Tumor αvβ3 Integrin Is a Therapeutic Target for Breast Cancer Bone Metastases

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Abstract
In breast cancer bone metastasis, tumor cells stimulate osteoclast-mediated bone resorption, and bone-derived growth factors released from resorbed bone stimulate tumor growth. The αvβ3 integrin is an adhesion receptor expressed by breast cancer cells and osteoclasts. It is implicated in tumor cell invasion and osteoclast-mediated bone resorption. Here, we hypothesized that the therapeutic targeting of tumor αvβ3 integrin would prevent bone metastasis formation. We first showed that, compared with mock-transfected cells, the i.v. inoculation of αvβ3-overexpressing MDA-MB-231 breast cancer cells in animals increased bone metastasis incidence and promoted both skeletal tumor burden and bone destruction. The direct inoculation of αvβ3-overexpressing transfectants into the tibial bone marrow cavity did not however enhance skeletal tumor burden and bone destruction, suggesting that αvβ3 controls earlier events during bone metastasis formation. We next examined whether a nonpeptide antagonist of αvβ3 (PSK1404) exhibits meaningful antitumor effects in experimental breast and ovarian cancer bone metastasis. A continuous PSK1404 treatment, which inhibited osteoclast-mediated bone resorption in an animal model of bone loss, substantially reduced bone destruction and decreased skeletal tumor burden. Importantly, a short-term PSK1404 treatment that did not inhibit osteoclast activity also decreased skeletal tumor burden and bone destruction. This dosing regimen caused a profound and specific inhibition of bone marrow colonization by green fluorescent protein, αvβ3-expressing tumor cells in vivo and blocked tumor cell invasion in vitro. Overall, our data show that tumor αvβ3 integrin stands as a therapeutic target for the prevention of skeletal metastases. [Cancer Res 2007;67(12):5821–30]

Introduction
Bone metastases are common complications of breast cancer (1). Most often osteolytic, or, to a lesser extent, osteoblastic or mixed, bone metastases can be fatal or may rapidly impede the quality of life (1). Bone-residing breast cancer cells do not directly destroy bone (1, 2). Instead, they secrete molecules, such as parathyroid hormone-related protein, interleukins (IL-6, IL-8, and IL-11), and prostaglandins that stimulate the activity of bone-resorbing cells (osteoclasts), leading to osteolysis (1, 3–6). These observations (1–6) have provided the rationale for using bisphosphonates (as inhibitors of osteoclast-mediated bone resorption) in the treatment of breast cancer patients with bone metastases (1). Yet, these treatments are only palliative and do not provide a life-prolonging benefit to patients with advanced disease. Molecular mechanisms involved in bone colonization by breast cancer cells need therefore to be understood to develop new therapies directed toward early bone metastatic processes.

Integrins constitute a family of cell surface receptors that are heterodimers composed of noncovalently associated α and β subunits (7). Some studies support the concept that integrins mediate metastasis in bone (8–12). Bone colonization by prostate cancer cells is mediated by αvβ3 integrin (8). The expression of αvβ3 integrin by breast cancer cells has been also associated with bone metastasis (9–12). For instance, by in vivo selection of MDA-MB-231 breast cancer cells, we have isolated a cell subpopulation (called B02) that only metastasizes to bone and constitutively overexpresses αvβ3 integrin (9). Similarly, the de novo expression of αvβ3 in 66c4 breast cancer and Chinese hamster ovary (CHO) ovarian cancer cells that metastasize to lungs, but not to bone, is sufficient to promote their dissemination to bone (9, 10). Finally, αvβ3 integrin cooperates with bone sialoprotein (BSP) and matrix metalloproteinase-2 (MMP-2) in promoting osteotropic cancer cell invasion (11, 12). These observations (9–12) were in line with a previous study showing that bone-residing breast cancer metastases express elevated levels of αvβ3 integrin compared with primary breast carcinomas (13). Osteoclasts also express αvβ3 integrin (14), and selective inhibitors of αvβ3 have been shown to inhibit osteoclast-mediated bone resorption in animal models of osteoporosis (15, 16) and malignant osteolysis (17, 18). For instance, the treatment of animals with an anti-β3 antibody blocks the formation of osteolytic lesions caused by PC-3 prostate cancer cells that do not express αvβ3 integrin (17). The preventive treatment of animals bearing MDA-MB-435 breast cancer cells with a peptidomimetic inhibitor of αvβ3 also reduces bone destruction (18).

Here, we present in vivo evidence that tumor αvβ3 integrin participates in the development of experimental breast cancer bone metastases and that a selective αvβ3 nonpeptide antagonist not only inhibits osteoclast-mediated bone resorption in animal models of bone metastasis but also blocks bone colonization by αvβ3-expressing cancer cells.

