[CANCER RESEARCH 64, 4693–4698, July 15, 2004]

Advances in Brief

CX3CR1-Fractalkine Expression Regulates Cellular Mechanisms Involved in Adhesion, Migration, and Survival of Human Prostate Cancer Cells

Shannon A. Shulby,[1] Nathan G. Dolloff,[1] Mark E. Stearns,[2] Olimpia Meucci,[1] and Alessandro Fatatis[1]

Departments of [1]Pharmacology and Physiology and [2]Pathology, Drexel University, College of Medicine, Philadelphia, Pennsylvania

Abstract

Chemokines and their receptors might be involved in the selection of specific organs by metastatic cancer cells. For instance, the CXCR4-SDF-1α pair regulates adhesion and migration of breast as well as prostate cancer cells to metastatic sites. In this study, we present the first evidence for the expression of CX3CR1—the specific receptor for the chemokine fractalkine—by human prostate cancer cells, whereas human bone marrow endothelial cells and differentiated osteoblasts express fractalkine. The adhesion of prostate cancer cells to human bone marrow endothelial cells in flow conditions is significantly reduced by a neutralizing antibody against fractalkine, and they migrate toward a medium conditioned by osteoblasts, which secrete the soluble form of the chemokine. Finally, fractalkine activates the PI3K/Akt survival pathway in human prostate cancer cells.

Introduction

It is well recognized that the organ selectivity of cancer metastasis depends on the relative contribution of congenial growth conditions present in specific tissues, as well as the anatomy of vascular patterns, which would distribute cancer cells predominantly to some organs rather than others (1). Cancer cells can elude the filtering action of the most proximal microcapillaries. Therefore, it is possible that the arrest of circulating cancer cells in the capillary beds of distant organs is not simply the result of mechanical trapping but involves specific interactions between the surface of cancer cells and the endothelial wall (2). Chemokines and their receptors have been proposed to play a role in the homing of cancer cells to selected organs. In fact, a recent study strongly suggests the involvement of CXCR4/SDF-1α in the metastasis of breast cancer cells (3). Prostate cancer cells, which show high propensity to metastasize to the skeleton, also express CXCR4 receptors, whereas SDF-1α affects their adherence, migration, and invasion (4). It is therefore likely that other chemokines might be involved in the adhesion and migration of circulating prostate cancer cells. A very interesting candidate is fractalkine, which differs from all other chemokines because it is expressed as a transmembrane protein (5). Thus, binding to plasma membrane-anchored molecules is not necessary for fractalkine to resist the shear forces of the blood flow and to interact with circulating cells expressing its receptor CX3CR1. Interestingly, the NH_2-terminal chemokine domain is tethered to the plasma membrane by a mucine-like stalk and has intrinsic cell-adhesive properties. In addition, fractalkine can be cleaved into a soluble fragment containing the majority of the chemokine domain and capable of attracting immunocompetent cells (5).

Received 11/3/03; revised 5/7/04; accepted 5/20/04.

Grant support: NIH Grants GM067892 (A. Fatatis), CA076639-07 (M. Stearns), and DA15014-01 (O. Meucci) and from W. W. Smith Charitable Trust Grant A0302 (O. Meucci).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Alessandro Fatatis, Department of Pharmacology and Physiology, Drexel University College of Medicine, 245 North 15th Street, New College Building, MS488, Philadelphia, PA 19102. Phone: (215) 762-8534; Fax: (215) 762-2299; E-mail: alessandro.fatatis@drexel.edu.

The aim of this study was to test the possibility that fractalkine could be involved in attracting prostate cancer cells to the bone marrow. The expression of CX3CR1 in breast cancer was recently ruled out (3), whereas in prostate cancer cells it has never been described.

Here, we produce evidence, for the first time, that CX3CR1 is expressed by human prostate cancer cells, whereas human bone marrow endothelial cells express plasma membrane-bound fractalkine. In addition, we found that human osteoblasts also express full-length fractalkine, can cleave it from their membrane as a soluble form and attract prostate cancer cells. Finally, the activation of CX3CR1 on prostate cancer cells by fractalkine recruits the PI3K/Akt survival signaling pathway with efficiency comparable with that of SDF-1α.

Materials and Methods

Cell Lines. The 2X N.I PC-3 (PC3 N) and bone metastatic PC-3 ML sublines were derived from human prostate PC-3 parent cell line as described previously (6). Human MDA PCa2b, DU-145, and LNCaP prostate cells and hFOB 1.19 human osteoblasts were from the American Type Culture Collection (Manassas, VA). The human nonmalignant prostate epithelial cell line was from Cambrex BioScience (Walkersville, MD). Human embryonic kidney cells (HEK-293) expressing the rat (V28) CX3CR1 receptor (HEK-D2 and HEK-V5 clones) were obtained as described previously (7). The human bone marrow endothelial cell line HBME-1 was a gift of Dr. Kenneth J. Pienta.

