CX3CR1 Is Expressed by Prostate Epithelial Cells and Androgens Regulate the Levels of CX3CL1/Fractalkine in the Bone Marrow: Potential Role in Prostate Cancer Bone Tropism

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Abstract

We have previously shown that the chemokine fractalkine promotes the adhesion of human prostate cancer cells to bone marrow endothelial cells as well as their migration toward human osteoblasts *in vitro*. Thus, the interaction of fractalkine with its receptor CX3CR1 could play a crucial role *in vivo* by directing circulating prostate cancer cells to the bone. We found that although CX3CR1 is minimally detectable in epithelial cells of normal prostate glands, it is overexpressed upon malignant transformation. Interestingly, osteoblasts, stromal and mesenchymal cells derived from human bone marrow aspirates express the cell-bound form of fractalkine, whereas the soluble form of the chemokine is detected in bone marrow supernatants. To investigate the mechanisms regulating the levels of soluble fractalkine in the bone marrow, we focused on androgens, which play a critical role in both prostate cancer progression and skeletal metastasis. Here, we show that dihydrotestosterone dramatically increases the cleavage of fractalkine from the plasma membrane of bone cells and its action is reversed by nilutamide—an antagonist of the androgen receptor—as well as the wide-spectrum inhibitor of matrix metalloproteases, GM6001. However, dihydrotestosterone was unable to induce fractalkine-cleavage from human bone marrow endothelial cells. Thus, androgens could promote the extravasation of CX3CR1-bearing cancer cells on a fractalkine concentration gradient, while leaving unaltered their ability to adhere to the bone marrow endothelium. In conclusion, our results indicate that CX3CR1, fractalkine, and the enzymes responsible for its cleavage might represent suitable targets for therapies aiming to counteract skeletal secondary tumors from prostate adenocarcinoma. [Cancer Res 2008;68(6):1715–22]

Introduction

The mechanisms by which cancer cells traveling through the circulatory system arrest into distant organs are still a matter of debate. However, it is likely that in addition to vascular patterns, unique characteristics of the endothelial cells lining the capillary beds of the colonized tissues play a role in cancer organ-tropism (1, 2). For instance, specific adhesion macromolecules might be expressed only by compatible cancer and endothelial cells, thus promoting the preferential, albeit not exclusive, arrest of cancer cells into a specific tissue. However, simply adhering to the endothelial wall will not suffice to invade an organ. Thus, similar to leukocytes and hematopoietic stem cells, cancer cells may migrate from the luminal side of the endothelial cells into the surrounding tissue in response to chemotactic molecules released by stromal cells. Chemokines—a family of chemotactic cytokines composed of more than 45 members (3, 4)—have been implicated in both the adhesion and extravasation of disseminated cancer cells (5). For instance, a recent seminal article shows that CXCL12/SDF-1α—by signaling through its receptor CXCR4—is involved in chemotactic and invasive responses of breast cancer cells *in vitro* and their induction of lung metastases in a mouse model (6).

The SDF-1/CXCR4 pair seems to exert a predominant role in cancer cell migration and chemoattraction, and its effect on the adhesion of cancer cells to bone marrow endothelium has also been proposed. However, because SDF-1 is a soluble chemokine, its presentation to circulating cancer cells must depend on its binding to cellular proteoglycans located on the surface of endothelial cells (7). Although this mechanism seems to be widely accepted (8–11), the resistance to the shear stress of the blood flow offered by SDF-1 while tethered to the endothelial wall raises some concerns (12). In addition, the proadhesive effect of SDF-1 is exerted indirectly, by inducing the expression of integrins and VCAM-1 through CXCR4 activation, similar to melanoma and small cell lung cancer cells (10, 13). Unlike SDF-1, fractalkine possesses intrinsic cell-adhesive properties and is constitutively expressed by human bone marrow endothelial (HBME) cells as a trans-membrane protein (14), an ideal feature to withstand the shear forces of the blood flow. In fact, immune cells expressing CX3CR1 directly bind and firmly adhere to fractalkine anchored to the surface of endothelial cells (15, 16). Interestingly, we have recently shown that human prostate cancer cells—studied in a dynamic adhesion assay and under physiologic flow conditions—attach to bone marrow endothelial cells in a fractalkine-dependent fashion (17). Furthermore, we found that fractalkine is also expressed by human osteoblasts in culture, which can cleave and release it into its soluble form and chemoattract prostate cancer cells *in vitro* (17). These results suggest that CX3CR1 and fractalkine could play a role in the prostate cancer cell adhesion and extravasation steps of skeletal metastasis; therefore, we decided to foster the translational significance of these previous observations by investigating the expression of this chemokine/receptor pair in prostate gland tissue specimens and bone marrow aspirates from human donors.

This study also aimed to determine whether the cleavage of fractalkine in its soluble form by human bone marrow cells could

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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be regulated by androgens. A recent clinical trial shows that the inhibitor of the androgen receptor bicalutamide induces a 33% reduction in the incidence of bone metastases in patients with localized or locally advanced prostate cancer (18), a likely consequence of the fact that bicalutamide negatively affects the survival of androgen-dependent cancer cells. As the androgen receptor is expressed by bone cells including osteoblasts (19–21), its activation could increase fractalkine cleavage and availability in the marrow stroma—by stimulating members of the ADAM matrix metalloproteinases (MMP) family (22, 23)—thereby promoting the establishment of a chemoattractant gradient for CX3CR1-expressing cells. Consequently, inhibition of the androgen receptor in cells of the bone marrow could also reduce fractalkine cleavage, thereby counteracting the extravasation of prostate cancer cells into the bone microenvironment.

