells, we used a direct injection approach to ensure uniform seeding of the tissues with the tumor cells. The injection of 1 × 10⁶ DU145, PC3, or PC3-HB1 cancer cells directly into implanted human bone fragments resulted in grossly evident

Table 1 Circulating cell colonization assay

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Human bone</th>
<th>Human lung</th>
<th>Human intestine</th>
<th>Mouse lung*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DU145</td>
<td>0/12</td>
<td>0/12</td>
<td>0/7</td>
<td>5/12 (12.5)</td>
</tr>
<tr>
<td>LNCaP</td>
<td>0/12</td>
<td>0/6</td>
<td>0/5</td>
<td>1/12 (6)</td>
</tr>
<tr>
<td>PC3</td>
<td>5/19b</td>
<td>0/16</td>
<td>0/5</td>
<td>2/19 (4.5)</td>
</tr>
<tr>
<td>PC3-HB1</td>
<td>0/12</td>
<td>0/12</td>
<td>ND</td>
<td>0/12</td>
</tr>
</tbody>
</table>

*Volume of individual prostate cancer cell lung nodules was less than 1 mm³ in all of the cases.

b Average PC3 bone tumor volume = 258.0 mm³.

ND, not done.

Fig. 1. SCID-hu tissue implants. Histological appearance of human fetal tissues 4 weeks after implantation into SCID mice. A, the marrow compartment of human bone implants contained mainly stromal elements and some residual hematopoietic cells, and evidence of new bone formation could be seen (arrow). ×40. B, implanted human lung with alveolar structures (arrow) and columnar epithelium lining bronchioles (arrowhead). ×40. C, implanted human fetal intestine appeared healthy, with a well-differentiated epithelial lining (arrow). ×40. No evidence of necrosis was seen in any tissue examined.
tumors in a majority of mice (Table 2). The bone tumors were easily palpable through the skin and could be measured with calipers.

To test the accuracy of tumor measurement before sacrifice, MRI was performed on two mice bearing PC3 tumors in human bone tissue. The tumors appeared as regions of high signal intensity on T2 weighted images (Fig. 3). Residual bony tissue could be discerned as dark punctate areas within the tumor mass. Measurements taken from these images agreed with direct measurement of the same bone tumors harvested the following day.

The average size of bone tumors formed by PC3 cells was larger than tumors formed by DU145 ($P < 0.05$), which indicated more rapid growth of PC3 cells (Table 2). Injection of $1 \times 10^4$ LNCaP cells did not result in detectable bone tumors (Table 2), however increasing the inoculum size to $5 \times 10^4$ cells resulted in tumors in 6 (75%) of 8 mice. The bone tumors formed by LNCaP cells were also large and similar in size to the PC3 and PC3-HB1 bone tumors ($P > 0.5$ for both comparisons). In contrast, no tumors were formed in any group after s.c. injection of the same low number of cancer cells, which indicated that the human bone environment enhanced prostate tumor growth in vivo.

Histological examination of the bone tumors formed by each of the cell lines showed large, poorly differentiated tumors with a variable degree of stromal-epithelial interaction (Fig. 4, A, B, and C). DU145 cells formed solid, cellular tumor masses without central necrosis (Fig. 4A). A limited desmoplastic stromal response was present at the tumor-normal tissue interface. In contrast, the tumors formed by PC3 cells showed a striking desmoplastic stromal response with large areas of central necrosis (Fig. 4C). Fragments of necrotic bone could be seen within the tumor masses. The differential distribution of tumor and stromal cells was more clearly demonstrated by immunostaining for cytokeratin (Fig. 4, D, E, and F). Tumors formed by DU145 and PC3 also appeared to be osteolytic in nature. In DU145 tumors, areas of bone resorption could clearly be seen adjacent to tumor cell nests (Fig. 4A, arrow); and in many of the PC3 tumors, the calcified bone tissue was almost totally replaced by tumor cells and fibrous stroma (Fig. 4C). LNCaP cells formed solid tumors in bone with a moderate desmoplastic stromal response (Fig. 4B). The tumors formed by LNCaP cells appeared to be of a mixed osteoblastic/osteolytic type (Fig. 4B), and regions of bone resorption and deposition of new bone matrix (arrow) could be seen. The level of total PSA was also measured in serum samples taken from two mice bearing LNCaP tumors in human bone tissues and was found to average 4.5 ng/ml. This result further confirmed the presence of LNCaP tumor in these mice and suggested that LNCaP cells retained the ability to produce PSA while growing in a human bone microenvironment in vivo.