Cell Cultures. PC-3 subclones, CX3CR1-transfected HEK-293 and DU-145, were cultivated in DMEM containing 10% fetal bovine serum (FBS). Geneticin (800 μg/ml) was added to the culture medium of stably transfected HEK-293 cells. LNCaP cells were grown in RPMI 1640 containing 10% FBS. MDA PCa2b were grown in F12K containing 20% FBS and a mixture of trophic factors, as indicated by American Type Culture Collection. The human nonmalignant prostate epithelial (PrEC) and HBME-1 cells were grown in their respective prostate and endothelial media obtained from Cambrex Bio Science. hFOB 1.19 human osteoblasts were grown in DMEM:Ham's F-12 containing 10% FBS and Geneticin (400 μg/ml) at the temperature of 34°C. Differentiation was achieved growing them at 39°C for at least 2 days, as described previously (8).

Treatment of Prostate Cancer Cells with Chemokines. Cells were incubated with culture medium deprived of serum for 4 h and then exposed to 50 nM fractalkine or 20 nM SDF-1α for different time periods at the temperature of 37°C. Cell lysates were obtained as described previously (9).

SDS-PAGE and Western Blotting. SDS-PAGE and Western blot analysis were performed as previously described (9), with few modifications. Blotted membranes were blocked and incubated with primary antibodies using Non Animal Proteins blocker (Genotech, St. Louis, MO). Antibodies against phospho-Akt (Ser^{473}, 1:2000), total Akt (1:4000), and phospho-glycogen synthase kinase-3β (Ser^{9}, 1:2000) were from Cell Signaling Technology (Beverly, MA). Antibodies against human CX3CR1 (0.3 μg/ml) and human fractalkine (1 μg/ml) were from Torrey Pines Biolabs (Houston, TX). Primary antibody binding was detected using horseradish peroxidase-conjugated Protein A (Sigma, St. Louis, MO) at 1:10,000 dilution. Chemiluminescent signals were obtained using SuperSignal West Femto reagents (Pierce, Rockford, IL) and detected with the ChemiDoc imaging system and relative software (Bio-Rad, Hercules, CA). Densitometry analysis was performed using the UN-SCAN IT software (Silk Scientific, Orem, UT).

Immunofluorescence. Cells were grown onto 15-mm glass coverslips and fixed with 4% paraformaldehyde but not permeabilized. This approach allowed us to impede the access of primary antibodies to the intracellular space, thus detecting proteins expressed on the cell surface. The primary antibodies against CD31 (NeoMarkers, Fremont, CA) and fractalkine (Torrey Pines Biolabs) were both used at 1:50 dilution. The antibody against human CX3CR1 was used at 0.3 μg/ml and obtained from Torrey Pines Biolabs. Cy3 and FITC-conjugated secondary antibodies were from Jackson ImmunoResearch (West Grove, PA). Fluorescent images were acquired using a charge-coupled device MicroMax 1300YHS digital camera (Roper Scientific, Trenton, NJ) connected to an IX-70 microscope (Olympus, Melville, NY) and managed by a computer equipped with the Metamorph software (Universal Imaging Corporation, Downingtown, PA).

Flow Chamber Cell Adhesion Assay. HBME-1, PC3-ML, and NIH-3T3 cells were labeled with 5-chloromethylfluorescein diacetate green, 5-(and-6)-(((4-chloromethyl)benzoyl) amino) tetramethylrhodamine orange or 7-amino-4-chloromethylcoumarin blue CellTrackers (Molecular Probes, Eugene, OR), respectively. HBME-1 and NIH-3T3 cells were plated on 24 $\times$ 50-mm glass coverslips coated with collagen and fibronectin and grown as monolayers (BD Biosciences, San Jose, CA). Coverslips were mounted in a parallel-plate RC-27N flow chamber (Warner, Hamden, CT). A flow adhesion saline buffer (320 mOsm) containing 1% bovine serum albumin was used to incubate the cell monolayers for 10 min. In some experiments, HBME-1 cells were incubated for 30 min with 5 μg/ml of a neutralizing antibody directed against human fractalkine (R&D Systems, Minneapolis, MN) before starting the perfusion. PC3-ML cells were resuspended (2×10^5/ml) and then perfused through the chamber using a tachometer-regulated microperfusion pump (Bioptechs, Butler, PA). The flow rate used for our experiments produced a shear force of 1.0 dyne/cm^2, calculated as described previously (10). After allowing PC3-ML cells to flow in the chamber for 5 min, the perfusion rate was increased to bring the shear force to 10 dynes/cm^2 to wash off nonadherent cells. Images were stream-acquired every 2 s with the same set up previously described for the immunofluorescence experiments using the Metafluor software (Universal Imaging Corporation).

ELISA Assay for Fractalkine. The soluble form of fractalkine was detected in cell supernatants using the Elisa DuoSet kit from R&D Systems according to the manufacturer protocol.

Cell Migration Assay. Cells labeled with 5-chloromethylfluorescein diacetate green CellTracker were plated on the top of FluoroBlock inserts (8-μm pore diameter; BD Biosciences) at the concentration of 80×10^3/insert. The inserts were then transferred into a 24-well plate. The wells of each 24-well plate contained serum-free culture medium with or without 50 nM fractalkine. In parallel experiments, differentiated human osteoblasts were plated to confluence in each well and incubated with serum-free culture medium for conditioning the day before the transfer of the inserts with prostate cancer cells. In some experiments, the human osteoblasts were incubated with 5 μg/ml of a neutralizing antibody directed against human fractalkine (R&D Systems) 30 min before the transfer of the inserts and throughout the entire assay. Cells were then allowed to migrate for 24 h after their transfer to the 24-well plate. When the inserts were examined using a Wallac Victor2 microplate reader (Perkin-Elmer, Boston, MA) set with bottom illumination and detection, only the fluorescent cells which migrated through the membrane were detected as the insert membrane blocks fluorescent light. The autofluorescence deriving from wells containing only medium or osteoblast monolayers was subtracted from the readings obtained from the inserts carrying the fluorescent cells.