Materials and Methods

Cell lines and cell cultures. The human bone marrow mesenchymal stem cells (hMSC), human bone stromal cells (hBSC), and normal human osteoblasts (NHOst)—all from male donors—were from Lonza Walkersville, Inc. The HBME-1 cells—originally isolated from a male donor by the group of Dr. Pienta (24)—were a gift from Dr. Mark Stearns (Drexel University College of Medicine, Philadelphia, PA). The hMSC were grown in medium containing 10% mesenchymal cell growth supplements, 2% L-glutamine, and 0.1% penicillin/streptavidin in mesenchymal stem cell basal medium (Lonza Walkersville, Inc.). The hBSC were grown in α-MEM containing 25% fetal bovine serum (FBS), 1% α-inositol, 1% β-mercaptoethanol, 1% folic acid, and 0.1% gentamicin. The NHOst were grown in osteoblast basal medium, containing 10% of FBS, 0.1% ascorbic acid, and 0.1% gentamicin in osteoblast basal medium (Lonza Walkersville, Inc.). The HBME-1 cells were grown in medium-199 containing 10% FBS, 10% human serum (Wisent, Inc.), and 1 ng/mL of β-fibroblast growth factor, 5 units/mL of heparin (Sigma-Aldrich), and 0.1% gentamicin.

Immunohistochemistry and tissue array analysis. Tissue microarrays were obtained from U.S. Biomax, Inc. For the detection of human CX3CR1, we used tissue arrays containing cores from 156 different cases of prostate adenocarcinoma, 10 specimens of normal tissue adjacent to cancer, and 31 normal prostate glands in total (PR802, PR242, PR483, PR951, PR481, and BC19111). For the detection of human fractalkine, we used an array containing bone marrow tissue cores from 27 different donors ranging from 30 to 75 years of age (PR802). Tissue arrays were deparaffinized with Histosolve (Dako Cytomation) and rehydrated with decreasing concentrations of ethanol. Endogenous peroxidase was quenched using methanol and hydrogen peroxide. Antigen retrieval was performed using DakoCytomation Target Retrieval Solution in a 95°C water bath for 30 minutes. Arrays were first blocked with 10% normal donkey serum in TBS for 60 minutes and then incubated in either antihuman CX3CR1 (Abcam 7201) at 3.3 μg/mL or antihuman fractalkine (R&D Systems AXV02) at 10 μg/mL in TBS at 4°C overnight. Primary antibody binding was detected using either biotin-conjugated donkey anti-rabbit or biotin-conjugated donkey anti-goat (Jackson ImmunoResearch), respectively, at 20 μg/mL in TBS for 2 hours. Signals were amplified with Vectastatin Elite ABC kit and visualized using the chromogenic visualization 3,3′-diaminobenzidine kit (Vector Laboratories). The tissue arrays were then dehydrated using increasing concentrations of ethanol and Histosolve. Permount solution (Fisher Scientific) was used for mounting.

Processed microarrays were examined using two different approaches: (*a*) tissue core staining was quantified and analyzed by acquiring a digital image of the entire array and processing it using the Metamorph software (Molecular Devices). Color images were converted to 8-bit gray scale images and gray pixel values from negative controls (primary antibody omitted) were averaged and subtracted from the gray level reading of each specimen exposed to the primary antibody; (*b*) cellular staining for each specimen was evaluated by light microscopy, using a Nikon Optiphoto-2 microscope connected to an Olympus DP-70 CCD color camera. For CX3CR1 detection, staining intensity was scored based on a scale in which negative specimens were graded 0 and strongly positive samples were graded 3 (also see Table 1). All the images were acquired using identical lamp settings and a shutter speed of 1/350 seconds without averaging.

Confocal microscopy. Cells were grown on 15 mm glass coverslips and fixed using a pH shift formaldehyde protocol, to avoid cell shrinkage and preserve the three-dimensional cellular organization. The same antibody used for Western blotting against fractalkine was employed for confocal microscopy analysis. A CY3-conjugated secondary antibody (Jackson ImmunoResearch), was used to detect the primary antibody. Samples were imaged using a Nikon PCM 2000 connected to a Nikon Diaphot 300 inverted microscope mounting a 60× 1.40 numerical aperture oil immersion objective. Images were collected using a step size of 0.5 μm along the *z* axis and at a resolution of 1,280 × 1,280 using the C-Imaging software.

Cell treatment with androgens and inhibitors. Cells were grown to ~90% confluence in their respective media. Prior to being exposed to dihydrotestosterone, used at 100 nmol/L, cells were moved in a culture medium containing charcoal-stripped FBS for 12 to 24 hours. For the experiments involving pharmacologic inhibitors, cells were preincubated for 30 minutes with either the androgen receptor inhibitor nilutamide (Sigma-Aldrich), used at 1 μmol/L, or the MMP inhibitor GM-6001 (Chemicon), used at 25 μmol/L, before treatment with dihydrotestosterone.

SDS-PAGE and Western blotting. Cell lysates were obtained using a lysis buffer containing Igepal CA-630 and DTT (Sigma), phosphatase, and protease inhibitor cocktails (Calbiochem). Protein concentrations of cellular lysates were determined using the bicinchoninic acid protein assay (Pierce). Samples containing equivalent amounts of proteins were resolved by SDS-PAGE using 10% polyacrylamide gels. The proteins were transferred onto Immobilon polyvinylidene difluoride membranes (Millipore Corporation). Membranes were then incubated in blocking buffer containing 25% non–animal protein blockers (Genotech) and 0.1% Tween 20 in TBS for 1 hour at room temperature. The membranes were then incubated overnight at 4°C with a rabbit polyclonal anti-fractalkine antibody specific for the chemotactic domain of human fractalkine (Torrey Pines Biolabs) at a concentration of 1 μg/mL in TBS containing 0.1% Tween 20. Horseradish peroxidase–conjugated protein A (Sigma-Aldrich) was used at 0.1 μg/mL to detect the primary antibody. Signal was detected using SuperSignal West Femto chemiluminescence reagents (Pierce) and visualized with a FluorChem imaging system (Alpha Innotech).

