Membrane Microvesicles as Actors in the Establishment of a Favorable Prostatic Tumoral Niche: A Role for Activated Fibroblasts and CX3CL1-CX3CR1 Axis

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Abstract

Tumor microenvironment is enriched in plasma membrane microvesicles (MV) shed from all cell types that constitute the tumor mass, reflecting the antigenic profile of the cells they originate from. Fibroblasts and tumor cells mutually communicate within tumor microenvironment. Recent evidences suggest that tumor-derived MVs (TMV) exert a broad array of biological functions in cell-to-cell communication. To elucidate their role in cancer-to-fibroblast cell communication, TMV obtained from two prostate carcinoma cell lines with high and weak metastatic potential (PC3 and LnCaP, respectively) have been characterized. TMV exhibit matrix metalloproteinases (MMP) and extracellular MMP inducer at their surface, suggesting a role in extracellular matrix degradation. Moreover, TMV not only induce the activation of fibroblasts assessed through extracellular signal-regulated kinase 1/2 phosphorylation and MMP-9 up-regulation, increase motility and resistance to apoptosis but also promote MV shedding from activated fibroblasts able in turn to increase migration and invasion of highly metastatic PC3 cells but not LnCaP cells. PC3 cell chemotaxis seems, at least partially, dependent on membrane-bound CX3CL1/fractalkine ligand for chemokine receptor CX3CR1. The present results highlight a mechanism of mutual communication attributable not only to soluble factors but also to determinants harbored by MV, possibly contributing to the constitution of a favorable niche for cancer development. [Cancer Res 2009;69(3):785–93]

Introduction

After cellular stimulation in the broad sense of the term, including initiation of a death program by apoptosis, the asymmetric distribution of membrane phospholipids is no longer maintained and the cell releases submicrometric membrane fragments (0.1–1 μm), termed microvesicles (MV), into its environment. MV can be detected in the peripheral blood of mammals. They are characterized by the presence of procoagulant phospholipids (phosphatidylserine, PS) at their surface, which confer a possible prothrombotic potential, and antigens characteristic of their cellular origin (1). It has been shown that the quantity of generated MV is directly proportional to the degree of cellular activation including apoptosis *in vitro*, and that it is a quasi-universal feature of eukaryotic cells (2, 3). MV contain cytoplasmic components and genetic material. In cardiovascular disorders, numerous studies have led to consider MV genuine pathogenic markers (4), able to remotely initiate or amplify a deleterious process, depending on the cellular type and stimulus at their origin determining their composition. More generally, MV may be considered multifunctional bioeffectors in intercellular communication, and their roles have been investigated in hemostasis, inflammation, immunity, and angiogenesis (1), but only scarce information is available in cancer.

It has to be emphasized that MV or tumor-derived MV (TMV) considered in this study are different from exosomes, another category of smaller vesicles resulting from the endosomal cycle, able to elicit biological responses in various pathophysiologic circumstances, including cancer (5). Exosomes have a more homogeneous protein composition than MV or TMV and do not generally express PS and are viewed as antigen-presenting vehicles (5, 6).

Fibroblasts are associated with tumor cells at all stages of cancer development. It has been proposed they play a central part in promoting migration and invasion of cancer cells, when activated (7). Fibroblasts are thought instrumental in creating a niche for cancer cells, and the activated phenotype has been associated to cancer progression and metastasis, probably sustained through reciprocal signals. Fibroblasts have the capacity to proliferate and/or are kept under an activated state that can support or initiate epithelial hyperplasia and eventual tumorigenesis (8). However, the mechanism leading to fibroblast activation is not yet elucidated, as well as their structural and functional contributions to cancer progression.

CX3CL1/fractalkine is constitutively expressed at low levels in epithelial cells, and is up-regulated in activated epithelia and endothelia (9–11). CX3CL1 exists as both a membrane-bound form promoting firm cell-cell adhesion and a soluble form acting as a chemoattractant for cells expressing its cognate receptor CX3CR1 (9). The expression of CX3CR1 was detected in human prostate cancer cells and the association of CX3CR1-CX3CL1 chemokine members regulates adhesion, migration, and survival of the latter (12).

Several studies have reported a role for MV in the communication between cancer cells and their environment, but data concerning the effect of MV in cancer-stromal cell interactions

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

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are missing. Because MV are present in body fluids and within tumor microenvironment, cells could "interpret" MV variations to mutually balance their behavior. In this context, we propose a model where not only cancer cells influence the fate of stromal cells via TMV, but in turn, cancer cells are influenced by MV shed from stromal cells exposed to TMV. Two prostatic carcinoma cell lines PC3 and LnCaP were selected to produce TMV, with respect to their differential metastatic potential, high and low, respectively. Because fibroblasts are major components of the tumor stroma, healthy fibroblasts were used as target cells. The CX3CR1-CX3CL1 axis was considered in these TMV-fibroblast and fibroblasts MV-tumor cell exchanges of information. The results provide a new insight on the crosstalk between tumor and stromal cells mediated by MV, which could be part of the mechanisms governing tumor development.

Materials and Methods

Reagents

Fetal bovine serum (FBS) was from Invitrogen. Other cell culture reagents were from Cambrex, actinomycin D (Act.D), staurosporine (St), etoposide (VP-16), 4′,6-diamidino-2-phenylindole (DAPI), propidium iodide (PI), PKH-26 and antibody (Ab) to β-actin, neutralizing goat Ab anti-CX3CL1, and control goat IgG were from Sigma. z-Val-Ala-Asp.fluoromethyl ketone (z-VAD.fmk) and U1026 ([1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene]) were from Calbiochem. Ripa Lysis Buffer was from Upstate Biotech.

Monoclonal Abs to matrix metalloproteinase (MMP)2, MMP3, MMP7, MMP9, and polyclonal Abs to MMP13, MMP14 were from LabVisionCorporation. Polyclonal Ab to phosphorylated ERK1/2, Ab anti-EMMPRIN, Ab anti-CX3CR1, and donkey peroxidase-conjugated anti-goat IgG were from SantaCruz, polyclonal Ab against ERK1/2 from CellSignaling, goat peroxidase–conjugated anti-rabbit IgG purchased from Bio-Rad, sheep peroxidase–conjugated anti-mouse IgG from AmershamBiosciences, and streptavidine-FITC conjugate from Rockland. Monoclonal Ab to prostate-specific membrane antigen (PSMA) was from Invitrogen, and polyclonal Ab anti-CX3CL1 was from TorreyPines Biolabs. Neutralizing Ab anti-CX3CR1 was from AbnovaCorporation, and control mouse IgG was from SouthernBiotech. Human blood coagulation factors, chromogenic substrate pNAPEP (Biopep), Annexin-V (AV), and AV-biotin were the same as those previously used (13).