Materials. THP-1 human monocytic cell extracts were from Santa Cruz Biotechnology (Santa Cruz, CA). Full-length fractalkine was from R&D Systems, fractalkine (chemokine domain), and SDF-1α were from Peprotech (Rocky Hill, NJ). Geneticin and culture media were from Invitrogen-Life Technologies, Inc. (Carlsbad, CA), unless otherwise indicated. FBS was from Hyclone (Logan, UT).

Statistics. Experimental results are expressed as mean $\pm$ SE. Statistical significance was determined with the one-way ANOVA test.

Results and Discussion

To test the possibility that fractalkine could contribute to the selective colonization of the bone marrow by prostate cancer, human cell lines derived from different metastatic sites, as well as PrEC cells, were probed for the expression of CX3CR1, the only known receptor for fractalkine.

Human Prostate Cancer Cells Express CX3CR1. First, we tested two different PC3 human prostate cancer cell sublines, PC3-N and PC3-ML, which have been previously selected on the basis of their invasiveness *in vitro* and metastatic potential *in vivo* (6). PC3-N cells are unable to migrate through a Matrigel-coated membrane *in vitro*, as well as induce metastases *in vivo* when injected in SCID mice. In contrast, PC3-ML cells are highly invasive *in vitro* and induce skeletal metastases in SCID mice in >80% of cases. Western blot analysis was performed on cell extracts from PC3-N and PC3-ML. We found that both PC3-N and PC3-ML cells express CX3CR1 (Fig. 1*A*). Extracts from the human monocytic THP-1 cells, which constitutively express CX3CR1, were included in the experiments as positive control. In addition, two different clones of human embryonic kidney cells (HEK-293) permanently transfected with CX3CR1 (7), and known to express different levels of CX3CR1 mRNA (Fig. 1*B*), were also tested along with the PC3 sublines. The expression levels of the protein detected in the two CX3CR1-transfected HEK 293 clones were in agreement with their different levels of mRNA for CX3CR1.

From this first set of data, it seems that CX3CR1 expression does not correlate with the metastatic phenotype because the expression levels of the chemokine receptor were similar in PC3-ML and PC3-N cells. On the other hand, if CX3CR1 was involved in the migration of

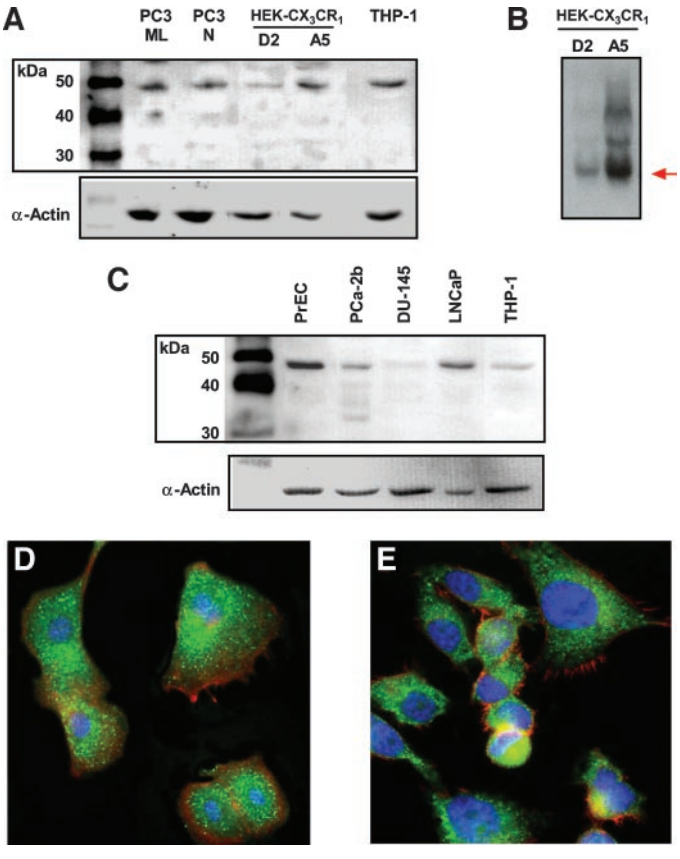

Fig. 1. Western blot analysis of CX3CR1 expression by both PC3-ML and PC3-N sublines (*A*). THP-1 cells, which constitutively express CX3CR1, were used as positive control, together with two HEK-293 cell lines permanently transfected with CX3CR1 and expressing different levels of mRNA for this chemokine receptor (*B*). CX3CR1 is also expressed in the other bone-derived prostate cell line PCa-2b in LNCaP cells, as well as in the human nonmalignant prostate epithelial cells (PrEC). In contrast, DU-145 cells express negligible levels of the receptor (*C*). CX3CR1 was also detected by indirect immunofluorescence in PrEC (*D*) and PC3-ML cells (*E*). Cells fixed in paraformaldehyde were not permeabilized, and thus, CX3CR1 was recognized at the plasma membrane level (FITC, *green*). A general staining for α-actin and a nuclear counterstaining were obtained using Cy3-conjugated phalloidin (*red*), and Hoechst 33342 (*blue*), respectively.

prostate cells to the skeleton, these two PC3 sublines would be equally able to localize to the bone marrow. In line with this assumption, previous experiments by Stearns *et al.* (6) indicated that both PC-3 ML and PC3-N were able to reach the skeleton of SCID mice within 24–36 h after their injection, although at 72 h, only the PC3-ML cells were still detected.