To control for the possibility that any implanted human tissue could enhance tumor growth, prostate cancer cell lines were also injected into implanted human lung tissues. Tumor deposits formed in a portion of implanted human lung tissues directly injected with prostate tumor cells, and in all of the cases, the tumor nodules were extremely small in comparison with tumors formed in the bone implants (Table 2). For all of the three cell lines, the tumors that grew in implanted lung tissue consisted of solid masses of tumor cells with intervening vasculature (Fig. 4, G, H, and I). Little very interaction with stromal cells could be seen, and many of the tumor nodules were surrounded by a thin capsule.

To control for species specificity, prostate cancer cell lines were injected into s.c. implanted mouse bone fragments. For each cell line, four mouse bone implants were injected, and in no case did a bone tumor result (Table 2). This provided further evidence of the importance of human-humans stromal-epithelial interactions with regard to prostate cancer cells growing within bone.

<table>
<thead>
<tr>
<th>Cell Line (no. of cells injected)</th>
<th>Human bone$^a$</th>
<th>Human lung$^a$</th>
<th>Mouse bone implant</th>
<th>Subcutaneous</th>
</tr>
</thead>
<tbody>
<tr>
<td>DU145 ($1 \times 10^4$)</td>
<td>7/9 (113.5 ± 26.0)</td>
<td>4/9 (4.0 ± 1.0)</td>
<td>0/4 0/4</td>
<td>0/7 0/7</td>
</tr>
<tr>
<td>LNCaP ($1 \times 10^4$)</td>
<td>0/7</td>
<td>5/10 (10.5 ± 1.5)</td>
<td>0/4 0/4</td>
<td>0/8 0/8</td>
</tr>
<tr>
<td>LNCaP ($5 \times 10^4$)</td>
<td>0/6 (305.5 ± 62.5)</td>
<td>ND$^b$</td>
<td>ND 0/6</td>
<td>ND</td>
</tr>
<tr>
<td>PC3 ($1 \times 10^4$)</td>
<td>16/18 (407.5 ± 106.5)</td>
<td>3/14 (2.5 ± 1.0)</td>
<td>0/4 0/4</td>
<td>0/8 0/8</td>
</tr>
<tr>
<td>PC3-HB1 ($1 \times 10^4$)</td>
<td>10/10 (235.5 ± 92.0)</td>
<td>3/10 (1.0 ± 0.5)</td>
<td>ND 0/6</td>
<td>ND</td>
</tr>
</tbody>
</table>

$^a$ Tumor volume (in parentheses) expressed in mm$^3$ ± SE

$^b$ ND, not done.
DISCUSSION

When designing animal models of human prostate cancer metastasis, it is important to take into account the possible contribution of human-human stromal-epithelial interactions to the establishment and growth of prostate tumors in the target bone tissue. The results of this study demonstrate that circulating human prostate cancer cells were able to specifically and preferentially colonize implanted human bone tissue in SCID mice, and that human bone provided a more favorable growth environment for human prostate cancer cells than either human lung or mouse bone tissue.