Cell Cultures

Human prostate adenocarcinoma cell lines PC3 and LnCaP were obtained from the American Type Culture Collection. PC3 cells were cultured in DMEM supplemented with 10% (v/v) heat-inactivated FBS, 100 μg/mL streptomycin and 100 U/mL penicillin, and LnCaP in RPMI 1640 supplemented with 10% heat-inactivated FBS, L-glutamine (2 mmol/L), Hepes (10 mmol/L), sodium pyruvate (1 mmol/L), 1% (v/v) nonessential amino acids, 100 μg/mL streptomycin, and 100 U/mL penicillin. Normal human fibroblasts were a kind gift of Dr. J. Ceraline (Université Louis Pasteur, Strasbourg, France) and cultured in DMEM supplemented with 10% heat-inactivated FBS and gentamicin (20 μg/mL). All cell types were incubated in a humidified 5% CO_2 atmosphere at 37°C. Cell viability and agent cytotoxicity was checked by trypan blue exclusion. All experiments were carried out at 80% to 90% cell confluence. PC3 and LnCaP were incubated without or with Act.D (0.5 μg/mL), St (0.33 nmol/L), and VP-16 (50 μmol/L) to produce TMV (see below). Normal human fibroblasts were cultured without or with 10, 30, or 50 μg/mL protein of different TMV preparations, isolated from nontreated and activated PC3 and LnCaP cells. Apoptotic fibroblasts were evaluated after 48 h of incubation with TMV (1.8% in control fibroblasts, 3% and 3.5% in fibroblasts with 30 or 50 μg/mL of TMV, respectively).

Isolation of TMV and MV from Cell Cultures

PC3 and LnCaP cells were used for TMV production. Briefly, cells at 80% to 90% confluence were treated with apoptogenic reagents for 24 h (14). Supernatants from both cell cultures were obtained by centrifugation at 750 *g* for 5 min and then at 1,500 *g* for 15 min to remove cells and large debris, respectively. TMV from supernatants were pelleted and washed by 3 centrifugation steps (45 min at 14,000 *g*, room temperature) and recovered in 500 μL HBSS (2). MV from conditioned medium (CM) were obtained from cultured untreated or TMV-activated fibroblasts. After incubation without or with TMV for 48 h, supernatants from fibroblast cultures were processed as described above. Double determination of MV was carried out by measuring their PS content by a functional prothrombinase assay (2) and protein content by Bradford's method (Bio-Rad) with bovine serum albumin (Sigma) as standard. TMV and fibroblast-derived MV isolated from at least five independent preparations were analyzed for each experimental condition. $TMV_{PC3\ Act.D}$ PS content was verified to be stable during 48 h of incubation in culture medium.

It has to be indicated that because no ultracentrifugation procedure (100,000 *g* or higher speed) has been used, exosomes are not expected to be present in MV or TMV preparations (6).

Detection of PSMA Antigen in MVs

Biotinylated anti-PSMA Ab was insolubilized onto streptavidin-coated microtitration plates and incubated with TMV from PC3 and LnCaP cells. Captured PS^+ MV (PS^+-MV) were quantified by prothrombinase assay (2).

Membrane Interaction Assay

To examine the kinetics of interaction between TMV and fibroblasts, the latter were incubated with labeled TMV from Act.D-treated PC3, ($TMV_{PC3\ Act.D}$). Briefly, $TMV_{PC3\ Act.D}$ (50 μg/mL protein) were previously labeled with the phospholipid dye, PKH-26 (1 μmol/L), for 10 min at room temperature in the dark, and extensively washed in HBSS/FBS 1:1 by 2 centrifugation steps (45 min at 14,000 *g*). The $TMV_{PC3\ Act.D}$ labeling was stable during 48 h of incubation in culture medium. Fibroblasts were incubated with labeled $TMV_{PC3\ Act.D}$, and at indicated time points, culture medium containing unbound $TMV_{PC3\ Act.D}$ was removed from fibroblasts growing in monolayers. Adherent cells were detached and centrifuged. Cells were resuspended in 300 μL of HBSS for flow cytometry analysis. Unbound labeled-$TMV_{PC3\ Act.D}$ were evaluated by flow cytometry using a FACScan flow cytometer (Becton Dickinson), and analyses were conducted using CELLQuest software. For fluorescence microscopy, cells were cultured in monolayers, incubated with labeled $TMV_{PC3\ Act.D}$ for different incubation times at 37°C, then washed and stained with DAPI. Images (magnification, ×400) were captured with a videographic system and acquired by using Spot software from Diagnostic Instruments, Inc.

Western Blots

After incubation with TMV as described above, fibroblasts were washed thrice with HBSS to eliminate unbound-TMV, and lysed in Ripa Lysis Buffer. Cell lysates were clarified by centrifugation (13,000 *g* × 3 min, 4°C), and protein content was determined with Bradford protein assay. For detection of TMV-associated metalloproteinases and EMMPRIN, TMV were resuspended in HBSS and samples containing 20 μg protein were separated by 10% SDS-PAGE. Separated proteins were then blotted onto Hybond-enhanced chemiluminescence nitrocellulose membrane (AmershamBiosciences). Blots were probed with specific Ab and developed with the corresponding horseradish peroxidase–conjugated secondary Ab. Proteins were visualized by enhanced chemiluminescence reagents and exposed to CL-XPosure films (Pierce); β-actin staining was used as control. Densitometry analyses were conducted using QuantityOne software (Bio-Rad).

Zymography Assay

After incubation with TMV as described before, fibroblasts were lysed in lysis buffer. Samples were separated by 10% SDS-PAGE containing 0.2% gelatin B (Sigma) under nonreducing conditions. Gels were washed in 2.5% Triton X-100 for 1 h at room temperature followed by incubation in reaction buffer [50 mmol/L Tris-HCl (pH 7.5), 200 mmol/L NaCl, 5 mmol/L $CaCl_2$, and 1 μmol/L $ZnCl_2$] for 40 h at 37°C. Gels were stained with 0.25% Coomassie blue (Sigma) and scanned for determination of gelatinolytic activity.

Determination of Apoptotic Cells by AV/PI Labeling

Fibroblasts were treated with TMV (50 μg/mL protein) and apoptosis was induced simultaneously by Act.D (0.5 μg/mL) in the presence or absence of z-VAD.fmk (50 μmol/L) or U0126 (10 μmol/L) for 24 h. Culture medium was removed from fibroblasts growing in monolayers. Adherent cells were trypsinized, detached, combined with floating cells from the original culture medium, and centrifuged. Cells were resuspended in HBSS containing 1 mmol/L $CaCl_2$, and incubated for 10 min with AV-biotin (4 μg/mL) and PI (6 μg/mL) at room temperature. Cells were then stained for 5 min with streptavidin-FITC (3 μg/mL), at room temperature in the dark. FITC and PI fluorescence were recorded by flow cytometry. FITC fluorescence was plotted as FL-1 versus PI fluorescence as FL-2, and both were reported in fluorescence intensity arbitrary units under the same settings as described in "Membrane interaction assay."