Materials and Methods
Immunohistochemistry. Eight pairs of human primary breast carcinomas and their bone metastases were selected from the tumor bank of the...
Centre Léon Bérard (Lyon, France). The tumor and metastatic material was fixed in Bouin Hollande and then embedded in paraffin. Four-micrometer-thick tissue sections were deparaffinized and rehydrated, and endogenous peroxidase activity was blocked in a sterile water solution containing 5% hydrogen peroxide. Tissue sections were then pretreated in a citrate buffer (pH 6.0) for 50 min at 95°C in a water bath. After antigen retrieval in citrate buffer, tissue sections were incubated for 1 h at room temperature with mouse monoclonal antibody (mAb) 52Z1 directed against the β3 integrin subunit [1:200 dilution in PBS containing 2 mg/mL bovine serum albumin (BSA; Chemicon)]. After washing, tissue sections were incubated with a biotinylated secondary antibody bound to a streptavidin-peroxidase conjugate (LSAB+ kit, DAKO), and the signal was developed with diaminobenzidine. Tissue sections were then counterstained with hematoxylin, dehydrated, and mounted.

Scoring of β3 integrin immunostaining in breast cancer tissue specimens. The immunostaining intensity was evaluated independently by two investigators (L.T. and S.G.). The intensity of the staining was scored arbitrarily as follows: negative (−), weak (+), moderate (2+), and strong (3+). In case of disagreement between examiners, slides were reviewed and a consensus opinion was obtained.

Cell lines and transfection. Human MDA-MB-231 breast carcinoma cells were obtained from the American Type Culture Collection. Characteristics of CHO-β3wt cells, stably transfected to de novo express αβ3 integrin, mock-transfected CHOdhfr cells, and MDA-MB-231/B02 cells, constitutively overexpressing integrin α5β3, were described elsewhere (9). CHO-β3wt, CHOdhfr, and MDA-MB-231/B02 cell lines were stably transfected to express green fluorescent protein (GFP) as described previously (19). MDA-MB-231 cells were transfected with a full-length human β3 cDNA (20) with the use of TransFast (Promega) to overexpress α5β3 integrin in these cells. Stable transfectants were selected with geneticin (1 mg/mL for 4 weeks).

Selection of the clones was obtained after growing the cells for 2 weeks in the presence of puromycin (2 μg/mL). Two transfectants overexpressing α5β3 integrin (clones #30.1 and #14.3) and one transfectant expressing the empty vector (clone EV1.4) were used in the present study. Transfectants and cell lines were routinely cultured in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (FBS; Bio-Media) and 1% (v/v) penicillin/streptomycin (Life Technologies) at 37°C in a 5% CO2 incubator.

Reverse transcription, standard, and quantitative PCR. Total RNA from MDA-MB-231 transfectants was extracted using Total RNA Isolation System (Promega). cDNA was synthesized using Moloney murine leukemia virus-1 (Promega). Primers for human β3 integrin subunit were designed from the β3 gene (National Center for Biotechnology Information accession number J02703) using nucleotides 1956–1961 as the forward primer and nucleotides 2259–2237 as the reverse primer. PCRs were run using a program consisting of 40 cycles of 95°C for 15 s, 64°C for 6 s, and 72°C for 15 s. Human β3 mRNA was quantified by real-time PCR using the Master SYBR Green 1 kit (Roche Diagnostics). The fluorescence data were quantitatively analyzed by using serial dilution of control samples included in each reaction to produce a standard curve. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression was analyzed in parallel to confirm the use of equal amount of cDNAs in each reaction. Results were expressed as the ratio of β3 to GAPDH gene expression in each transfectant.

Antibodies and nonpeptidic αβ3 integrin antagonist. Function-blocking mouse mAb LM609 directed against αβ3 integrin and mouse mAbs directed against β3 integrin subunit (clone SZ21) and αβ3 (clone P1F6) and αβ3 (clone E7P6) integrins were purchased from Chemicon. Phycocyanin-conjugated antimouse IgG and mouse mAbs directed against α1 (clone HP2B6), α2 (clone G9), α3 (clone M-KID2), α5 (clone HP2/1), α5 (clone SAM-1), α6 (clone GoH3), β1 (clone K20), and β1 (clone ASC-3) integrin subunits were purchased from Coulter/Immunotech. Mouse mAb MOPC21 was obtained from ICN Pharmaceuticals.

Nonpeptidic αβ3 integrin antagonist PSK1404 has been described as a member of a new series of highly potent and selective RGD peptideomimetic αβ3 antagonists that contain acylguanidines as an arginine replacement (21). PSK1404 is selective for α5β3 (IC50 for fibronogen binding to human recombinant α5β3 integrin, 10,000 nmol/L; IC50 for vitronectin binding to human recombinant α5β3 integrin, 2 nmol/L; ref. 22).