We then tested CX3CR1 expression in the PCa-2b cell line, also derived from a prostate cancer skeletal metastasis (11), the prostate cancer cell lines DU-145 and LNCaP obtained from brain and lymph node metastases, respectively, and PrEC cells. We found that all cell types tested express CX3CR1, with the exception of DU-145 cells in which very low levels, if any, of the chemokine receptor were detected (Fig. 1*C*). CX3CR1 expression at the plasma membrane level in the two PC3 sublines and in normal prostate epithelial cells was first established using an antibody for the extracellular portion of the receptor and detected by indirect immunofluorescence. All of the cell types in which CX3CR1 expression was assessed by Western blot analysis also stained positive for the chemokine receptor. The antibody staining for CX3CR1 in PrEC and PC3-ML cells is shown in Fig. 1, *D* and *E*. We conclude from these data that the expression of CX3CR1 is a usual feature of the prostate epithelium because it is observed in nonmalignant cells, as well as in cancer cells derived from metastases localized to different tissues, including the bone.

Human Bone Marrow Endothelial Cells Express Fractalkine. To hypothesize a role for CX3CR1 in the localization of prostate cancer cells to the skeleton, fractalkine should be expressed by the bone marrow endothelium. The expression of this chemokine has never been investigated in bone marrow endothelial cells. However, it is known that mRNA levels for fractalkine in human endothelial cells from different tissues such as aorta, lung, and umbilical vein are very low in the inactivated state but are markedly increased after stimulation with tumor necrosis factor or interleukin 1 (3). Fractalkine on the endothelial surface will be then recognized by circulating leukocytes. Although this mechanism makes perfect sense for the trafficking of immune-competent cells during the inflammatory response, it seems unlikely to be relevant in metastasis because there is no evidence of inflammation-driven adhesion and extravasation of circulating cancer cells. Consequently, to represent a suitable adhesion target for circulating prostate cancer cells, bone marrow endothelial cells should constitutively express fractalkine on their surface.

We therefore investigated fractalkine expression in the HBME-1 cells. As shown in Fig. 2*A*, we found that these cells express fractalkine in the absence of cytokine stimulation. The 85-kDa protein detected in HBME-1 comigrated with the full-length recombinant fractalkine used as a standard in the same Western blot. In addition, HBME-1 cells stained positive to an antibody directed against the extracellular chemokine domain of fractalkine and detected by indirect immunofluorescence (Fig. 2*B*).

These results suggest that fractalkine-expressing bone marrow endothelial cells could represent a preferential target for circulating prostate cancer cells expressing CX3CR1.

Bone-Derived Prostate Cancer Cells Adhere to Bone Marrow Endothelial Cells under Flow Conditions. In a recent study, Lehr and Pienta (12) compared the adhesion of prostate cancer cells to human bone marrow and umbilical vein endothelial cells and shed light on the tendency that prostate cancer cells have to preferentially adhere to endothelial cells from the bone marrow. In a subsequent study, Taichman *et al.* (4) presented data that strongly suggest the involvement of CXCR4-SDF-1α in this process. The current model of SDF-1α-induced cell adhesion requires that this chemokine, produced by stromal cells, is bound and presented toward the luminal side by proteoglycans located on endothelial cells. SDF-1α stimulation of CXCR4 expressed by circulating cells such as hematopoietic progenitors would then activate selectins