Cell Migration

Chemotaxis of fibroblasts. The chemotactic motility of fibroblasts was estimated using 24-well Transwell plates with polyethylene-terephthalate filters (8-μm pore size). The lower wells of Boyden chambers (Becton Dickinson) were loaded with DMEM supplemented with 10% FBS as chemotactic factor. After preincubation of fibroblasts with TMV or without (control) for 30 min, they were harvested and resuspended in DMEM containing 0.1% FBS, and loaded at 1×10^5 cells/mL into each of the upper wells. The chamber was incubated at 37°C for 24 h. After incubation, cells were fixed with methanol and stained with crystal violet (1%, w/v). Nonmigrating cells were removed from the upper surface of the filter, and chemotaxis was visualized by microscopy. Images were captured with a videographic system and acquired by using Spot software. Chemotaxis was quantified by counting cells that migrated to the lower side on 4 random fields of the filter at low magnification (×200). Data are expressed as mean ± SE of at least three separate experiments for each condition.

Chemotaxis of cancer cells. To estimate the migration capacity of cancer cells toward fibroblast-CM (CM-Fb), fibroblasts were incubated without (control) or with $TMV_{PC3\ Act.D}$ at 37°C for 48 h. CM-Fb were collected and placed in the lower wells with or without Ab to CX3CL1 or irrelevant Ab (8 μg/mL), and used as chemoattractant for cancer cells. PC3 and LnCaP cells were trypsinized and loaded in the upper well of Boyden chambers with or without Ab to CX3CR1 or irrelevant Ab (25 μg/mL), and allowed to migrate for 24 h at 37°C. After incubation, samples were analyzed as described above.

Trans-Matrigel Chemoinvasion Assay

To evaluate the ability of PC3 cells to cross a basement membrane, the Matrigel chemoinvasion assay (Becton Dickinson) was used. The lower chambers were loaded with culture medium from TMV-activated fibroblasts (CM-Fb TMV) or CM from untreated fibroblasts (CM-Fb NT) used as control. CM-Fb was prepared as described above. Cancer cells were loaded into the upper compartment at 3×10^5 cells/chamber and incubated at 37°C for 48 h. After incubation, samples were analyzed as detailed above.

Statistical Analysis

Results were expressed as mean ± SE of at least three separate experiments performed at different culture stages and/or passages, with no obvious difference attributable to the culture conditions. Paired Student's t test was used for statistical analysis. A P value of <0.05 was considered significant.

Results

Kinetics of interaction between tumor cell–derived MVs and fibroblasts. Incubation of fluorescent $TMV_{PC3\ Act.D}$ (labeled by PKH-26) with fibroblasts resulted in time-dependent transfer of fluorescence to fibroblasts (Fig. 1*A*). The increase of fluorescence intensity observed in fibroblasts was accompanied by a decrease of fluorescence intensity of labeled $TMV_{PC3\ Act.D}$ counterpart, according to the incubation time. After 48 h at 37°C, most of

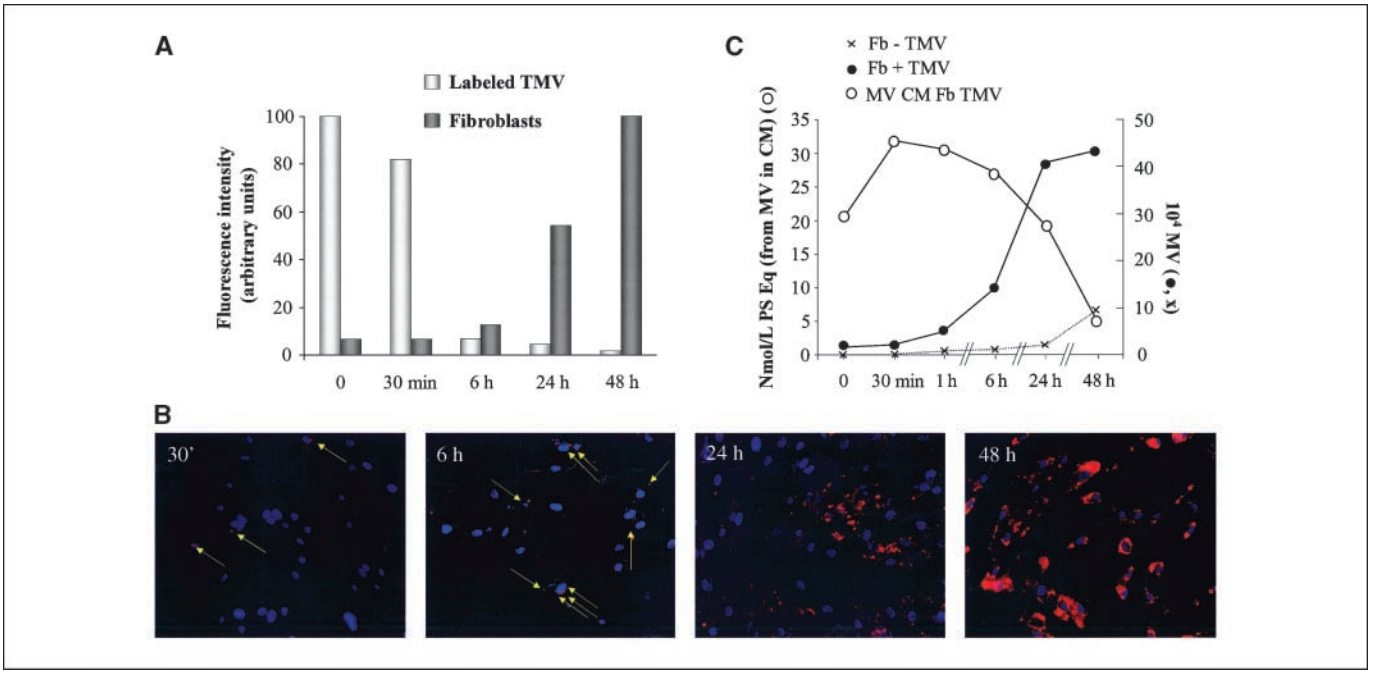

Figure 1. MV interaction with fibroblasts. *A,* interaction of labeled $TMV_{PC3\ Act.D}$ and fibroblasts in function of incubation time as assessed by flow cytometry. There is an increase of fluorescence in the fibroblast population at the expense of that of $TMV_{PC3\ Act.D}$, corresponding to the transfer of fluorescence from $TMV_{PC3\ Act.D}$ to fibroblasts (n = 3). *B,* fluorescence microscopy in the rhodamine channel (magnification, ×400) showing the transfer of the fluorescent probe PKH-26 from labeled $TMV_{PC3\ Act.D}$ to fibroblasts growing in monolayers. Nuclei of cells were labeled with DAPI dye (*blue fluorescence*). These images are representative of three experiments, performed under the same conditions. *Yellow arrows,* labeled $TMV_{PC3\ Act.D}$. *C,* relationship of procoagulant MV harboring PS (○) with number of MV (●) measured in CM. After capture onto AV, MV PS content was measured as nanomolar PS equivalents (nmol/L PS Eq) by functional prothrombinase assay (see Materials and Methods). Numbers of MV in CM of fibroblasts were estimated by flow cytometry. *Black crosses,* the basal release of MV from fibroblasts, e.g, in the absence of $TMV_{PC3\ Act.D}$. Histograms and fluorescence images are representative of three separate experiments.