Detection of soluble fractalkine. Fractalkine in both human bone marrow aspirates and cellular supernatants were detected using the ELISA DuoSet kit (R&D Systems), after removal of the cellular fraction and as suggested by the manufacturer. Briefly, plates were coated and incubated overnight with the capture antibody at 4 μg/mL in saline solution. After washing and the addition of blocker (1% bovine serum albumin) the samples were added to the wells. The biotinylated detection antibody was used at 500 ng/mL, followed by streptavidin-horseradish peroxidase. A tetramethylbenzidine substrate solution was incubated for 30 minutes to detect the streptavidin bound to the detection antibody. The chromogenic reaction was then stopped with 50 μL of sulfuric acid and absorbance determined at 450 nm with correction set to 570 nm. A standard curve was generated using fractalkine as purified recombinant protein and sample concentrations were calculated using a linear regression equation.

Statistical analysis. Experimental results are expressed as mean ± SE. Statistical significance was determined with the one-way ANOVA test. Statistical significance was assumed at $P \leq 0.05$.

Results and Discussion

We hypothesize that circulating prostate cancer cells are able to locate to the bone marrow using a mechanism similar to that used by leukocytes migrating to inflamed tissues and involving CX3CR1 and its ligand fractalkine expressed on the surface of prostate and endothelial cells, respectively. A further step in the metastatic cascade could then be completed when prostate cancer cells adhering to the bone marrow endothelium respond to a gradient of

Table 1. Intensity and distribution of CX3CR1 detection in either normal, adjacent to cancer, or malignant prostate gland tissues

Tissues	0	0–1	1–2	2–3	Total
Normal	17	9	5	0	31
Adjacent to cancer	0	2	8	0	10
Malignant	53	32	54	17	156

NOTE: Staining intensity was scored based on a scale in which negative specimens were graded (0) and strongly positive samples were graded (3). To evaluate distribution, tissues with complete lack of staining were scored as (0); tissues prevalently negative for CX3CR1 displaying some areas of positive staining were scored as (0–1); tissues showing uniform staining for CX3CR1 were scored as either (1–2) or (2–3), based on the intensity of the signal observed.

chemoattractant molecules, which would drive their extravasation into the bone microenvironment. Fractalkine, in its soluble form—after being cleaved from the surface of bone marrow cells—could likely be involved in this process, as we have previously shown by an *in vitro* chemotaxis approach (17). To emphasize the clinical relevance of our model, we investigated CX3CR1 and fractalkine expression in human samples of prostate gland and bone marrow, respectively.

A first set of experiments was conducted to establish whether the CX3CR1 receptor for fractalkine was expressed by *ex vivo* specimens and employed microarrays assembled with tissue cores of prostate glands that were either normal or affected by adenocarcinoma. CX3CR1 expression was observed in normal prostate tissues, although the highest levels of signal intensity were detected exclusively in specimens from malignant glands (Table 1). The expression of CX3CR1 by the epithelial cells was confirmed by light microscopy (Fig. 1*A–C*; Supplementary Data 1); the specificity of the antibody used in this study for CX3CR1 was validated by the lack of staining observed in human tissue samples from heart, combined with the strong signal detected in samples of human lung, in agreement with previous studies by others (ref. 25; Supplementary Data 1).

These results suggest that the expression of the CX3CR1 chemokine receptor is a common feature of the prostate epithelium—as we previously reported using primary prostate cells in culture (17)—although malignant transformation seems to increase its expression levels (Table 1). Interestingly, specimens of morphologically normal prostate gland adjacent to cancer tissue were always found positive for CX3CR1 (Table 1). However, a relevant number of specimens from both normal and malignant prostate stained negative for CX3CR1. This could be due to histologic heterogeneity and different areas of the same gland might display diverse patterns of CX3CR1 expression. However, it could be alternatively hypothesized that CX3CR1 expression is a feature of selected