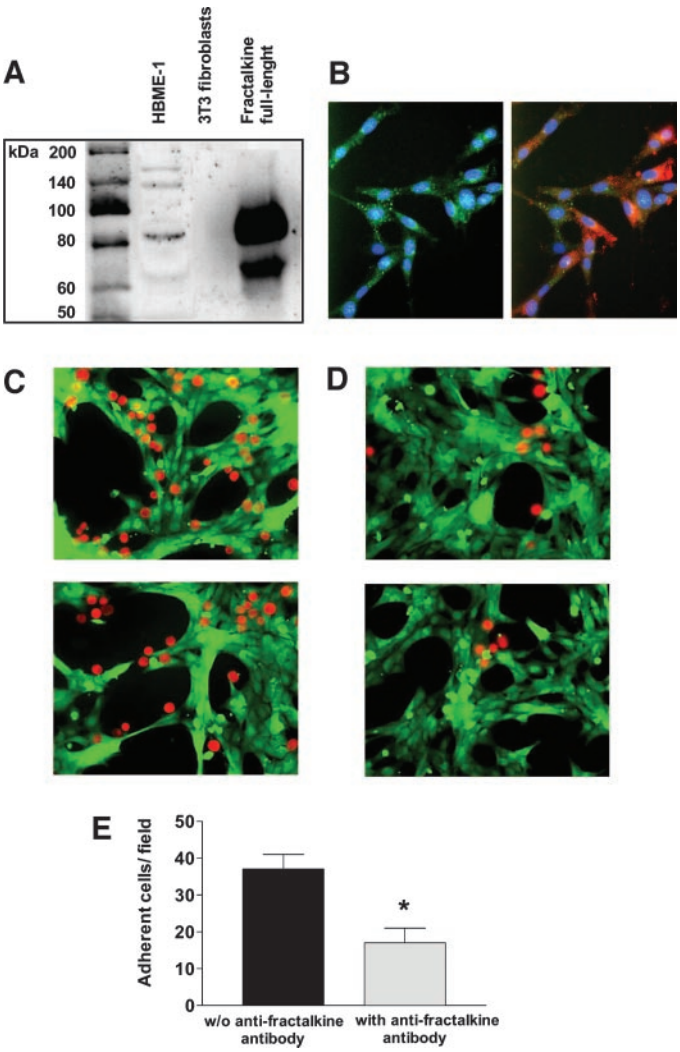

Fig. 2. Western blot analysis of fractalkine expression in HBME-1 cells. Full-length recombinant fractalkine (90 kDa) was used as positive control. NIH-3T3 fibroblasts do not express fractalkine (17) and were included as negative control (*A*). HBME-1 cells stained positive to the immunofluorescence detection of the endothelial-specific antigen CD31 (FITC, *green*) and fractalkine (Cy3, *red*). Blue nuclear counterstaining was obtained with Hoechst 33342 (*B*). PC3-ML cells (*red*), tested in flow conditions, adhere to HBME-1 cells (*green*), as shown by two representative experiments (*C*, *top and bottom panels*). The preincubation of HBME-1 cells with a neutralizing antibody directed against human fractalkine significantly reduced the number of adherent PC3-ML cells (*D*, *top and bottom panels*). The efficiency of PC3-ML cells adhesion to HBME-1 cells in the absence or presence of the fractalkine neutralizing antibody (*E*) was calculated by averaging data from 10 different microscopic fields derived from three experiments performed for each condition. Data are means $\pm$ SE; *, $P < 0.005$.

and integrins, which are responsible for the cell capture (13). Therefore, to study SDF-1α-induced adhesion *in vitro*, CXCR4-expressing cells are conventionally incubated with the chemokine before exposing them to endothelial cells (4). In contrast, fractalkine functions itself as an adhesion molecule and its transmembrane expression on the endothelial surface would directly provide docking sites for CX3CR1-expressing prostate cells. To test this model, we conducted flow adhesion assays using fluorescently labeled cells. A suspension of PC3-ML cells was perfused onto a monolayer of HBME-1 cells using flow rates, resulting in a shear force similar to that recorded in bone marrow microvessels *in vivo* (see "Materials and Methods" and Ref. 14). Fig. 2*C* shows that after washing off the nonadherent or weakly adherent cells, a relevant number of PC3-ML cells were firmly attached to the surface of HBME-1 cells at the end of each experiment. Interestingly, when PC3-ML cells were perfused onto a monolayer of NIH-3T3 fibroblasts, which do not express fracta-