Western immunoblotting. The immunodetection of the β3 integrin subunit in MDA-MB-231 transfectants was done as described previously (9).

Flow cytometry analysis. Cell surface expression of integrins by MDA-MB-231 transfectants was analyzed via a Galaxy flow cytometer (DAKO) as described previously (9).

Cell adhesion, invasion, and proliferation assays. All of the different experimental procedures were essentially as described (6, 9). Briefly, tumor cells resuspended in RPMI 1640 containing 1% (v/v) BSA (0.12 × 106 cells/mL) were plated in 100-mm bacteriologic Petri dishes dotted with BSA. After a 3-h incubation at 37°C, nonadherent cells were removed, and adherent cells were fixed, stained, and counted via microscope. Cell invasion experiments were done using Bio-Coat cell migration chambers (Becton Dickinson) coated with basement membrane Matrigel (30 μg/filter). Tumor cells resuspended in RPMI 1640 containing 1% (v/v) BSA were added to the upper chamber (5 × 105 cells/0.5 mL), and the chemoattractant (10% FBS) was placed in the lower chamber (0.75 mL/well). After a 24-h incubation at 37°C, the noninvading cells were removed and the invading cells on the under surface of the 8-μm-diameter pore size filter were fixed, stained, and counted via microscope. For cell proliferation experiments, tumor cells resuspended in complete RPMI 1640 were seeded in 96-well plates (500 cells/100 μL/well). After a 24-h incubation at 37°C, growing cells were washed and further cultured in complete medium for 5 days. Cell proliferation was measured spectrophotometrically at 550 nm using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (9).

Animals. All procedures involving animals, including housing and care, methods of euthanasia, and experimental protocols, were conducted in accordance with a code of practice established by the local ethical committee (CREA, Lyon, France). These studies were monitored on a routine basis by the attending veterinarian to ensure continued compliance with the proposed protocols. Four-week-old female BALB/c homozygous (nu/nu) athymic mice and sham-operated or ovariectomized wild-type (WT) BALB/c mice of 4 weeks of age were obtained from Charles River.

Animal models of bone metastasis. Bone metastasis experiments in animals were conducted as described previously (6, 9, 19). Briefly, MDA-MB-231 transfectants overexpressing integrin α5β3 (3 × 105 cells in 100 μL PBS) were inoculated into the tail vein of anesthetized nude mice. Alternatively, MDA-MB-231/B02 (5 × 106 cells in 100 μL PBS) or CHO-β3wt cells (106 cells in 100 μL PBS) were inoculated i.v. into animals. Radiographs of anesthetized animals were taken weekly with the use of MIN-R2000 films (Kodak) in an MX-20 cabinet X-ray system (Faxitron X-ray Corp.). The area of osteolytic lesions was measured using a Visiobal 2000 computerized image analysis system (Explora Nova) and the extent of bone destruction per animal was expressed in square millimeter as described previously (6, 9, 19). Metastatic animals were killed by cervical dislocation at day 21 (CHO-β3wt cells), day 30 (MDA-MB-231/B02 cells), or day 42 (MDA-MB-231 transfectants) after tumor cell inoculation, and bones were collected for histologic analysis.

Animal model of intraosseous tumorigenesis. For intraosseous xenograft experiments in mice, a small hole was drilled with a 30-gauge sterile needle through the tibial plateau with the knee flexed. Using a new sterile needle fitted to a 50-μL sterile Hamilton syringe (Hamilton Co.), a single-cell suspension (3 × 106 cells in 30 μL PBS) of α5β3-overexpressing MDA-MB-231 cells (clone #30.1) or mock-transfected cells (clone EV1.4) was then carefully injected in the bone marrow cavity. The progression of osteolytic lesions was monitored by radiography as described above. Animals were killed 42 days after tumor cell inoculation, and bones were collected for histologic analysis.

Animal models of homing of GFP-expressing tumor cells to bone. MDA-MB-231/B02 cells (5 × 105 in 100 μL PBS) and CHO-β3wt and mock-transfected CHO cells (106 cells in 100 μL PBS) that had been stably transfected with the gene encoding GFP were injected into the tail vein of anesthetized animals. None of these mice had radiographic evidence of bone destruction on the day they were killed. For each mouse, long bones were collected and the bone marrow was flushed out. Bone marrow cells
were then analyzed by flow cytometry for the detection of GFP-expressing cells.