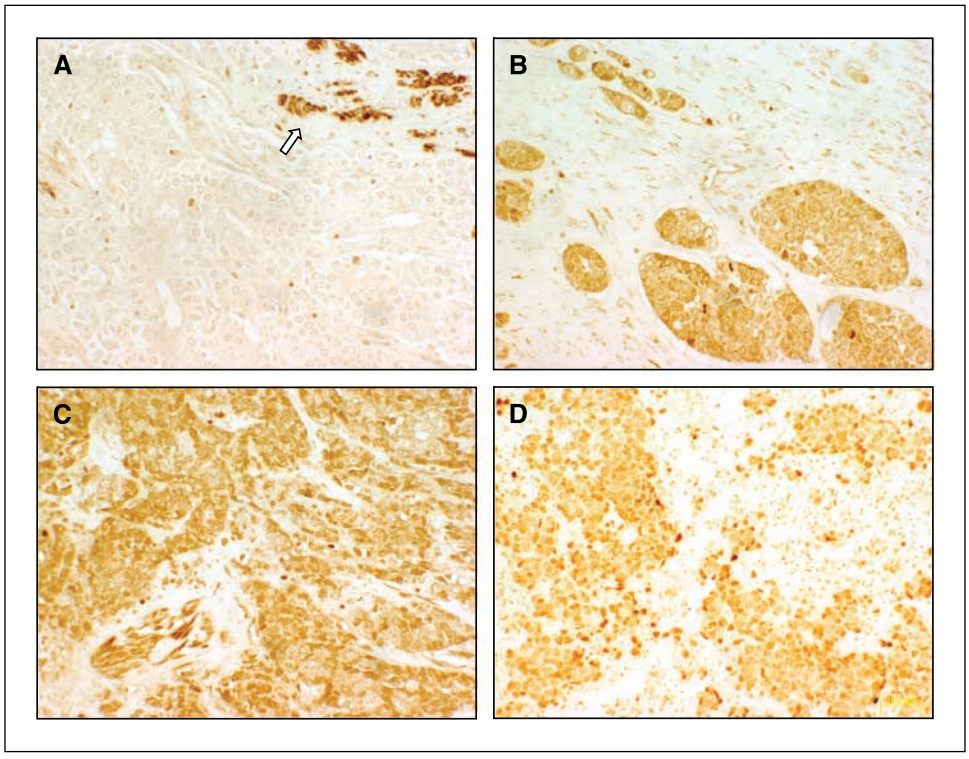

Figure 1. Expression of CX3CR1 in prostate glands from human donors. Normal and malignant prostate glands stain either negative (*A*) or show different staining intensities and distributions for CX3CR1 (*B* and *C*). As previously reported by others (34), smooth muscle cells were consistently found positive for CX3CR1 (*white arrow*). CX3CR1 expression was also detected in tissue cores of skeletal metastases obtained from subjects affected by prostate adenocarcinoma (*D*). Original magnification, ×200.

epithelial prostate phenotypes which, in case of malignant transformation, would thereby acquire a higher propensity to locate to fractalkine-expressing secondary tissues. The information regarding the postsurgery follow-up for the donors of each prostate tissue included in the microarrays we used was not available; therefore, the possible correlation between CX3CR1 expression and the occurrence of bone secondary tumors could not be addressed in the present study. However, we tested tissue cores of skeletal metastases from prostate adenocarcinoma obtained from four different donors and found them all positive for CX3CR1 expression (Fig. 1*D*).

According to a widely accepted model for the establishment of metastasis, a further step following the adhesion of circulating cancer cells to the endothelial wall is their migration and invasion into the surrounding tissue in response to chemoattractant molecules. We have previously shown that fractalkine could promote *in vitro* migration of CX3CR1-expressing cells (17); the production of this chemokine by bone-resident cells could therefore play a role in the invasion of the bone tissue by prostate cancer cells. To ascertain whether fractalkine is present in the bone marrow, we employed tissue arrays including samples from 27 different human donors and detected the chemokine with a signal intensity ranging from weak to moderate (Fig. 2*A*). The specificity of the primary antibody was validated using cores from human kidney tissue, which expresses fractalkine as well as liver, which is negative for this chemokine (ref. 14; Supplementary Data 2). Using a complementary approach, we isolated cellular fractions from bone marrow aspirates and detected the membrane-associated form of the chemokine by Western blotting (Fig. 2*B, top*). The cleavage of the full-length form of fractalkine from these cells would accumulate the soluble and chemoattractant form of the chemokine into the stroma and therefore induce CX3CR1-expressing cancer cells to extravasate into the bone marrow. To test this idea, we measured soluble fractalkine in human bone marrow aspirates—deprived of their cellular fraction—and found them positive for the chemokine in concentrations averaging 2 ng/mL (Fig. 2*B, bottom*). Interestingly, similar concentrations of this chemokine induce the *in vitro* migration of prostate cancer cells through a Matrigel-coated membrane (17). It should be emphasized that the fractalkine concentrations we measured *ex vivo* necessarily represent average values, and because of the inherent compartmentalization of the human bone marrow, it is likely that higher levels of this chemokine may exist in selected regions of the intact tissue.

The next series of experiments were conducted using single cell types isolated from human bone marrow and available through a commercial source. We used primary osteoblasts (NHOst), bone mesenchymal stem cells (hMSC), and bone stromal cells (BSC) and found them all positive for fractalkine expression, with BSC expressing the most, and NHOst the least, of the full-length form of the chemokine (Fig. 2*C*). In addition to the hematopoietic cell lineage, these three cell types represent the most relevant cellular fraction within the bone marrow microenvironment. The fact that fractalkine is ubiquitously expressed among these cells suggests that significant levels of this chemokine could be produced within the marrow regardless of the changes in the bone