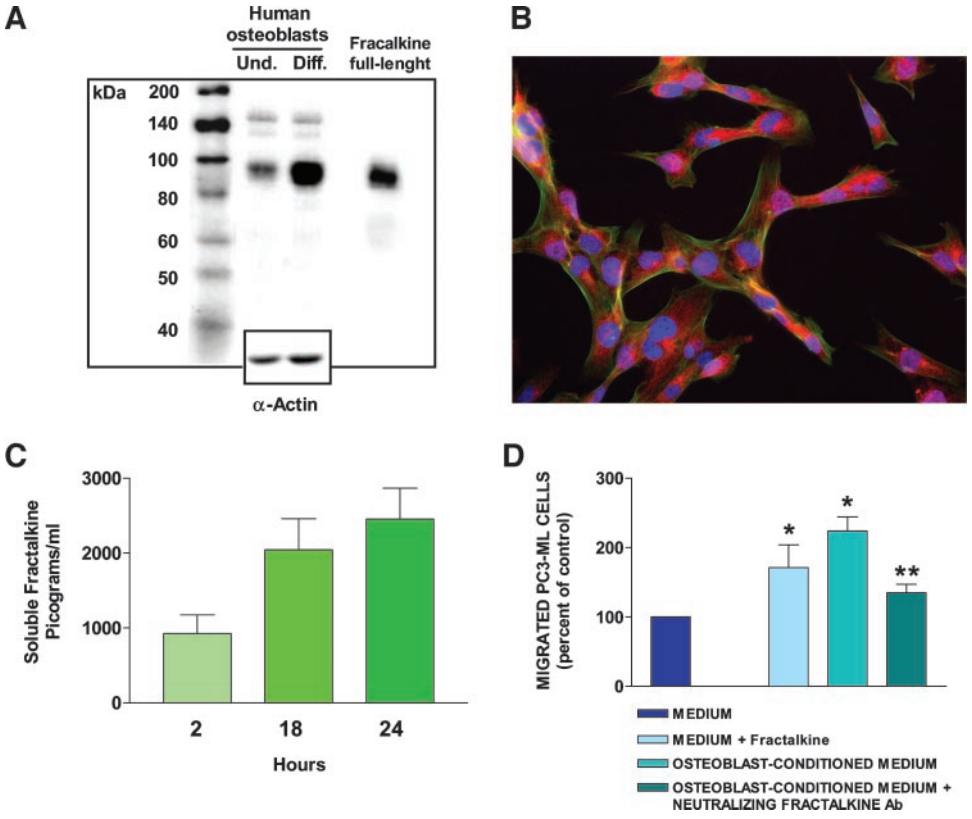

Fig. 3. Western blot analysis of fractalkine expression in undifferentiated and differentiated human osteoblasts. Full-length recombinant fractalkine was used as positive control. Differentiated osteoblasts expressed higher levels of the chemokine (*A*). Fractalkine was also detected in differentiated osteoblasts by indirect immunofluorescence (Cy3, *red*). A general staining for α-actin and a nuclear counterstaining were obtained using FITC-conjugated phalloidin (*green*) and Hoechst 33342 (*blue*), respectively (*B*). Time-dependent accumulation of fractalkine in the culture medium of differentiated osteoblasts measured by ELISA (data are means ± SE of three separate experiments; *C*). Fractalkine-containing and osteoblast-conditioned media were both able to chemoattract PC3-ML cells, whereas a neutralizing antibody against fractalkine almost completely blocked PC3-ML cell migration (data are means ± SE of three separate experiments; *, $P < 0.005$ *versus* medium alone, **, $P < 0.005$ *versus* osteoblast-conditioned medium; *D*).

lkine (Fig. 2*A*; Ref. 15), a negligible number of cells attached (data not shown). To establish the specificity of this process, additional experiments were performed in which HBME-1 cells were incubated with a neutralizing antibody for fractalkine before being perfused with PC3-ML cells. In these conditions, a significant lower number of PC3-ML cells attached to HBME-1 cells. (Fig. 2, *D* and *E*). Although these experiments suggest that additional molecular mechanisms may regulate the adhesion of prostate cancer cells to HBME cells, they also provide strong evidence for the involvement of the CX3CR1-fractalkine pair in this process.

Human Osteoblasts Express and Release Fractalkine, whereas PC3-ML Cells Migrate toward Fractalkine-Containing or Osteoblast-Conditioned Medium. 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 (16). As fractalkine can promote migration of CX3CR1-expressing cells, the production of this chemokine by bone-resident cells such as osteoblasts could therefore play a role in the invasion of the bone tissue by prostate cancer cells. We found that human osteoblasts express fractalkine and differentiated cells expressed higher levels of this chemokine (Fig. 3*A*). In addition, differentiated osteoblasts stained positive to the immunofluorescence detection of membrane-bound fractalkine (Fig. 3*B*).

It is worth to remember that the vascular sinusoids of the bone marrow are lined with endothelial cells having fenestrae of 60 Å in diameter and lacking a basement membrane (17). Thus, prostate cancer cells in the blood stream might bypass the endothelial layer and starting to colonize the bone tissue through a direct adhesion to osteoblasts.

On the other hand, fractalkine could also be released by osteoblasts and produce a concentration gradient capable of attracting prostate cells into the bone tissue. We therefore measured the levels of soluble fractalkine released by differentiated human osteoblasts in culture. Fig. 3*C* shows the time-dependent accumulation of soluble fractalkine in the osteoblast medium, detected by an ELISA assay. Within a 24-h interval, ~2 ng/ml of the chemokine were shed from the osteoblast membrane. This phenomenon occurred in resting conditions, *i.e.*, in the absence of any evident stimulation of metalloproteinase activity, and it seems therefore dependent on the constitutive ADAM10 (15) metalloproteinase, rather than the ADAM17-inducible isoform (18).

The evidence for the shedding of fractalkine from osteoblasts prompted us to ascertain whether the soluble form of this chemokine could induce the migration of prostate cancer cells. The results shown in Fig. 3*D* indicate that both soluble fractalkine and culture medium conditioned by differentiated osteoblasts for 24 h were able to attract PC3-ML cells *in vitro*. Interestingly, although it is very likely that additional factors released by osteoblasts could result chemoattractant for PC3-ML cells in our experiments, the increase in cell migration induced by soluble fractalkine alone was comparable with that induced by the osteoblast-conditioned medium and the use of a neutralizing antibody against fractalkine almost completely prevented the migration of PC3-ML cells (Fig. 3*D*). Finally, DU-145 cells, which express very low levels of CX3CR1 (see Fig. 1*C*), did not show a significant migration toward human osteoblast-conditioned medium (15 ± 6% above control, data not shown).

In conclusion, human osteoblasts express and secrete fractalkine and the highly metastatic prostate cancer cells PC3-ML migrate with similar efficiency toward both soluble fractalkine and osteoblast-conditioned medium.