**PSK1404 treatment protocols.** For the treatment of animals, we used a continuous or a short-term treatment protocol with nonpeptide $\alpha_{v}\beta_{3}$ integrin antagonist PSK1404. In the continuous treatment protocol, PSK1404 administration (10 mg/kg, twice daily, s.c.) to BALB/c nude mice was initiated at the time of tumor cell inoculation and continued until day 21 (CHO-$\beta_{3}$wt), day 30 (MDA-MB-231/B02), or day 42 (MDA-MB-231 transfectants). The progression of osteolytic lesions was monitored weekly by radiography. Sham-operated and ovariectomized BALB/c mice (used here as an animal model of osteoporosis) received a similar PSK1404 dosing regimen for 30 days to evaluate, in the absence of tumor cells, the effect of the drug on ovariectomy-induced bone loss. At the end of each protocol, animals were killed by cervical dislocation and bones were collected for histologic analysis.

**Bone histology.** Hind limbs from animals were fixed and embedded in methylmethacrylate. Seven-micrometer sections of undecalcified long bones were then cut with a microtome (Polycut E, Reichert-Jung) and stained with May-Grunwald-Giemsa or Goldner’s trichrome. Histologic analysis was performed by light microscopy. The extent of bone destruction and skeletal tumor burden was also evaluated by histomorphometry.

**Figure 1.** Tumor $\alpha_{v}\beta_{3}$ integrin expression and bone metastasis. A, immunohistochemical detection of $\beta_{3}$ integrin subunit in the primary breast carcinoma and bone metastasis of the same patient. Magnification, x20. Immunohistochemistry was done using $\beta_{3}$-specific, mouse mAb SZ21. Almost all breast cancer cells were strongly positive for $\beta_{3}$ integrin protein expression. B, overexpression of $\alpha_{v}\beta_{3}$ in MDA-MB-231 breast cancer cells. PCR: $\beta_{3}$ and GAPDH mRNA expression in parental MDA-MB-231 cells, mock-transfected MDA-MB-231 cells (EV#1.4), and $\alpha_{v}\beta_{3}$-overexpressing clones #30.1 and #14.3 cells. Reverse transcription-PCR fragments were separated on a 2% agarose gel and stained with ethidium bromide. Numbers correspond to real-time PCR quantification data of the $\beta_{3}$ mRNA copy number relative to that of GAPDH mRNA for each clone (mean ± SD; $P<0.001$ for #30.1 and #14.3 clones when compared with parental and EV#1.4 cells). Western blotting (WB): cell extracts (40–50 μg proteins/lane) were electrophoresed on a 7% Laemmli SDS-polyacrylamide gel under nonreducing conditions and then transferred to polyvinylidene difluoride membranes and immunoblotted with mAbs against human $\beta_{3}$ integrin (clone SZ21) or tubulin (used as an internal control for equal protein loading). Fluorescence-activated cell sorting (FACS): FACS analysis for cell surface expression of $\alpha_{v}\beta_{3}$ in parental MDA-MB-231 cells, EV#1.4 cells, and clones #30.1 and #14.3. The detection was done using mouse mAb LM 609 that recognizes the $\alpha_{v}\beta_{3}$ complex (red histograms). Background fluorescence was detected with isotype-matched negative control mouse mAb MOPC21 (white histograms). Y axis, fluorescence intensity (log scale). X axis, the number of cells per channel (events). D, intraosseous growth of EV#1.4 and #30.1 cells. Cell suspensions ($3 \times 10^{6}$ cells) were injected intratibially. Forty-two days after tumor cell inoculation, the extent of bone destruction and skeletal tumor burden was the same in animals bearing EV#1.4 or #30.1 tumors. Bar, 200 μm.
analyses were done on longitudinal medial sections of tibial metaphysis by using a Visiolar 2000 computerized image analysis system as described previously (6, 9, 19).

**Statistical analysis.** All data were analyzed with the use of StatView software (version 5.0; SAS Institute, Inc.). Statistical analyses were carried out by doing an unpaired Student’s t test or ANOVA followed by a Fisher’s protected least significant difference test. P values <0.05 were considered statistically significant.

**Results**

Expression of $\beta_3$ integrin subunit in pairs of human primary breast carcinomas and their matching bone metastases. As a first step toward evaluating tumor $\alpha_v\beta_3$ integrin in breast cancer bone metastasis, we did immunohistochemistry on the primary breast carcinoma and bone metastases of the same patient using mAb SZ21 directed against the $\beta_3$ integrin subunit. Eight pairs of primary breast tumors and their matching bone metastases were studied. As shown in Fig. 1A, all of the matching primary breast and metastatic tumors expressed $\beta_3$, and the immunostaining was always homogenous (80–90% of the tumor cells were positive for $\beta_3$). The scoring of $\beta_3$ staining intensity in pairs of primary tumors and bone metastatic specimens showed a moderate-to-strong staining in tumor cells from seven of eight patients, whereas a weak staining of tumor cells was observed in the primary tumor and matching bone metastatic lesion from one patient. As opposed to what has been reported previously by Liapis et al. (13), we did not notice any increase in the $\beta_3$ immunostaining intensity of bone-residing breast cancer cells when compared with that observed in tumor cells from matched primary carcinomas.