Circulating PC3 cells formed tumors in 5 (26%) of 19 human bone implants but in only 2 (11%) of 19 mouse lungs. In addition, the human bone tumors were very large compared with the microscopic mouse lung tumors, and we found no other metastatic deposits in the native mouse organs. This is remarkable for several reasons: (a) i.v.-injected tumor cells must pass through the mouse lung capillary beds before entering the general circulation; and (b) the mass of human tissue available to the circulating cancer cells is quite small in comparison with the mouse skeleton and other organs. These observations further support the notion that the colonization of human bone involved species- and tissue-specific mechanisms and was not due to the passive lodging of tumor cells in the bone. Studies on other tumor cell lines have clearly demonstrated that selective colonization of specific target organs is not simply the result of nonspecific trapping.

Fig. 3. MRI. T2-weighted spin echo MR images (TE = 60 ms, TR = 1225 ms) of a mouse bearing a PC3 tumor in implanted human bone tissue. A, coronal section. B, axial section. Arrows, tumor location.

Fig. 4. End organ growth assay. Representative photomicrographs demonstrating histological features of tumors formed in human bone tissue (A–F) and human lung tissue (G–I) 6 weeks after the direct injection of human prostate cancer cell lines. A, DU145 cells formed solid tumors with evidence of osteolytic activity (arrow). ×200. B, bone tumors formed by LNCaP also showed a desmoplastic stromal response, and evidence of both osteoblastic (arrow) and osteolytic activity (arrowhead) could be seen by routine staining. ×100. C, PC3 cells formed solid osteolytic tumors, and bone tissue was often completely destroyed. ×100. A strong desmoplastic stromal response was visible in these tumors, revealed both by routine H&E staining (C) and by red staining for cytokeratin (F). ×200. DU145, LNCaP, and PC3 cell lines all formed small solid tumors in human lung tissue and demonstrated little stromal-epithelial interaction (G, H, and I, respectively). ×100.
of tumor cells by the organ vasculature. One of the best studied examples is the B16 melanoma cell line, in which sublines have been isolated that preferentially metastasize to the lung and liver (20). Similarly, in vitro selection combined with repeated in vivo passing of the PC3 human prostate cancer cell line has resulted in sublines that reliably colonize distinct organ sites (7), and sublines of the LNCaP cell line with the ability to metastasize to bone have been isolated (9). A natural selection process in the original patients may also account for the differential ability of the three cell lines used in our study to colonize human bone implants; PC3 cells were derived from a metastatic bone lesion (21), whereas the LNCaP and DU145 cell lines were derived from lymph node and brain metastases, respectively (22, 23).

The ability of circulating PC3 cells to colonize human bones in 25% of our mice is also remarkable considering that we used routinely cultured PC3 cells at a low passage number after purchase, without any prior selective pressure. In previous studies, metastasis of PC3 cells to bone has been achieved, but only after repeated rounds of in vivo selection (7, 24) or through forced seeding of the lumbar vertebræ by simultaneous tail vein injection and caval occlusion (11). Taken in this light, our observed tumor rate in human bone clearly exceeds what would be expected for the metastasis of PC3 cells to mouse bone.

The colonization of bone marrow by prostate cancer cells is likely to involve adhesion to specific ligands found either on endothelial or parenchymal cells or within the extracellular matrix of the bone marrow environment. It has been demonstrated that various bone components can stimulate tumor cell adhesion and chemotaxis (25), and recently Kosteniuk et al. (26) found that prostate cancer cells adhere to type I collagen in osteoblast extracellular matrix via the α3β1 integrin. In addition, Pasqualini and Ruoslahti (27) have suggested the existence of organ-selective vascular address molecules. Using in vivo screening of random peptides, they identified amino acid sequences that specifically target the vascular endothelium of target organs, through binding to either integrins or other undefined molecules. It is possible that prostate tumor cells use a similar mechanism to adhere specifically to endothelium in human bone, allowing colonization and eventual tumor formation. Such a mechanism has been demonstrated for the homing of lymphocytes and granulocytes to specific tissue sites (28). Conversely, a lack of expression of these important molecules by DU145 and LNCaP could explain the inability of these circulating cells to colonize the human bone implants. We are currently investigating specific cell-surface molecules that may allow PC3 cells to colonize human bone.