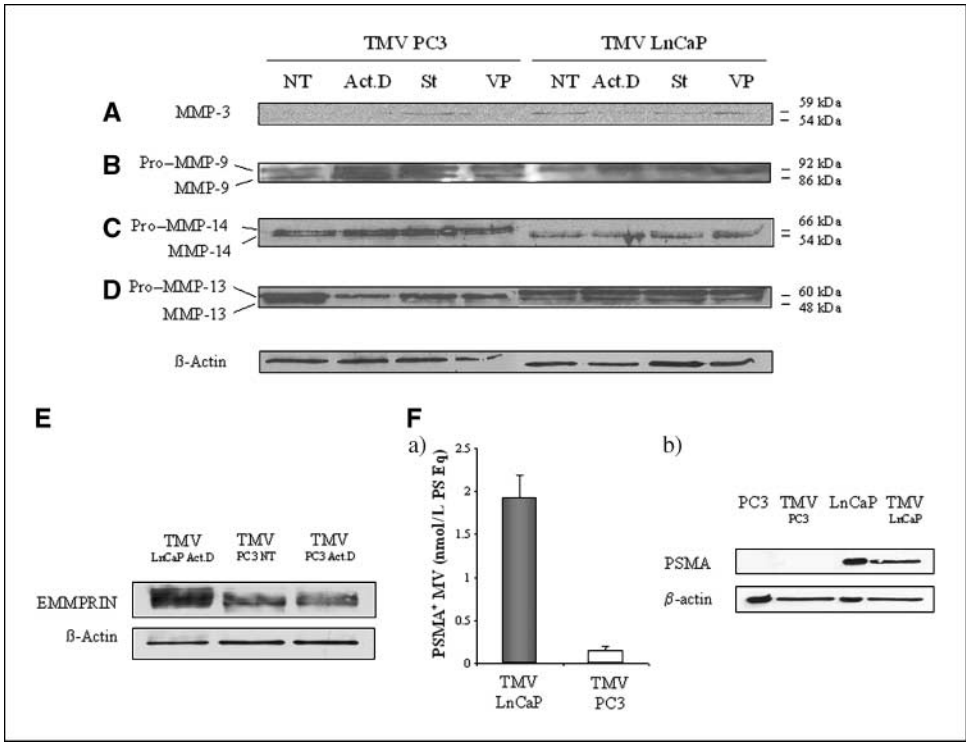

Figure 2. Immunoblot analysis of MMP, EMMPRIN, and PSMA expression in different samples of TMV. TMV generated from untreated PC3 and LnCaP cells (*NT*), and after different activation treatments, with Act.D, St, and VP16 (*VP*). MMP-3 (*A*), MMP-9 (*B*), MMP-13 (*C*), and MMP-14 (*D*) expression profiles were assessed by Western blot analysis. Three determinations yielding similar results were performed. For all MMP, a β-actin control was included. *E,* EMMPRIN was also detected by Western blots in $TMV_{PC3\ NT}$ or $TMV_{PC3\ Act.D}$ and $TMV_{LnCaP\ Act.D}$. β-actin staining was used as control. *F-a,* detection of PSMA in TMV. Biotinylated monoclonal Ab to PSMA was insolubilized onto streptavidin-coated microtitration plates and incubated with TMV from PC3 and LnCaP cells (2). Captured TMV were quantified by prothrombinase assay, and values were expressed as nanomolar PS equivalents (nmol/L PS Eq). *F-b,* PSMA expression in untreated PC3 and LnCaP cells (noted as PC3 and LnCaP, respectively) and $TMV_{PC3\ Act.D}$ and $TMV_{LnCaP\ Act.D}$ was assessed by Western blot.

$TMV_{PC3\ Act.D}$ fluorescence disappeared at the benefit of fibroblasts, suggesting transfer from $TMV_{PC3\ Act.D}$ to fibroblasts. This was confirmed by fluorescence microscopy analysis (Fig. 1*B*), in which nuclei of fibroblasts were labeled with DAPI (*blue fluorescence*) and $TMV_{PC3\ Act.D}$ with PKH-26 (*red fluorescence*). The time-dependent interaction of $TMV_{PC3\ Act.D}$ and fibroblasts resulted in MV release from fibroblasts evidenced by increased MV counts occurring in the culture medium measured by flow cytometry, and by a method of MV capture onto AV (2) to detect the presence of PS through its procoagulant potential. As shown in Fig. 1*C*, there was an inverse relationship between the number of MV and their PS content. The decrease of PS proportion can be explained by the limited PS content of a given cell type (15). In the absence of $TMV_{PC3\ Act.D}$, the basal release of fibroblast MV remained stable and close to the detection limit of the assay (~1.5 nmol/L PS Eq) during 48 h. Hence, TMV treatment is indeed necessary to elicit MV release from fibroblasts.

Characterization of tumor cell–derived MVs. MMP are endopeptidases that play pivotal roles in promoting tumor progression, including angiogenesis, growth, local invasion, and subsequent distal metastasis (16). The MMP enrichment was evaluated in TMV shed from PC3 and LnCaP cell lines, untreated (control) and activated by different agonists. For MMP-3, only a very weak signal could be detected in all different preparations of TMV (Fig. 2*A*). MMP-9 and MMP-14 were strongly expressed in $TMV_{PC3\ Act.D}$ (Fig. 2*B* and *C*). This was the case for MMP-13 in TMV from unstimulated PC3 cells (*NT*; $TMV_{PC3\ NT}$) and LnCaP cells treated with Act.D ($TMV_{LnCaP\ Act.D}$; Fig. 2*D*). For this reason, only the three different preparations of TMV with strong expression of MMP were considered for assessment of effects on fibroblasts. MMP-7 and MMP-2 were not detected in TMV (data not shown). Because the MMP inducer EMMPRIN has been evidenced at the surface of TMV stemming from tumor cells (17), its presence was verified at the surface of TMV from PC3 and LnCaP cells. As shown, in Fig. 2*E*, EMMPRIN is mainly expressed in all TMV ($TMV_{PC3\ NT}$, $TMV_{PC3\ Act.D}$, and $TMV_{LnCaP\ Act.D}$). In addition, PSMA expressed by normal and neoplastic epithelial cells negatively regulates migration and invasion (18). Using a method of MV antigenic capture and quantification (2), PSMA could be detected mainly in $TMV_{LnCaP\ Act.D}$ (Fig. 2*F-a*), which was confirmed by Western blot (Fig. 2*F-b*). Hence, TMV present differential expression patterns of MMP depending on the conditions of stimulation. All of them harbor procoagulant PS and tissue factor (TF) activity at their surface (data not shown), and $TMV_{LnCaP\ Act.D}$ display a stronger expression of PSMA.