Overexpression of $\alpha_v\beta_3$ integrin in breast cancer cells increases the incidence and formation of osteolytic lesions in animals. Because immunohistochemical data indicated a possible role of $\alpha_v\beta_3$ integrin in the pathogenesis of breast cancer bone metastases, human MDA-MB-231 breast cancer cells were stably transfected to overexpress $\alpha_v\beta_3$ integrin. Two transfectants (clones $\#30.1$ and $\#14.3$) were selected based on their specific and high expression of $\beta_3$ subunit mRNA (real-time PCR) and protein (Western blotting) when compared with that observed in mock-transfected (clone EV#1.4) and parental MDA-MB-231 cells (Fig. 1B). The flow cytometry analysis of clones $\#30.1$ and $\#14.3$ for the cell surface expression of $\alpha_v\beta_3$ integrin confirmed Western blot data (Fig. 1B) and showed that $\alpha_v\beta_3$ integrin overexpression did not modify the cell surface expression levels of $\alpha_5\beta_3$, $\alpha_v\beta_6$, $\alpha_v$, $\alpha_2$, $\alpha_3$, $\alpha_5$, $\beta_3$, and $\beta_4$ integrins (Supplementary Fig. S1).

MDA-MB-231 transfectants were then injected into the tail vein of animals to examine the contribution of $\alpha_v\beta_3$ integrin in the development of bone metastases *in vivo*. The overexpression of $\alpha_v\beta_3$ in breast cancer cells was associated with a higher bone metastasis incidence in animals (Table 1). The follow-up by radiography of metastatic animals bearing $\alpha_v\beta_3$-overexpressing tumors also showed a 2-fold increase in bone destruction compared with that of mice bearing EV#1.4 tumors (Fig. 1C; Table 1).

Histomorphometric analysis of hind limbs with metastases showed that animals bearing $\alpha_v\beta_3$-overexpressing tumors had statistically significantly lower ratios for bone volume relative to tissue volume (BV/TV), indicating a higher bone destruction compared with mice bearing mock-transfected EV#1.4 tumors (Fig. 1C; Table 1). The skeletal tumor burden relative to soft tissue volume ratio (TB/STV) in animals bearing $\alpha_v\beta_3$-overexpressing tumors was also markedly increased compared with mice bearing EV#1.4 tumors (Fig. 1C; Table 1). In sharp contrast, when EV#1.4 and $\#30.1$ cells were directly injected into the tibial bone marrow cavity, mice bearing EV#1.4 or $\#30.1$ cells had a similar extent of bone destruction [$11.3 \pm 1.1 \text{ mm}^2 (n = 5)$ and $7.5 \pm 3.1 \text{ mm}^2 (n = 5)$, respectively] and skeletal tumor burden (Fig. 1D). The larger bone metastatic lesions in animals bearing $\alpha_v\beta_3$-overexpressing tumors were therefore not directly related to $\alpha_v\beta_3$ integrin overexpression but an indirect result of the greater number of $\alpha_v\beta_3$-expressing tumor cells residing in the bone marrow that stimulated osteoclast-mediated bone resorption.

All of the transfectants proliferated at a similar rate *in vitro*, irrespective of $\alpha_v\beta_3$ integrin expression (Supplementary Fig. S2A). In contrast, there was a 2- to 3-fold increase in the attachment of $\#30.1$ and $\#14.3$ cells to BSP when compared with that observed with EV#1.4 and parental MDA-MB-231 cells (Supplementary Fig. S2B). Similarly, there was a substantial gain in invasion of $\#30.1$ and $\#14.3$ cells when compared with EV#1.4 and parental cells (Supplementary Fig. S2C). The increased invasion of $\alpha_v\beta_3$-expressing transfectants was specifically and statistically significantly inhibited by anti-$\alpha_v\beta_3$ antibody LM609 when compared with that observed with negative control antibody MOPC21 and anti-$\alpha_v\beta_3$ antibody P1F6 (Supplementary Fig. S2D).