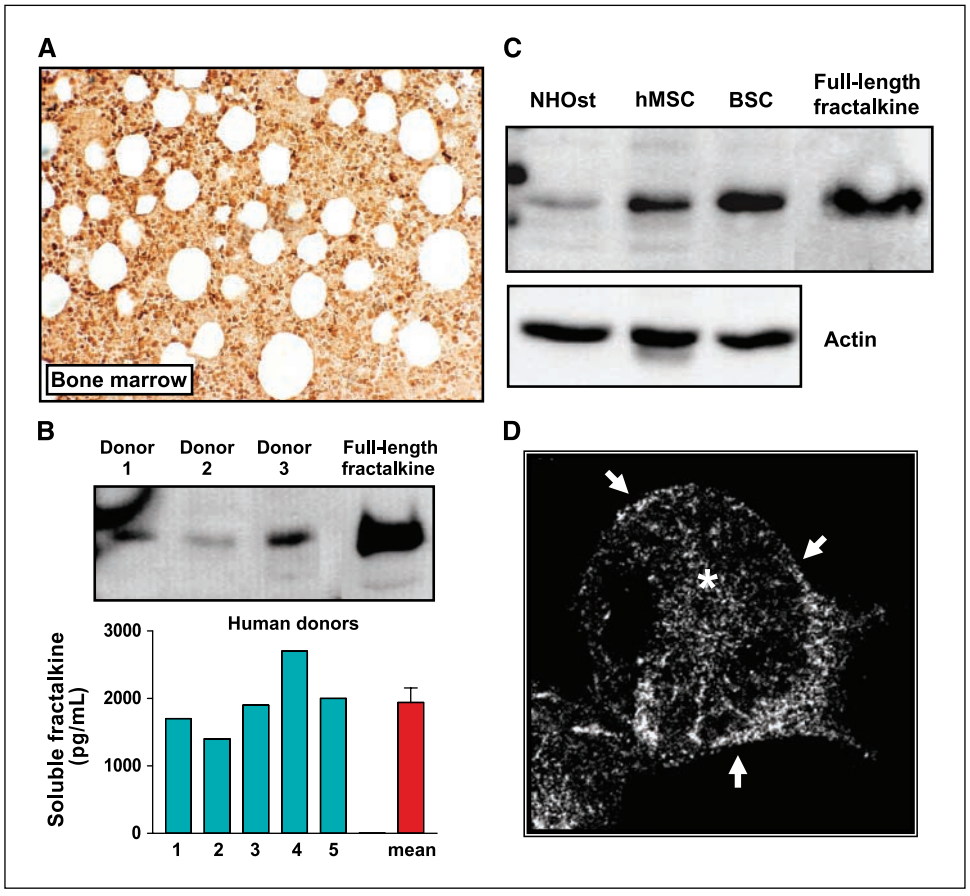

Figure 2. Detection of both cell-bound and soluble forms of fractalkine in human bone marrow. *A,* normal bone marrow stains positive for fractalkine expression, showing a staining diffused throughout the entire marrow stromal architecture. *B,* cells of the bone marrow obtained from aspirates of three human donors express the full-length and cell-bound forms of fractalkine when analyzed by Western blotting. A recombinant, full-length form of fractalkine was loaded along with the cell lysates in the same gel and used as a positive control (*top*). The supernatants of bone marrow aspirates from five different human donors were analyzed by ELISA and tested positive for the soluble form of fractalkine (*bottom*). *C,* when distinct human bone marrow cell types were analyzed by Western blotting, they were all found positive for the full-length form of fractalkine, with the lowest expression levels detected in osteoblasts (*NHOst*), the highest in BSC and hMSCs showing intermediate levels. *D,* the location of fractalkine at the plasma membrane level was confirmed by fluorescence confocal microscopy (*white arrows*) in the hMSC shown; *, a possible intracellular storage for the chemokine (26). Original magnification, ×600.