Phosphorylation of extracellular signal-regulated kinase 1/2, and active MMP-9 overexpression. Cell migration is critical for many physiologic processes and is often dysregulated in developmental disorders and pathologic conditions, including cancer. Constitutive activation of the extracellular signal-regulated kinase (ERK) and/or phosphatidylinositol-3 kinase/Akt signaling pathways is associated with neoplastic transformation of a variety of human tumor cells (19). The phosphorylation of ERK1/2 was assessed in untreated fibroblasts (*NT*) or treated with 10, 30, and 50 μg/mL of TMV. As illustrated in Fig. 3*A*, $TMV_{PC3\ NT}$ and $TMV_{PC3\ Act.D}$ induced an enhancement of ERK1/2 phosphorylation, this effect was dose-dependent, with a ~30% increase at 50 μg/mL TMV compared with control (*NT*). The effect of TMV on the expression of MMP in fibroblasts has been evaluated. Expression of MMP-9 (Fig. 3*B*) was higher in fibroblasts incubated with TMV (10, 30, and 50 μg/mL) for 24 hours, in a dose-dependent manner. Furthermore, MMP-9 was found active by zymography assay, as evidenced by the gelatinolytic activity detected in fibroblasts exposed to TMV (10, 30, and 50 μg/mL; Fig. 3*C*). These results indicate that TMV are able to activate signaling pathways in fibroblasts.

TMV promote migration of fibroblasts. TMV induce ERK1/2 phosphorylation and increase active MMP-9 expression in

fibroblasts, knowing that these actors are involved in tumor progression. In this line, the capacity of fibroblasts to migrate in the presence of TMV has been assessed. Figure 3*D* shows that after 24 hours, fibroblasts incubated with 30 and 50 μg/mL of $TMV_{PC3\ NT}$ or $TMV_{PC3\ Act.D}$, exhibited a 2- and 3-fold increase of migration, respectively. When fibroblasts were incubated in the presence of 30 and 50 μg/mL $TMV_{LnCaP\ Act.D}$, the migration was enhanced by only 1.2- to 1.6-fold, respectively, probably related to the lower metastatic potential of LnCaP cells. However, the apoptotic origin of TMV did not seem to have any influence.

TMV decrease chemosensitivity of fibroblasts. To assess the transfer of TMV-mediated chemoresistance to fibroblasts, the latter were incubated with 50 μg/mL $TMV_{PC3\ Act.D}$, and induced into apoptosis with Act.D (0.5 μmol/L, during 24 hours). Apoptosis ratios in the absence or presence of TMV were measured by double labeling of nonpermeabilized cells with AV and PI. Figure 4*A* and *B* show that Act.D-induced apoptosis of fibroblasts (12% of AV^+/PI^- events, reflecting the apoptotic population), was significantly reduced to 7.2% (AV^+/PI^- events) in the presence of TMV. TMV therefore protected fibroblasts from apoptosis by ~40%, probably through the promotion of partial chemoresistance to Act.D. To investigate the implication of caspases in Act.D-induced apoptosis, the pan-caspase inhibitor, z-VAD.fmk, was used. As reported in Fig. 4*B*, this inhibitor reduced the degree of apoptosis evoked by Act.D. At 50 μmol/L, z-VAD.fmk significantly inhibited apoptosis, by ~52%. z-VAD.fmk also prevented apoptosis in fibroblasts treated with TMV, by ~50%. As expected, these results confirm that caspases play a critical role in apoptosis induced by Act.D in fibroblasts but without a clear differential effect due to TMV.

Because the ERK pathway is implicated in many cellular processes, including apoptosis (20), and ERK1/2 is phosphorylated in fibroblasts after TMV treatment, it was examined whether chemoresistance induced by TMV in apoptotic fibroblasts was mediated by the activation of the ERK pathway. For this, an inhibitor of mitogen activated protein kinase, U0126 (21), was used. As shown in Fig. 4*C-a*, this inhibitor promoted an increase (~25%) of apoptosis evoked by Act.D in fibroblasts, and abolished the chemoresistance induced by TMV in apoptotic fibroblasts. These observations were confirmed by Western blot analysis indicating that ERK1/2 phosphorylation seems strictly correlated with apoptosis chemoresistance induced by TMV (Fig. 4*C-b*). This suggests that the ERK pathway could be involved in the partial chemoresistance elicited by TMV in Act.D-challenged fibroblasts.

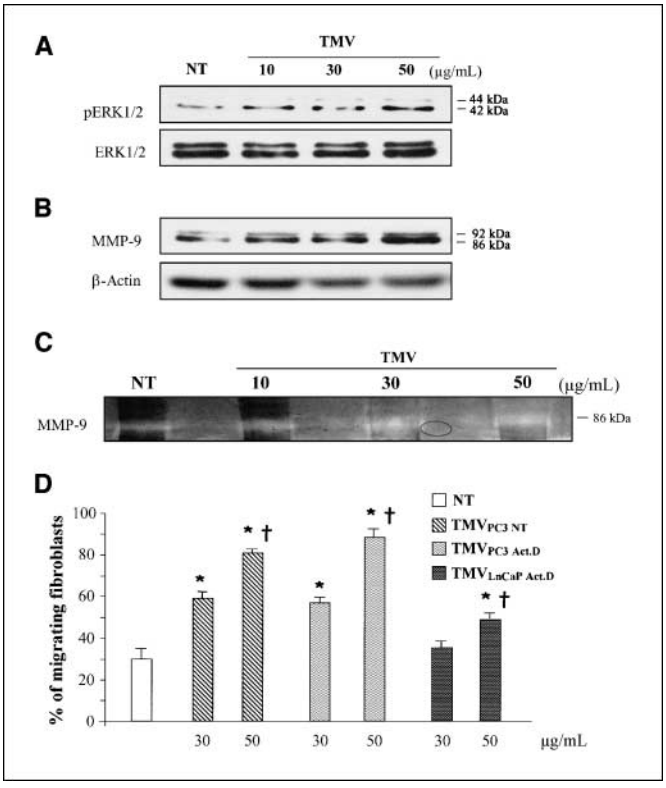

Figure 3. MMP-9 expression and ERK1/2 phosphorylation in fibroblasts, and their migration capacity after TMV stimulation. Fibroblasts, growing in monolayers, were treated for 24 h with medium as control or 10, 30, and 50 μg/mL of $TMV_{PC3\ Act.D}$. The degree of ERK1/2 phosphorylation (*A*) and MMP-9 expression (*B*) were explored by Western blotting. Total ERK1/2 (*A*) and β-actin (*B*) were included as respective controls. Three separated experiments were performed likewise and representative results are shown. *C,* zymographic assay was performed to detect MMP-9 gelatinolytic activity in fibroblasts treated by different concentrations of $TMV_{PC3\ Act.D}$ (0 as control, 10, 30, and 50 μg/mL). Three separated experiments were performed and representative results are shown. *D,* fibroblasts were incubated for 24 h in the absence (*NT*) or presence of $TMV_{PC3\ Act.D}$, $TMV_{PC3\ NT}$, and $TMV_{LnCaP\ Act.D}$ (30 or 50 μg/mL protein). *Columns,* mean of numbers of migrating cells counted in four random fields from three separate experiments performed in duplicate and expressed in %; *bars,* SE. *, $P < 0.05$, 30, or 50 μg/mL vs *NT*; †, $P < 0.05$, 50 μg/mL vs 30 μg/mL.