Effect of a nonpeptide $\alpha_v\beta_3$ integrin antagonist (PSK1404) on bone metastasis formation caused by $\alpha_v\beta_3$-expressing cancer cells. Because osteoclasts express $\alpha_v\beta_3$ integrin (14), we...
first assessed the antiresorptive potency of PSK1404 in a mouse model of bone loss caused by ovariectomy. Histomorphometric measurement of tibial metaphyses from placebo-treated ovariectomized wild-type BALB/c mice showed a bone loss, as judged by the marked reduction of the BV/TV ratio (40% reduction) compared with that of sham-operated animals (Fig. 2A). Bone loss induced by ovariectomy was completely prevented on continuous treatment of ovariectomized animals with PSK1404, when using a dosing regimen of 10 mg/kg, given s.c., twice daily for 28 or 3 d. Histomorphometric analysis of legs revealed that the bone volume relative to tissue volume was significantly decreased in ovariectomized animals. A continuous treatment (28 d) with PSK1404 completely prevented bone loss associated with ovariectomy, as opposed to a short-term PSK1404 treatment (3 d). *, *P < 0.001 using unpaired Student's t test. B, animals inoculated with B02 breast cancer cells were treated continuously with PSK1404 (10 mg/kg, twice daily, s.c.) or the vehicle from the time of tumor cell inoculation until day 30. Alternatively, PSK1404 administration was initiated 1 d before B02 tumor cell inoculation and discontinued 2 d later. Radiographic and histologic analyses were done 30 d after tumor cell inoculation. A continuous or a short-term PSK1404 treatment substantially reduced bone destruction and skeletal tumor burden.

Next, we validated the antiresorptive effect of PSK1404 in animal models of bone metastasis caused by #30.1 breast cancer cells, B02 breast cancer cells, or CHO-β3wt ovarian cancer cells. PSK1404 (at a dose of 2 × 10 mg/kg/d, given continuously until the end of the protocols) significantly reduced the extent of osteolytic lesions in the three different animal models of bone metastasis, as judged by radiography (Fig. 2B; Table 2). Histomorphometric analysis confirmed radiographic analysis and showed that bone destruction observed in animals bearing #30.1, B02, or CHO-β3wt cells was substantially reduced on PSK1404 treatment (Fig. 2B; Table 2). A PSK1404 treatment also markedly reduced skeletal tumor burden in animals when compared with the vehicle (Fig. 2B; Table 2).

We then examined whether a 3-day treatment with PSK1404, which did not prevent bone loss in ovariectomized animals (Fig. 2A), inhibits bone metastasis formation. Compared with the vehicle, a 3-day PSK1404 treatment statistically significantly decreased the extent of bone destruction and skeletal tumor burden in animals bearing B02 or CHO-β3wt tumors, whereas it had no inhibitory effect in animals bearing #30.1 tumors (Fig. 2B; Table 3).

Effect of a short-term treatment with nonpeptide αVβ3 integrin antagonist PSK1404 on bone marrow colonization by αVβ3-expressing cancer cells. The detection of fluorescence expressed by tumor cells is a highly sensitive method to detect the early development of bone metastasis (19). Our use of B02 or
CHO-β3wt cells that expressed a stably transfected gene encoding GFP (19) allowed us to examine whether mice treated for 3 days with PSK1404 displayed evidence of GFP-expressing cancer cells in the bone marrow on day 7 after tumor cell inoculation, at which time there was no radiographic evidence of osteolytic lesions (data not shown). We assumed that the detection of fluorescent cells by flow cytometry was an indication that cancer cells were present in the bone marrow.

Forward and side scatter (FSC/SCC) dot plot analysis of bone marrow cells of hind limbs from vehicle-treated animals showed that 15.5% of the bone marrow cells corresponded to B02/GFP cells (Fig. 3B). By contrast, the bone marrow cell population of hind limbs from mice treated with PSK1404 had only 1.8% of B02/GFP cells (Fig. 3B). There was an 88% reduction in B02/GFP tumor burden on PSK1404 treatment. Compared with the vehicle, PSK1404 also substantially decreased CHO-β3wt/GFP tumor burden by 95% (8.7% versus 0.4% of the bone marrow cell population; Fig. 3C), whereas it had no inhibitory effect on the tumor burden of GFP-expressing parental CHO cells that do not express αvβ3 (2.6% versus 3.4% of the bone marrow cell population; Fig. 3D). CHO-β3wt/GFP tumor burden in the bone marrow of hind limbs from vehicle-treated animals was 3.3-fold higher than that of mice bearing CHO/GFP cells, further indicating that αvβ3 integrin promoted bone colonization by cancer cells.

Table 2. Effect of a continuous treatment with nonpeptide αvβ3 integrin antagonist PSK1404 on the formation of experimental bone metastases

<table>
<thead>
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<th>Cell line*</th>
<th>Radiography</th>
<th>Histomorphometry</th>
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<tr>
<td></td>
<td>Osteolytic lesions (mm²/mouse)</td>
<td>BV/TV (%)</td>
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<tr>
<td></td>
<td>Vehicle</td>
<td>PSK1404</td>
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<td>#30.1</td>
<td>7.5 ± 1.7 (n = 5)</td>
<td>2.9 ± 2.2† (n = 6)</td>
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<td>B02</td>
<td>8.1 ± 3.5 (n = 15)</td>
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<td>CHO-β3wt</td>
<td>7.3 ± 2.9 (n = 13)</td>
<td>0.7 ± 0.8† (n = 8)</td>
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NOTE: All measurements were made at the end of the protocols. Data are expressed as the mean ± SD. n is the number of metastatic animals. n’ is the number of legs with bone metastasis.