The failure of circulating prostate cancer cells to form gross skeletal tumors in the mouse skeleton could be related to an inability of the cells to adhere to molecular targets in the mouse marrow compartment via mechanisms mentioned above. Alternatively, single tumor cells or small cell aggregates may have been present in the marrow but were unable to proliferate and form detectable skeletal tumors because of a lack of favorable environmental factors that are present in human bone tissue. In fact, results of earlier studies have shown that whether injected via intraarterial or i.v. routes, tumor cells could be detected in organ sites throughout the test animals, regardless of the ultimate formation of metastatic deposits (29). Rapid growth of human prostate cancer cells in mouse bone has been demonstrated after intrafemoral injection (30); however, larger numbers of prostate cancer cells were used in this study, making it difficult to determine whether tumor growth in that system was actually dependent on the bone marrow environment. Our observations show that a relatively small inoculum of $1 \times 10^6$ prostate cancer cells failed to grow when seeded directly into implanted mouse bone but formed tumors reliably in human bone implants. Therefore, both specific adhesion mechanisms and species-specific factors may help explain the difficulty in obtaining skeletal metastasis in other rodent models.

Once metastatic cells arrive in the bone marrow compartment, they must grow to form a tumor. Results from our end organ growth assay clearly showed that the three human prostate cancer cell lines preferred to proliferate and form tumors in the human bone environment in comparison with the human lung, mouse bone, and mouse s.c. environments. In 1889, Paget (4) noted that breast cancer consistently metastasized to specific organs. This observation led to the seed-and-soil hypothesis of metastasis, whereby certain organs provide a favorable environment for the growth of certain tumor cells. Our observations are consistent with the hypothesis of Paget and suggest that human bone provides a supportive environment for both the targeting and growth of human prostate cancer cells. Results of a number of studies have demonstrated the inductive capacity of bone and bone-derived factors on the growth of prostate cancer cells. Gleave et al. (31) noted that soluble factors produced by bone fibroblasts could induce tumor formation by LNCaP in nude mice, suggesting that paracrine interactions might be involved. Tissue culture studies have also shown that soluble factors derived from bone marrow (32) or osteoblast-like cells (33) could stimulate growth of various prostate cancer cell lines. Using coculture techniques, Lang et al. (34) found that close contact with bone marrow stromal cells in culture can stimulate the growth of cells derived from primary prostate tumors. Close physical interaction with bone cells has also been shown to enhance tumorigenicity of normally nontumorigenic LNCaP prostate cancer cells (35). s.c. tumors formed in nude mice after coinjection of LNCaP cells with either bone or prostate fibroblasts but not with kidney or lung fibroblasts. Our present observations also suggest that human bone provides a more supportive environment than human lung. A combination of the SCID-hu system and in vitro studies is likely to be useful in defining the factors important to prostate cancer growth in bone in vivo.

The choice to use fetal bones rather than adult bones in this study was based on the success of previous studies using this system (12, 13) and the observations of Heike et al. (36), who found that implanted adult human bone fragments did not stimulate an angiogenic response and required constant cytokine infusion to prevent degeneration and fibrosis of the marrow compartment. It is possible that some biological factors are different between adult and fetal bones. For example, hematopoietic cells from implanted human marrow retain some fetal characteristics (14). Nonetheless, the SCID-human bone system is still a valuable tool to begin addressing specific human-human interactions involved in bone metastasis, including growth factors, adhesion molecules, extracellular matrix components, and cell-cell contact. In addition, the surgical procedures are simple, measurements are easy and accurate, and the resulting tumors cause minimal discomfort to the animals, allowing longer-term studies. Use of the SCID-hu model may also allow more accurate prediction of the response of metastatic cancer cells in the bone to therapeutic agents (37) and will be helpful in the development of new therapies targeted at sites of interaction between prostate cancer cells and the human bone environment.

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Jeffrey A. Nemeth, John F. Harb, Ubirajara Barroso, Jr., et al.


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