Migration and invasion of PC3 cells toward the CM of stimulated fibroblasts. The above results provide evidence of the effect of TMV on the acquisition of an "activated phenotype" by treated fibroblasts. To further evaluate the consequences of the crosstalk between tumor and stromal cells mediated by TMV, the capacity of PC3 cell migration toward CM from untreated (CM-Fb NT) or TMV-stimulated fibroblasts (CM-Fb TMV) was examined. Basal migration of PC3 cells was observed in the presence of CM-Fb NT, this condition was taken as the control level of migration of PC3 cells (Fig. 5*A*). CM of fibroblasts incubated for 48 hours with TMV (30 and 50 μg/mL $TMV_{PC3\ Act.D}$), promoted significant migration of PC3 cells. This dose-dependent effect was increased 5- and 7-fold by CM of fibroblasts treated with 30 and 50 μg/mL TMV, respectively (Fig. 5*A-b* and *A-c*), with respect to control (Fig. 5*A-a*). Microscopic analysis of membranes from Boyden chambers was performed (Fig. 5*B*). TMV from LnCaP were also able to promote MV release from fibroblasts that stimulate PC3 migration as observed for PC3 TMV, but to a lower extent, with only ~40% of the effect of PC3 TMV, in possible relation with the lower metastatic potential of LnCaP cells (data not shown). The next question was is this effect on PC3 migration attributable to soluble factors secreted by fibroblasts in response to TMV treatment, or to MV released by fibroblasts? To address it, the different CM-Fb were centrifuged (see Materials and Methods) to pellet MV, and the corresponding supernatants were examined. These MV-free CM were noted CM without MV. They were incubated with PC3 cells in Boyden chambers for 24 hours and no increase of PC3 migration was observed (Fig. 5*A*). In addition, to exclude possible contamination by residual TMV (from Act.D-treated PC3 cells) in the CM-Fb TMV, migratory capacity of PC3 cells toward $TMV_{PC3\ Act.D}$ was controlled. Under this condition, PC3 cells did not migrate (Fig. 5*A-d* and *B-d*), reinforcing the fact that fibroblasts actively released MV after TMV uptake. Because neither fibroblast MV nor CM on their own were able to induce tumor cell migration that occurred only when together, it can be concluded to a cooperative effect between the two counterparts. Furthermore, as shown in Fig. 5*C*, CM-Fb TMV promoted invasion of PC3 cells, this effect being dependent on the concentration of

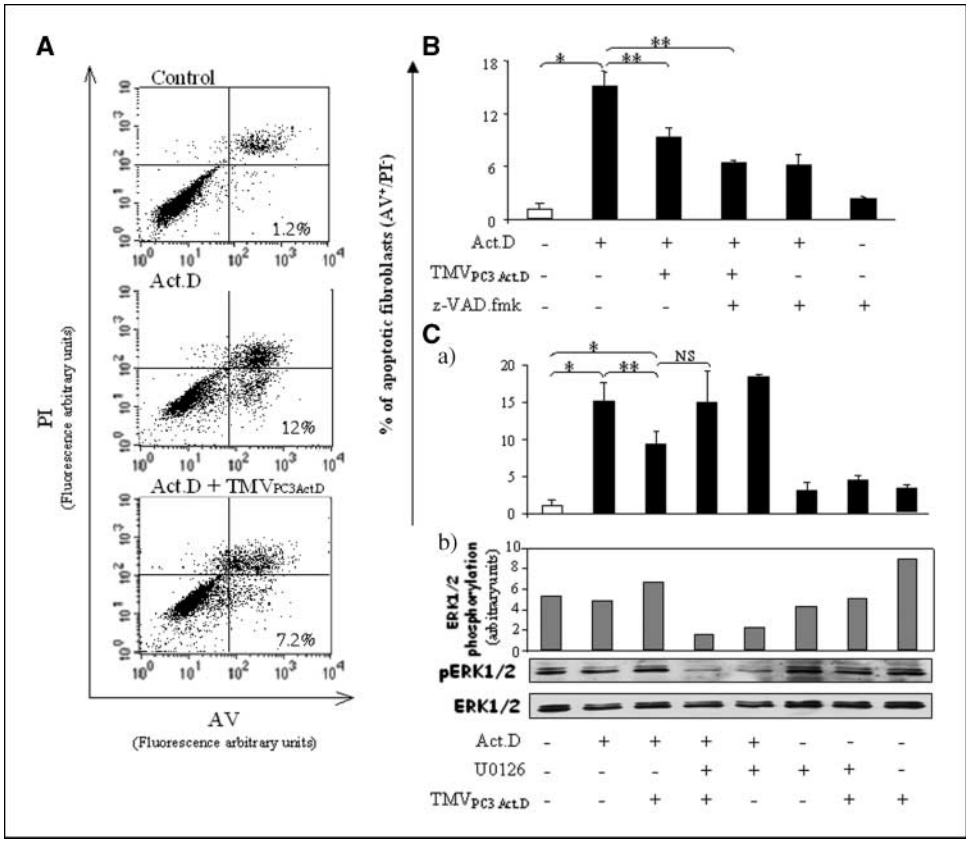

Figure 4. ERK-dependent acquisition of partial chemoresistance by fibroblasts after TMV treatment. *A,* dot plots of double labeling AV/PI to determine apoptotic population (AV^+/PI^-) of fibroblasts treated with TMV (50 μg/mL) and induced to apoptosis with Act.D (0.5 μg/mL) for 24 h. These dot plots are representative of five experiments performed under the same conditions. *B,* histograms represent % of apoptotic fibroblasts under the same conditions as described above in the presence or absence of z-VAD.fmk (50 μmol/L). *, $P < 0.05$; **, $P < 0.005$. *C-a,* histograms represent % of apoptotic fibroblasts under the same conditions as described above in the presence or absence of U0126 (10 μmol/L) for 24 h. These results are from five separate experiments. *, $P < 0.05$; *C-b,* Western blot and densitometry analysis of phosphorylated (*pERK1/2*) and total ERK1/2 (*ERK1/2*) under the same condition for (*C-a*). Histograms and blots are representative of three separate experiments. *NS,* not significant.

TMV used to stimulate fibroblasts, and returned to basal level when MV were eliminated from CM as described above. These results indicate that MV stemming from fibroblasts in response of TMV were essential to induce both migration and invasion of PC3 cells.

CX3CR1 is involved in the control of migration and invasion of PC3 cells toward CM of stimulated fibroblasts. Regarding the implication of CX3CR1-CX3CL1 axis, we have verified the expression of CX3CR1 in PC3 and LnCaP cells, fibroblasts. THP-1 cells that constitutively express CX3CR1 were included in the experiments as positive control. The presence of CX3CL1 was assessed in MV isolated from CM of fibroblasts treated with $TMV_{PC3\ Act.D}$ (MV CM-Fb TMV) toward control (22). As shown in Fig. 6*A-a*, CX3CR1 was detected in PC3 cells and fibroblasts but not in LnCaP cells. On the other hand, CX3CL1 was detected in MV from CM of fibroblasts treated by TMV and in $TMV_{PC3\ Act.D}$ (Fig. 6*A-b*). By neutralizing CX3CL1 in CM-Fb TMV or CX3CR1 in cancer cells with a specific Ab, migration of PC3 cells toward CM-Fb TMV was partially but significantly inhibited (Fig. 6*B*). No basal migration of PC3 cells in the presence of anti-CX3CL1 or anti-CX3CR1 was observed. In addition, no migration of LnCaP cells was induced by CM-Fb (data not shown), probably due to the absence of CX3CR1 expression in these cells. This suggests that CX3CR1 is implicated in the control of migration of PC3 tumor cells, when activated fibroblasts treated with TMV respond through the shedding of MV presenting a chemotactic potential for PC3 cells.