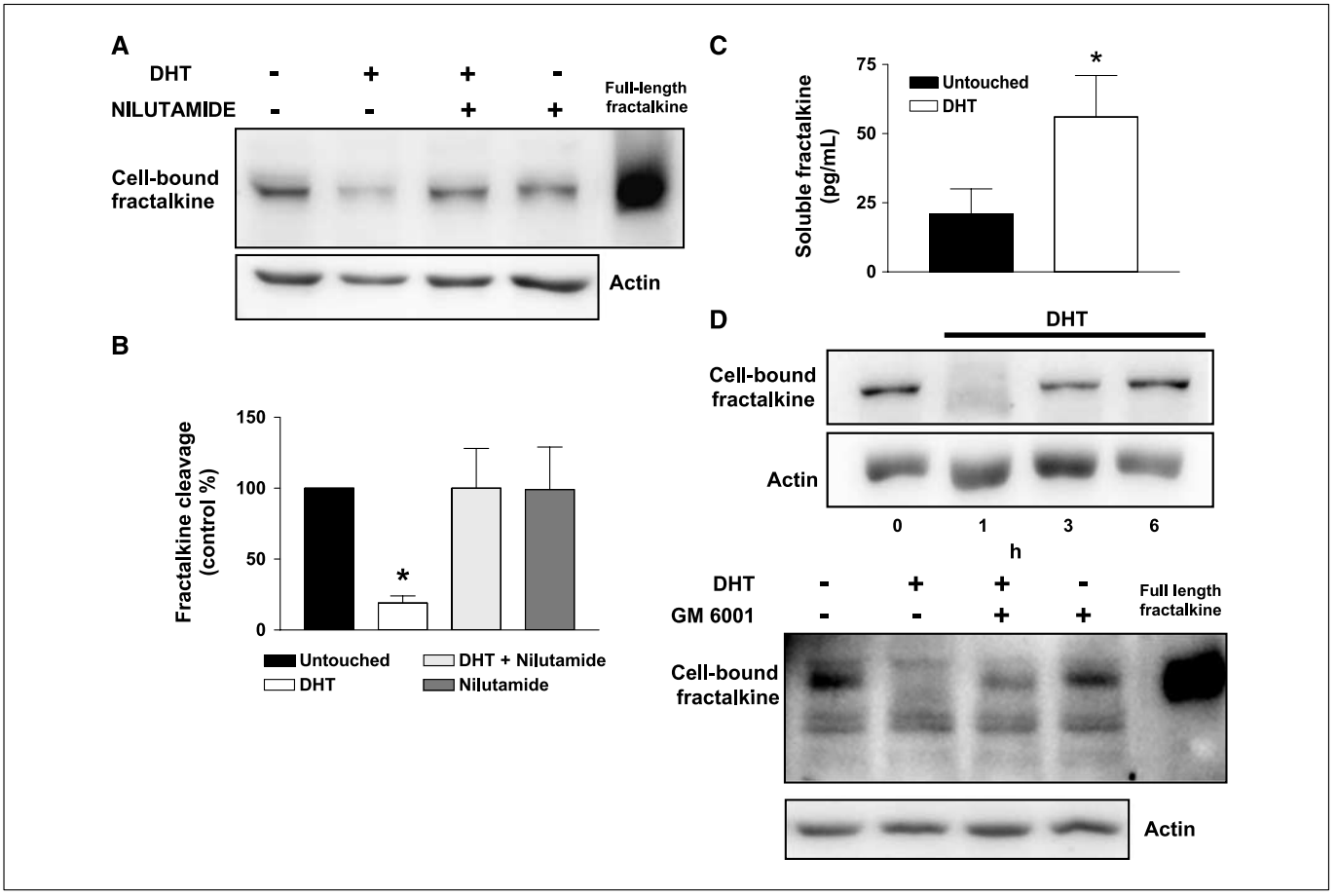

Figure 3. The cleavage of fractalkine from the cell surface is regulated by the androgen receptor. hMSCs from bone marrow were exposed to 100 nmol/L of dihydrotestosterone for 1 hour with or without previous 30-minute incubations with 1 μmol/L of nilutamide. The dramatic decrease in full-length fractalkine induced by 100 nmol/L of dihydrotestosterone was completely blocked by nilutamide, whereas nilutamide alone did not significantly affect the levels of cell-bound fractalkine. *A* and *B,* results are representative of five separate experiments (*, $P < 0.05$). An ELISA-based assay was used to detect the soluble form of fractalkine in hMSC supernatants and shows that dihydrotestosterone induces the cleavage of the membrane-bound chemokine into its soluble form. *C,* three separate experiments were conducted using supernatants of hMSC cells obtained from different donors and assessed in duplicate (*, $P < 0.05$). *D,* subsequently, fractalkine at the cell surface is restored in a time-dependent fashion (*top*). The broad-spectrum MMP inhibitor GM6001 partially blocked the decrease in cell-bound fractalkine induced by dihydrotestosterone, confirming that the reduction in this chemokine level is a consequence of cleavage from the cells and that MMPs are involved in this process (*bottom*). For Western blotting analysis, a recombinant and full-length form of fractalkine was loaded along with the cell lysates in the same gel and used as a positive control.

metabolic status and consequent variations in the relative number of each cell type, such as those occurring during bone turnover and remodeling. The conclusive demonstration that the full-length form of fractalkine is effectively exposed on the cell surface of bone-resident cells was obtained by confocal microscopy; in addition, we could also detect an intracellular staining for fractalkine, as previously described in different cell types by others (26). This storage compartment has been found to be involved in the recycling of the chemokine and is responsible for its translocation to the cell surface (Fig. 2*D*).

The chemokine-like domain of fractalkine is located atop a glycosylated mucin-like stalk connected to a transmembrane domain (14); its proteolytic cleavage and the ensuing ectodomain shedding are largely mediated by members of MMPs (22, 23), which are expressed by osteoblasts (19–21) and regulated by androgens (28–30). Interestingly, circulating androgens critically affect the clinical progression of prostate adenocarcinoma; accordingly, anti-androgen therapeutic approaches can significantly prolong the metastasis-free survival, as shown by a recent clinical trial (18). This circumstantial evidence prompted us to investigate whether the exposure of bone cells to androgens could increase the cleavage of the cell-bound form of fractalkine; we used hMSC as they express intermediate levels of the chemokine among the cell types tested and also represent the precursors of the majority of cell types found in the bone stroma. When hMSC were exposed to dihydrotestosterone (100 nmol/L) for 1 hour, the levels of cell surface fractalkine were dramatically reduced; this effect was indeed exerted through the stimulation of androgen receptors, such as the specific inhibitor nilutamide (31), which fully blocked the decrease in fractalkine induced by dihydrotestosterone (Fig. 3*A* and *B*). The removal of fractalkine from the cell surface after dihydrotestosterone exposure corresponded to its cleavage into the extracellular space, as shown by the increased levels of the soluble form of the chemokine in the cell supernatants after 24-hour exposure to the androgen (Fig. 3*C*). The cleavage of fractalkine has been reported as being coupled to the rapid relocation of the chemokine to the plasma membrane from intracellular storage sites. This recycling mechanism would finely regulate the availability of fractalkine at the cell surface as well as support the cellular needs for an increased release of its soluble form (26). Interestingly, we found

that the levels of fractalkine at the surface of hMSC—after being dramatically reduced following 1 hour of dihydrotestosterone exposure—were restored in a time-dependent manner despite the continuous exposure of the cells to the hormone (Fig. 3*D, top*). It seems plausible that this is due to an increased recycling of the chemokine, whereas the effect exerted by dihydrotestosterone on its cleavage is long-lasting.

A further indication that dihydrotestosterone could stimulate the cleavage of cell-bound fractalkine was obtained by using the broad-range MMPs inhibitor Ilomastat or GM6001 (32). This compound was able to partially block the reduction of the fractalkine from the cell surface induced by dihydrotestosterone in hMSC, thus pointing toward an involvement of MMPs in the cellular cleavage of this chemokine (Fig. 3*D, bottom*).