Fractalkine Stimulates CX3CR1 Receptors on Prostate Cancer Cells and Activates the PI3K/Akt Signaling Pathway with Efficiency Comparable with SDF-1α. This series of experiments was conceived to ascertain whether, in addition to its role in cell adhesion and migration, CX3CR1 expressed on prostate cancer cells could activate downstream signaling pathways. In consideration of the importance that cell survival has in the successful establishment of metastatic lesions (19), we examined the PI3K/Akt pathway. PC-3

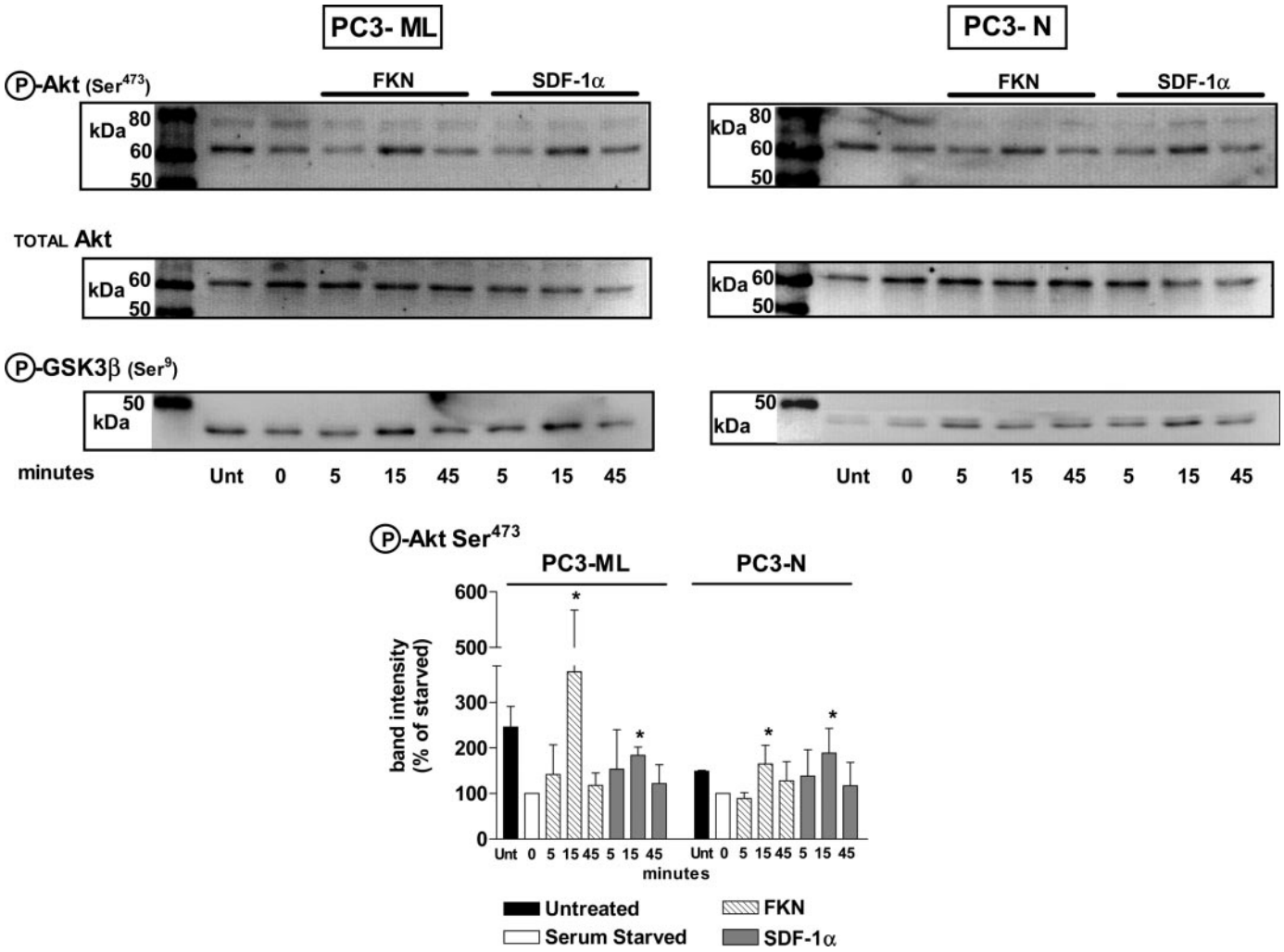

Fig. 4. Time-dependent phosphorylation of Akt induced by soluble fractalkine (FKN, 50 nM) in both PC3-ML and PC3-N, with an efficacy comparable with SDF-1α, (20 nM) as shown by Western blotting (*top*) and densitometry analysis (*bottom*). The intensity values from total Akt bands, obtained after membrane stripping, were used to normalize the phospho-Akt signals and compensate for possible variations in protein gel loading among samples. Each experiment was repeated three times. Data in the graph are means ± SE (*, $P < 0.005$). Glycogen synthase kinase-3β (GSK3β), a downstream target of Akt, is phosphorylated in a similar fashion, which additionally suggests Akt kinase activity.

ML and PC3-N cells were exposed for periods of time comprised between 5 and 45 min to fractalkine and the relative phosphorylation levels of Akt evaluated by Western blot analysis. In parallel experiments, cells were exposed to SDF-1α to compare CX3CR1 functioning to that of CXCR4. As shown in Fig. 4, Akt was clearly phosphorylated by both fractalkine and SDF-1α, with a peak observed at 15 min. In line with these results, the Akt downstream substrate glycogen synthase kinase-3β (GSK3β) was also phosphorylated in a similar fashion. These results indicate that CX3CR1 expressed in both PC3 sublines is functional and recruits crucial prosurvival effectors such as Akt and GSK3β in a way similar to CXCR4.