Discussion

Here, we propose a part for MV derived from the plasma membrane of tumor cells in the transfer of biological information between tumor and normal cells as a mode of communication within the tumor microenvironment. Evidence is provided that such TMV have a potential to alter normal cell function, fibroblasts here. TMV promote MMP-9 expression and ERK1/2 phosphorylation in stimulated fibroblasts that become procoagulant, through increase of TF activity expression and PS exposure (Supplementary Fig. S1*A* and S1*B*), and acquire partial chemoresistance. Furthermore, TMV induce migration of fibroblasts and release of MV that in turn promote migration and invasion of cancer cells. Finally, this effect is mediated by CX3CR1, at least partially.

MMP are implicated in extracellular matrix degradation, facilitating cancer cell migration and invasion, and favoring a crosstalk between tumor and stromal cells (23). MMP vehicled by membrane MV shed from tumor and endothelial cells (24–26) may precisely represent a way to interact with the microenvironment. We have therefore assessed the expression of members of this family of protease in TMV. TMV stemming from PC3 cells are enriched in MMP-9 and -14, whereas enrichment in MMP-13 was detected in TMV from LnCaP cells (27).

Treatment of fibroblasts by TMV induced dose-dependent ERK1/2 phosphorylation, known to regulate fundamental processes including proliferation, differentiation, and cell survival (28, 29). TMV from PC3 cells promoted higher migration capacity of fibroblasts than TMV from LnCaP cells, probably in relation with the cellular origin and/or enrichment in MMP-14 observed in TMV from PC3 cells. The implication of the ERK1/2 pathway in pro–MMP-9 expression has been reported by others (30, 31). Our results are in agreement with those obtained with platelet MV, described to induce increase of ERK1/2 phosphorylation and MMP-9 release in fibroblasts. The authors concluded that platelet MV participate

in the invasiveness of breast cancer cells (32). In addition, it has been suggested that platelet MV could contribute to the formation of new blood vessels in growing tumor, via the activation of mitogen-activated protein kinase pathway (25). Another molecule involved in cell migration and activation is PSMA, expressed by normal and neoplastic prostate epithelium, as negative regulator of invasion capability (18). PC3 and LnCaP cells have inverse invasion capability, possibly correlated to PSMA expression at the plasma membrane.

In the context of drug efflux, through the release of TMV for instance, the latter could play a relevant role in the attenuation of sensitivity to drug. In fact, there is a strict correlation between MV shedding, as a mechanism of drug expulsion, and anticancer drug resistance (33). We have observed that $TMV_{PC3\ Act.D}$ were able to decrease Act.D-induced apoptosis in fibroblasts, whereas $TMV_{PC3\ NT}$ had no effect (data not shown). In addition, ERK1/2 pathway is thought to be involved in cell proliferation and protect cells from apoptosis in normal and cancer cells (20), contributing to their drug-resistant nature (34). In the presence of ERK inhibitor, U0126, the effect of TMV on induced-apoptosis of fibroblasts was abolished, suggesting that ERK pathway is activated by TMV to support cell survival.

The interactions between tumor cells and the stroma, including fibroblasts, were described in the establishment of a tumor microenvironment favorable to cancer progression. The latter is thought to be enriched in MV released by cancer cells, platelets, monocytes, and lymphocytes infiltrating the tumor tissue (35). Fibroblasts are associated with tumor cells and, when activated, are believed to play a central role in migration and invasion of cancer cells (7). However, the mechanisms leading to fibroblast activation are far from being fully understood. Because fibroblasts represent the main constituents of the tumor stroma, it was relevant to assess the biological effects of TMV on this cell type. MV isolated from CM of TMV-treated fibroblasts were capable to promote the migration and invasion of cancer cells, suggesting that the activated state of fibroblasts can support the pathogenicity of