*#30.1, MDA-MB-231 breast cancer cells stably transfected to overexpress αvβ3; B02, bone metastatic clone of MDA-MB-231 cells constitutively overexpressing αvβ3 (9); CHO-β3wt, CHO cells stably transfected to de novo express αvβ3 (9). Animals inoculated with each of the different cell lines received a treatment with PSK1404 (10 mg/kg) or the vehicle, given s.c., twice daily from the day of tumor cell inoculation to the end of the protocol.

†P < 0.05, when compared with vehicle-treated animals using unpaired Student’s t test.

‡P < 0.001, when compared with vehicle-treated animals using unpaired Student’s t test.

Table 3. Effect of a short-term treatment with nonpeptide αvβ3 integrin antagonist PSK1404 on the formation of experimental bone metastases

<table>
<thead>
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<td>7.3 ± 2.9 (n = 13)</td>
<td>1.3 ± 0.6† (n = 6)</td>
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NOTE: All measurements were made at the end of the protocols. Data are expressed as the mean ± SD. n is the number of metastatic animals. n’ is the number of legs with bone metastasis.

Abbreviation: n.d., not done.

*#30.1, MDA-MB-231 breast cancer cells stably transfected to overexpress αvβ3; B02, bone metastatic clone of MDA-MB-231 cells constitutively overexpressing αvβ3 (9); CHO-β3wt, CHO cells stably transfected to de novo express αvβ3 (9). Animals inoculated with each of the different cell lines received a treatment with PSK1404 (10 mg/kg) or the vehicle, given s.c., twice daily for 3 d.

†P < 0.05, when compared with vehicle-treated animals using unpaired Student’s t test.

‡P < 0.001, when compared with vehicle-treated animals using unpaired Student’s t test.
Table 2. Effect of a continuous treatment with nonpeptide αvβ3 integrin antagonist PSK1404 on the formation of experimental bone metastases (Cont’d)

<table>
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<th>TB/STV (%)</th>
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<td>80.8 ± 25 (n’ = 13)</td>
<td>0.3 ± 0.5† (n’ = 8)</td>
<td>1.84 ± 0.57 (n’ = 13)</td>
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Table 3. Effect of a short-term treatment with nonpeptide αvβ3 integrin antagonist PSK1404 on the formation of experimental bone metastases (Cont’d)

<table>
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<th>TB/STV (%)</th>
<th>Tumor area (mm²)</th>
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<tr>
<td>1.98 ± 0.09 (n’ = 5)</td>
<td>n.d.</td>
<td>1.41 ± 0.19 (n’ = 15)</td>
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<tr>
<td>62.2 ± 8.7 (n’ = 15)</td>
<td>6.4 ± 1.1† (n’ = 7)</td>
<td>1.84 ± 0.57 (n’ = 13)</td>
</tr>
<tr>
<td>80.8 ± 25 (n’ = 13)</td>
<td>4.3 ± 0.7† (n’ = 6)</td>
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increased the adhesiveness and invasiveness of MDA-MB-231 cells in vitro. Our results are in accordance with the observation that