In conclusion, in this study, we present the first evidence for the expression of the fractalkine receptor by prostate cancer cells. We also found that human bone marrow endothelial cells and osteoblasts express fractalkine. This chemokine seems to be involved in the adhesion of prostate cancer cells *in vitro* because PC3-ML cells efficiently adhere to bone marrow endothelial cells under flow conditions. PC3-ML cells also migrate in response to a fractalkine gradient or a medium conditioned by human osteoblasts, which release soluble fractalkine. Furthermore, the activation of CX3CR1 in prostate cancer cells recruits an important antiapoptotic signaling pathways such as Akt/GSK3β (20).

These findings raise the possibility that the CX3CR1-fractalkine pair is involved in the location of circulating prostate cancer cells to the bone marrow, as well as their preferential migration and survival into the bone tissue.

Acknowledgments

We thank Dr. Kenneth J. Pienta (University of Michigan Medical Center, Ann Arbor, MI) for the HBME-1 cell line. We also thank Autumn Nelson (Program for Advanced Research, Central High School, Philadelphia, PA) and Brian Musser for excellent technical assistance.

References

1. Chambers AF, Groom AC, MacDonald IC. Dissemination and growth of cancer cells in metastatic sites. Nat Rev Cancer 2002;2:563–72.
2. Orr FW, Wang HH, Lafrenie RM, Scherbarth S, Nance DM. Interactions between cancer cells and the endothelium in metastasis. J Pathol 2000;190:310–29.
3. Müller A, Homey B, Soto H, et al. Involvement of chemokine receptors in breast cancer metastasis. Nature (Lond.) 2001;410:50–6.
4. Taichman RS, Cooper C, Keller ET, Pienta KJ, Taichman NS, McCauley LK. Use of stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone. Cancer Res 2002;62:1832–7.
5. Bazan JF, Bacon KB, Hardlman G, et al. A new class of membrane-bound chemokine with a CX3C motif. Nature (Lond.) 1997;385:640–4.
6. Wang M, Stearns ME. Isolation and characterization of 3 PC-3 human-prostatic tumor sublines which preferentially metastasize to select organs in SCID mice. Differentiation 1991;48:115–25.

7. Meucci O, Fatatis A, Simen AA, Miller RJ. Expression of CX3CR1 chemokine receptors on neurons and their role in neuronal survival. Proc Natl Acad Sci USA 2000;97:8075–80.
8. Harris SA, Enger RJ, Riggs BL, Spelsberg TC. Development and characterization of a conditionally immortalized human fetal osteoblastic cell line. J Bone Miner Res 1995;10:178–86.
9. Fatatis A, Miller RJ. Cell cycle control of PDGF-induced Ca^{2+} signaling through modulation of sphingolipid metabolism. FASEB J 1999;13:1291–301.
10. Kerfoot SM, Lord SE, Bell RB, Gill V, Robbins SM, Kubes P. Human fractalkine mediates leukocyte adhesion but not capture under physiological shear conditions; a mechanism for selective monocyte recruitment. Eur J Immunol 2003;33:729–39.
11. Navone NM, Olive M, Ozen M, et al. Establishment of two human prostate cancer cell lines derived from a single bone metastasis. Clin Cancer Res 1997;3:2493–500.
12. Lehr JE, Pienta KJ. Preferential adhesion of prostate cancer cells to a human bone marrow endothelial cell line. J Natl Cancer Inst (Bethesda) 1998;21:118–23.
13. Netelenbos T, van den Born J, Kessler FL, et al. Proteoglycans on bone marrow endothelial cells bind and present SDF-1 towards hematopoietic progenitor cells. Leukemia (Baltimore) 2003;17:175–84.
14. Mazo IB, Gutierrez-Ramos JC, Frenette PS, Hynes RO, Wagner DD, von Andrian UH. Hematopoietic progenitor cell rolling in bone marrow microvessels: parallel contributions by endothelial selectins and vascular cell adhesion molecule 1. J Exp Med 1998;188:465–74.
15. Hundhausen C, Misztela D, Berkhout TA, et al. The disintegrin-like metalloproteinase ADAM10 is involved in constitutive cleavage of CX3CL1 (fractalkine) and regulates CX3CL1-mediated cell-cell adhesion. Blood 2003;102:1186–95.
16. Moore MAS. The role of chemoattraction in cancer metastasis. Bioessays 2001;23: 674–6.
17. De Bruyn PPH. Structural substrates of bone marrow function. Semin Hematol 1981;18:179–93.
18. Garton KJ, Gough PJ, Blobel CP, et al. Tumor necrosis factor-alpha-converting enzyme (ADAM17) mediates the cleavage and shedding of fractalkine (CX3CL1). J Biol Chem 2001;276:37993–8001.
19. Fidler IJ. Critical determinant of metastasis. Semin Cancer Biol 2002;12:89–96.
20. Vivanco I, Sawyers CL. The phosphatidylinositol 3-kinase-Akt pathway in human cancer. Nat Rev Cancer 2002;2:489–501.