An androgen-induced increase of soluble fractalkine in the bone marrow could potentially promote the migration of CX3CR1-bearing cancer cells toward the concentration gradient and therefore their extravasation; however, androgens could equally promote the cleavage of this chemokine from bone marrow endothelial cells—which express androgen receptor (20)—thereby impairing the adhesion of circulating prostate cancer cells to the vascular wall of the marrow sinusoids. Therefore, the next experiments were conducted to ascertain the effects of dihydrotestosterone on the cleavage of fractalkine from HBME cells. Fractalkine was detected on the surface of HBME cells by confocal microscopy (Fig. 4*A*), thus confirming our previous results obtained by conventional immunofluorescence (17). However, cell surface fractalkine was not reduced by dihydrotestosterone exposure and although the soluble form of the chemokine could be detected in the supernatants of HBME cells, its levels were unaffected by dihydrotestosterone (Fig. 4*B* and *C*). The basis for the lack of fractalkine cleavage from HBME cells in response to androgens could be the different involvement in the cleavage process of the ADAM-17/TACE protease (22) in these cells as compared with hMSC. ADAM-17 has been implicated in the inducible cleavage of fractalkine (23), its activity is stimulated by dihydrotestosterone (29) and therefore would represent an ideal candidate for the release of soluble fractalkine from hMSC. Two forms of this metalloprotease are found in cells: a full-length precursor (120 kDa) and the mature form (100 kDa), which has been detected at the cell surface level (28, 33). When we tested hMSC and HBME cells for ADAM-17 expression, we found that the ratios of the two forms of the protease were dramatically different: whereas hMSC express higher levels of the mature ADAM-17 compared

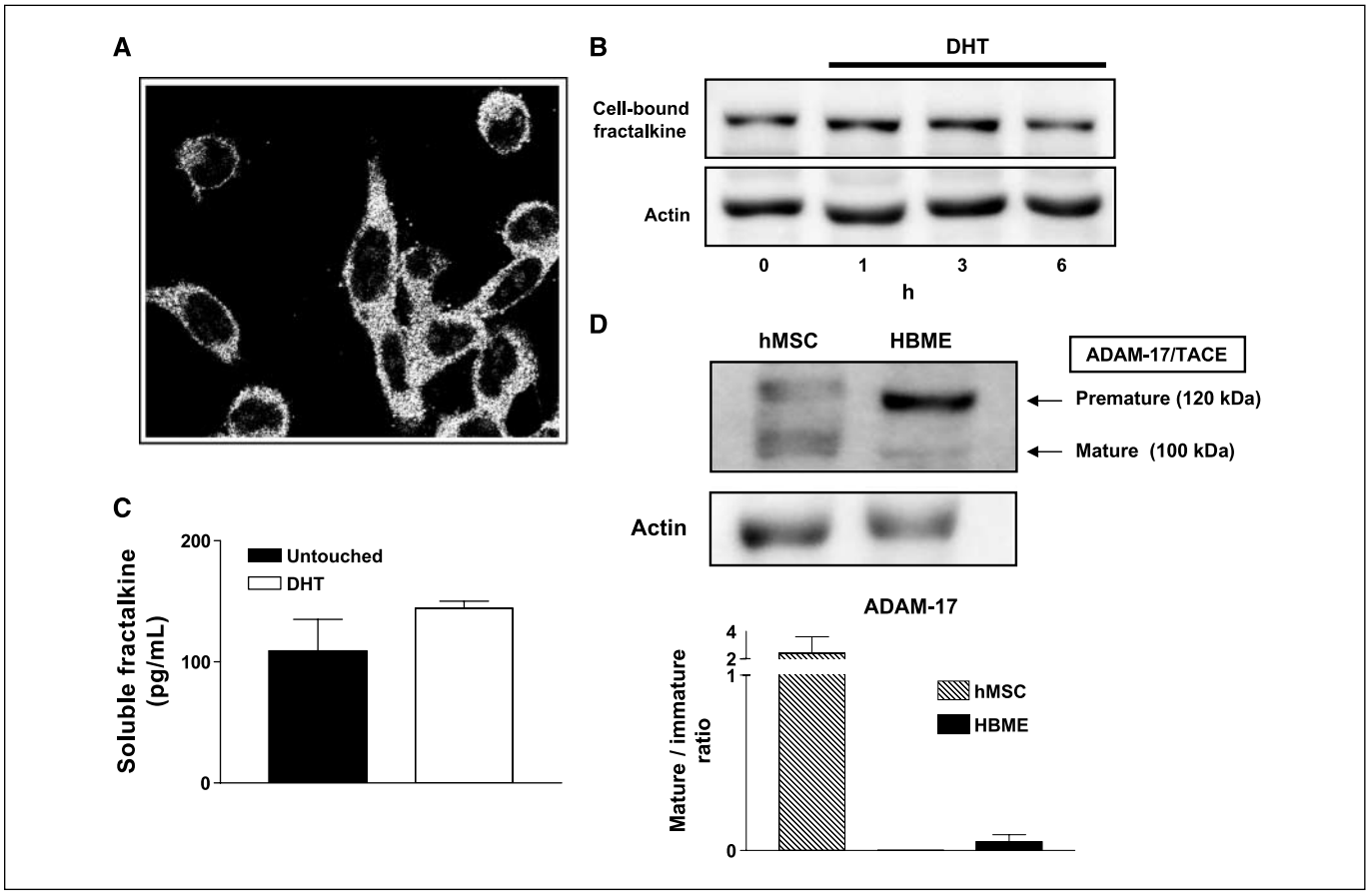

Figure 4. Expression of fractalkine by HBME cells and lack of effect of dihydrotestosterone on its cleavage. *A,* confocal microscopy analysis shows that fractalkine is expressed on the plasma membrane of HBME cells. The exposure of these cells to 100 nmol/L of dihydrotestosterone did not affect the levels of plasma membrane fractalkine, even after prolonged treatment. *B,* the results are representative of five separate experiments. *C,* the lack of reduction in fractalkine at the cell surface corresponds to the absence of dihydrotestosterone-induced cleavage of the cell-bound chemokine into its soluble form, as established by an ELISA assay performed using supernatants of HBME cells derived from five separate experiments and assessed in duplicate. *D,* the premature and mature forms of ADAM-17/TACE protease were detected in hMSC and HBME cells by Western blotting (*top*). The hMSC express significantly higher levels of the mature ADAM-17 as compared with the HBME cells, which show almost exclusive expression of the premature form of the protease (*bottom*).