Figure 3. Flow cytometry analysis of bone marrow cells isolated from vehicle- and PSK1404-treated animals bearing GFP-expressing tumor cells. A, FSC/SCC dot plots of bone marrow cells isolated from naive and metastatic animals. FSC/SCC variables were set using bone marrow cells from naive animals that did not receive tumor cells. A single-cell population is observed. In contrast, two cell populations are observed in the bone marrow from metastatic animals. B to D, animals receiving a 3-day treatment with PSK1404 or the vehicle were sacrificed 7 d after i.v. inoculation of either GFP-expressing B02 breast cancer cells that constitutively overexpress αvβ3 (B02/GFP), GFP-expressing CHO ovarian cancer cells stably transfected to express αvβ3 (CHO-h3wt/GFP), or GFP-expressing CHO ovarian cancer cells that do not express αvβ3 (CHO/GFP). Bone marrow cells were then analyzed by flow cytometry to detect the presence of fluorescent tumor cells. Top, FSC/SSC dot plots of bone marrow cells from metastatic animals. In metastatic animals, the normal bone marrow cell population is located in quadrant 3 (Q3), whereas tumor cells are in quadrants 2 and 4 (Q2 and Q4). Percentage of cells present in each quadrant. Bottom, flow cytometry histograms of bone marrow cells. Y axis, the number of cells per channel (events); X axis, the relative fluorescence intensity in arbitrary units (log scale).
critical for bone colonization by αvβ3-expressing cancer cells. Additional αvβ3-dependent mechanisms, such as tumor cell arrest during blood flow (29), may also be involved in bone metastasis formation. We therefore reasoned that a therapeutic approach that targets tumor αvβ3 could be an effective way to minimize bone colonization by breast cancer cells. We used a short-term preventive therapy with a nonpeptide αvβ3 integrin antagonist (PSK1404) to investigate whether it has the potential to inhibit bone metastasis formation. Notably, this short-term dosing regimen of PSK1404 did not inhibit bone resorption in ovariectomized animals, a feature that allowed us to uncouple the direct effects of PSK1404 to αvβ3-expressing tumor cells from osteoclast-mediated effects. We found that PSK1404 treatment of animals caused a profound and specific inhibition of the bone marrow colonization by αvβ3-expressing B02 and CHO tumor cells. As discussed previously, bone colonization by cancer cells likely involves early metastatic processes, such as tumor cell invasion. In this respect, PSK1404 blocked tumor cell invasion in vitro at concentrations that can be achieved in the plasma of metastatic animals, suggesting that a short-term preventive regimen of PSK1404 could inhibit tumor cell invasion in vivo. Moreover, it is interesting to notice that a short-term dosing regimen of PSK1404 not only decreased skeletal tumor burden but also reduced bone destruction in animals bearing αvβ3-expressing B02 or CHO tumor cells. These results are in accordance with the observation that tumor cells stimulate osteoclast-mediated bone resorption (1–6). They suggest that a short-term therapy with PSK1404 inhibits bone colonization by tumor cells, which in turn decreases the production of tumor-derived factors that are required for osteoclast-mediated bone resorption, thereby leading to a reduction of bone destruction.

Aside from our observation that a nonpeptide αvβ3 integrin antagonist may directly interfere with bone colonization by tumor cells, we used a continuous therapy with PSK1404 to determine whether this integrin antagonist also had the potential to directly inhibit osteoclast-mediated bone destruction. We found that PSK1404, at a dose that inhibited osteoclast-mediated bone resorption in ovariectomized animals, drastically reduced the formation of osteolytic lesions caused by three different αvβ3-expressing cancer cell lines. A continuous therapy with PSK1404 was even more effective than a short-term strategy for reducing bone destruction and skeletal tumor burden in animals bearing αvβ3-overexpressing MDA-MB-231 breast cancer cells (clone #30.1).

Our results are reminiscent of those obtained in a preventive study on animals bearing MDA-MB-435 breast cancer cells, in which a peptidomimetic inhibitor of αvβ3 (S247) reduces bone destruction and skeletal tumor burden (18). Likewise, the treatment of animals with an anti-β3 antibody blocks the formation of osteolytic lesions caused by PC-3 prostate cancer cells that do not express αvβ3 integrin (17). Thus, these results (this study and refs. 17, 18) highlight the importance of osteoclast αvβ3 integrin in mediating malignant osteolysis. These results are also in accordance with the “vicious cycle” theory (1), in which tumor cells stimulate osteoclast-mediated bone resorption and bone-derived growth factors released from resorbed bone stimulate tumor growth. They suggest, as observed previously for osteoclast inhibitors bisphosphonates and osteoprotegerin (30, 31), that the blockade of bone resorption by PSK1404 (S247 or the anti-β3 antibody) most probably deprives tumor cells of bone-derived growth factors that are required for tumor growth. Additional mechanisms, such as the inhibition of tumor angiogenesis, may also be involved in reducing skeletal tumor burden. Endothelial cell αvβ3 integrin is known to play a key role in tumor angiogenesis (32). We found that PSK1404 inhibited the formation of capillary-like tubes in vitro. The treatment of mice with an anti-β3 antibody also reduces angiogenesis in experimental prostate cancer bone metastases (17). Thus, a continuous regimen of PSK1404 may enable multiple inhibitory effects on cancer cells, endothelial cells, and osteoclasts, leading to inhibition of bone metastasis formation.

In conclusion, our study shows that αvβ3 integrin not only plays a causal role in the bone colonization by metastatic cells but also stands as a therapeutic target for the prevention of bone metastases.

Acknowledgments

Received 12/10/2006; revised 3/7/2007; accepted 4/3/2007.

Grant support: Institut National de la Sante et de la Recherche Medicale (the French National Agency for Health and Medical Research), Prokalia (Romainville, France), the French National League against Cancer, the Association for Cancer Research grants n°3502 and 7853, and the European Commission grant n° LSHC-CT-2004–503049 (P. Cézardin).

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We thank Dr. Larry Fisher (National Institute of Dental and Craniofacial Research, NIH, Bethesda, MD) for providing human recombinant BCP; technical platform CeCIL at the IFB62 for flow cytometry experiments; and Sophie Godillard and Julien Guglielm for excellent technical assistance.

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