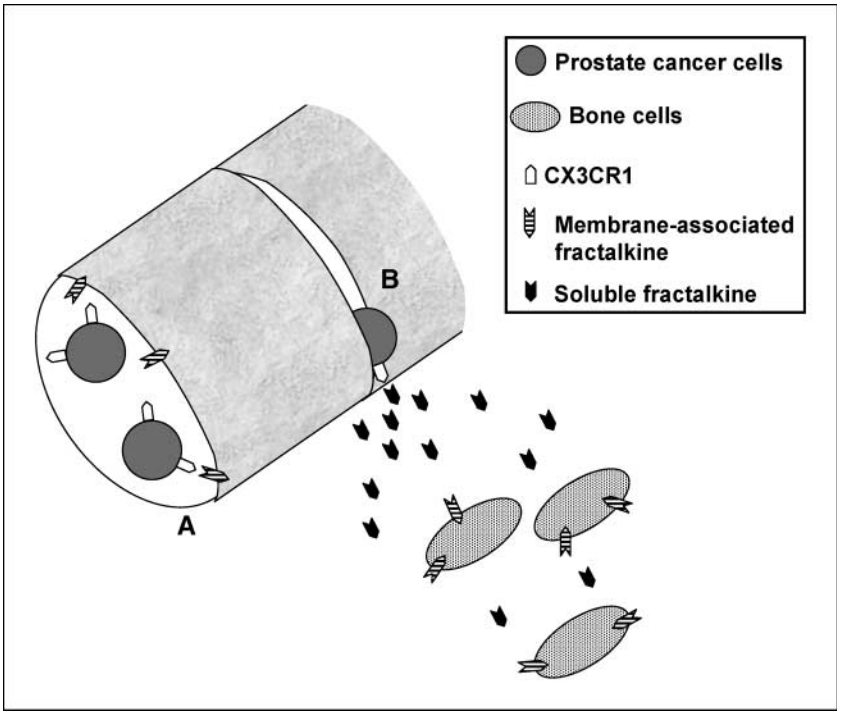

Figure 5. Possible roles of fractalkine and its receptor CX3CR1 in the adhesion of circulating prostate cancer cells to the bone marrow endothelium and their extravasation. *A,* the cell-bound fractalkine expressed on the surface of HBME cells directly functions as an adhesion molecule and allows CX3CR1-bearing cells to adhere to the vascular wall of bone marrow sinusoids. *B,* adherent cancer cells migrate into the marrow stroma following the concentration gradient of soluble fractalkine, produced and released by cells of the bone microenvironment.

with its immature precursor, the opposite was observed for HBME cells (Fig. 4*D*). The hMSC also showed higher overall levels of mature ADAM-17 as compared with HBME cells. The negligible expression of functional protease could therefore explain the lack of fractalkine cleavage from HBME cells exposed to dihydrotestosterone.

In summary, our study shows the expression of the chemokine receptor CX3CR1 in the epithelial cell compartment of the prostate gland. We also obtained the first demonstration that the chemokine fractalkine—the only ligand for CX3CR1 (3, 4)—is present in the bone marrow of adult human subjects. The plasma membrane–associated form of fractalkine is detected in osteoblasts, mesenchymal, and stromal cells and its cleavage leads to the accumulation of the soluble and chemoattractant form in the acellular fraction of the marrow. This phenomenon could produce a concentration gradient of this chemokine that is able to chemoattract CX3CR1-bearing cells—including prostate cancer cells—from the blood circulation and into the skeleton. Interestingly, the exposure of cells of the bone marrow stroma to dihydrotestosterone causes a dramatic increase in fractalkine cleavage. This process is blocked by nilutamide as well as inhibitors of MMPs, indicating that fractalkine cleavage involves an androgen receptor–dependent functional stimulation of these enzymes.

Thus, our results point to a possible scenario in which full-length fractalkine, exposed on the luminal side of the endothelial cells lining the bone marrow sinusoids, provides viable docking sites for circulating CX3CR1-expressing cancer cells (Fig. 5). Interestingly, we found that HBME cells do not cleave the plasma membrane–associated fractalkine in response to dihydrotestosterone *in vitro*; this would indicate that these cells provide CX3CR1-bearing cancer cells with docking sites on bone marrow sinusoids also in the presence of circulating androgens. The adhesion and extravasation of cancer cells are complex phenomena that depend on several molecules in general and chemokines in particular. However, based on our present and past studies (17), the involvement of CX3CR1-fractalkine interactions in cancer cell adhesion to selected endothelial cells seems very likely and might also participate in the extravasation of cancer cells into the bone marrow stroma (Fig. 5). This mechanism, based on the release of the soluble form of fractalkine from cells of the marrow stroma, seems to involve enzymes of the MMP family promoted by activation of the androgen receptor.

It should be emphasized that conclusive evidence for a determinant role of the CX3CR1/fractalkine pair in skeletal metastasis will be obtained using experimental approaches targeting these molecules by short hairpin RNA and employing *in vivo* animal models as well as transgenic knockout animals; these experiments are currently being conducted in our laboratory. However, the *ex vivo* and *in vitro* evidence we present in this study strongly suggests that the chemokine receptor, CX3CR1, its ligand fractalkine, and the enzymes responsible for the cleavage of this chemokine may represent suitable targets for a therapeutic intervention aiming to counteract skeletal metastasis from prostate cancer cells. Importantly, our results suggest the existence of an androgen receptor–dependent signaling mechanism modulating fractalkine cleavage from bone-resident cells and possibly underlying some of the effect of antiandrogen therapy in delaying the appearance of skeletal secondary tumors in patients affected by prostate adenocarcinoma.

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