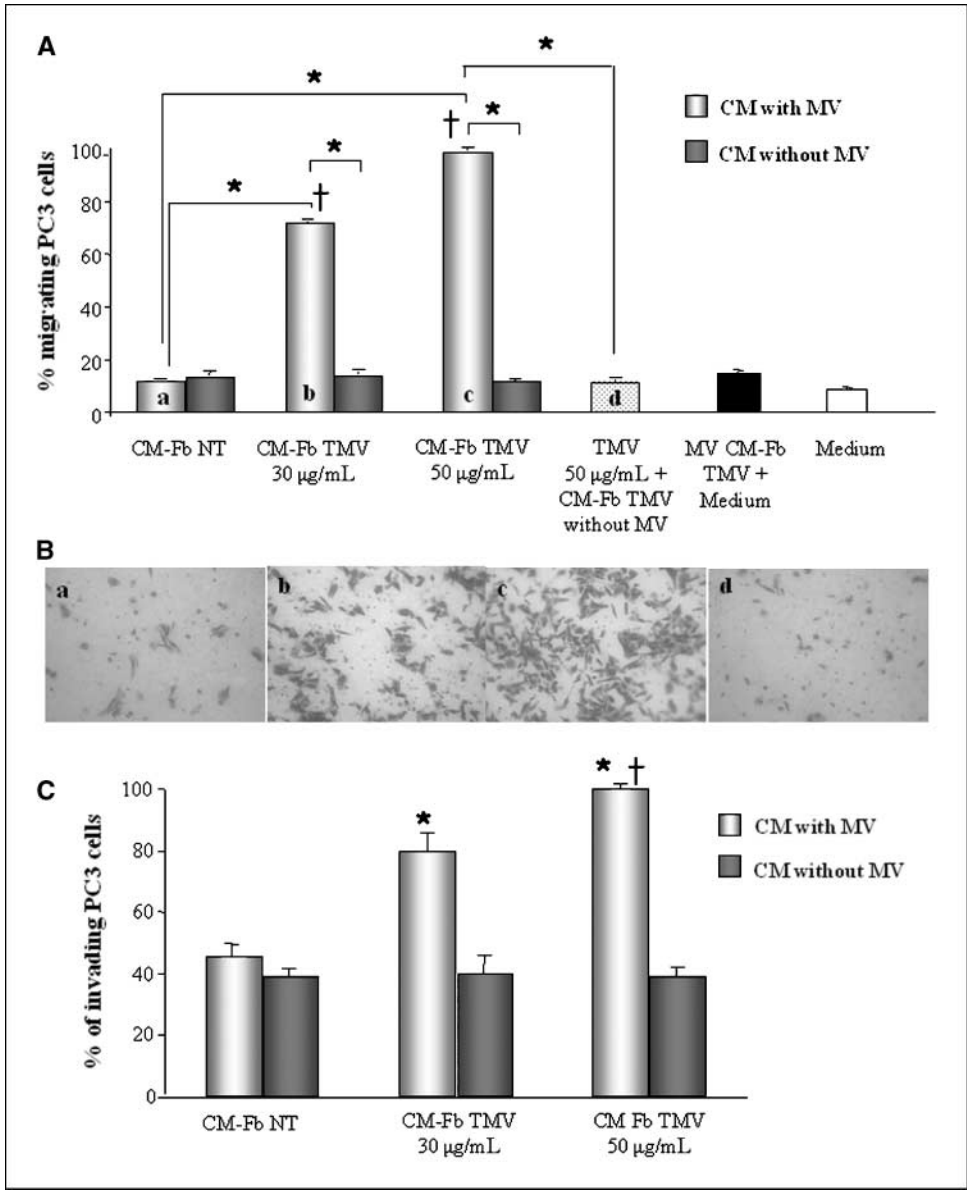

Figure 5. Migration and invasion of cancer cells toward CM of fibroblasts. *A,* histogram representing migrating PC3 cells toward CM of fibroblasts incubated for 48 h with $TMV_{PC3\ Act.D}$ (noted *CM-Fb TMV*), 30 or 50 μg/mL, or CM of untreated fibroblasts (*CM-Fb NT*), used as control. Migration of PC3 cells was tested against CM containing MV (noted *CM with MV*) or CM made free of MV by centrifugation (noted *CM without MV*). *Columns,* mean of experiments performed thrice in duplicate; *bars,* SE. *, $P < 0.05$ vs CM-Fb NT; †, $P < 0.0001$ CM-Fb TMV 50 μg/mL vs CM-Fb TMV 30 μg/mL. *B,* microscopic analysis of PC3 cells on the bottom surface of the Boyden chambers (crystal violet staining). The experiments were carried out thrice with four filters per condition and representative images are shown (magnification, ×200). The small letters correspond to the conditions of *A* above. *C,* invasion of PC3 cells toward CM of fibroblast cultures using a Matrigel chemoinvasion assay (see Materials and Methods). *Columns,* mean of experiments performed thrice in triplicate; *bars,* SE. *, $P < 0.05$, CM from 30 μg/mL or 50 μg/mL TMV-treated fibroblasts vs CM from untreated fibroblasts; †, $P < 0.05$, CM from 50 μg/mL TMV-treated fibroblasts vs CM from 30 μg/mL TMV-treated fibroblasts.

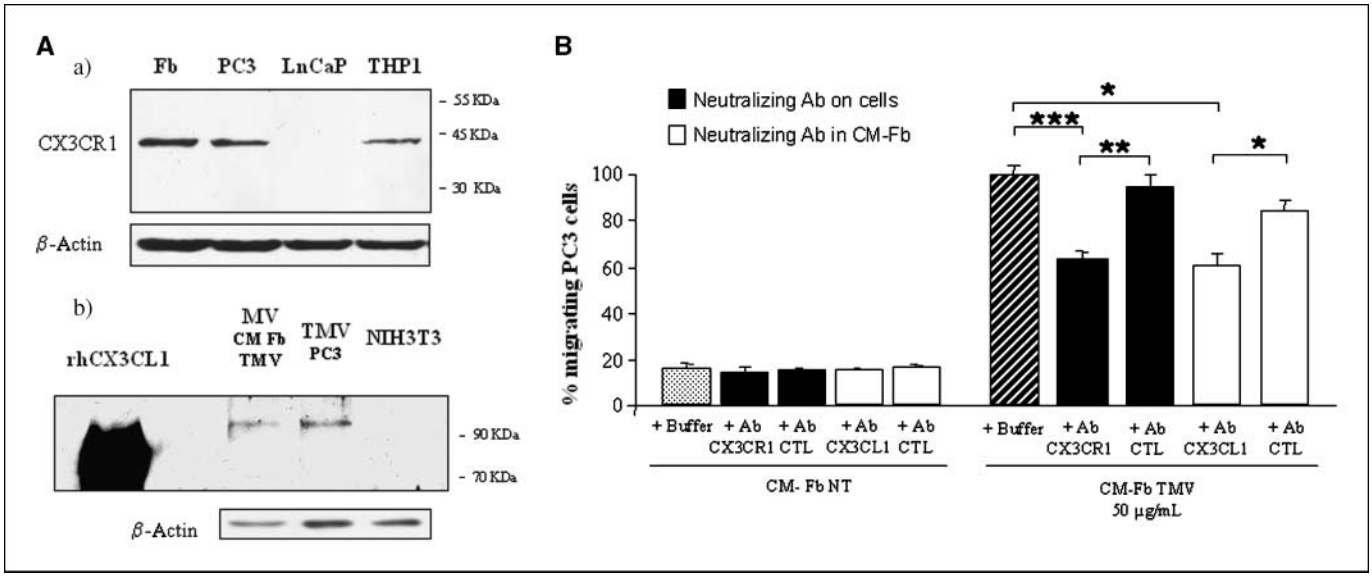

Figure 6. CX3CR1-dependent migration of cancer cells toward CM of fibroblast cultures. *Aa,* CX3CR1 expression was detected by Western blotting in untreated PC3 cells and fibroblasts but not in LnCaP cells. THP-1 cell lysate was used as positive control (36). *Ab,* CX3CL1 was detected by Western blotting in MV isolated from CM of fibroblasts treated with TMV from Act.D-treated PC3 cells (noted as *MV CM-Fb TMV*), and in $TMV_{PC3\ Act.D}$ (noted as *TMV PC3*), NIH3T3 fibroblast lysate was included as negative control for CX3CL1 (22) and recombinant human protein for CX3CL1 as positive control, β actin was included as loading control. Experiments were performed thrice. *B,* migration of PC3 cells toward CM of fibroblasts as previously described (Fig. 5*A*), and in the presence of a specific neutralizing Ab to CX3CR1 (25 μg/mL) or irrelevant IgG (*Ab CTL*) in PC3 cells (■), and in the presence of neutralizing Ab to CX3CL1 (8 μg/mL) or irrelevant IgG in the CM of fibroblasts (□). In control, basal migration of PC3 cells toward CM of fibroblasts nontreated (noted *CM-Fb NT*) in the presence of anti-CX3CL1 or anti-CX3CR1 Abs or irrelevant IgG in the CM of fibroblasts. *Columns,* mean of experiments performed thrice in duplicate; *bars,* SE. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$.

cancer through the release of MV. CX3CR1 seems to play a part in this process. The association with its cognate ligand could regulate adhesion, migration, and survival of these cells (12). Because CX3CR1 expression was verified in human prostate cancer cells and CX3CL1 is present in MV isolated from CM of TMV-treated fibroblasts, the blockade of CX3CR1 or CX3CL1 by specific Ab induced partial, but significant, reduction of tumor cell migration. Even PC3 TMV contain CX3CL1 likewise fibroblast MV (Fig. 6*A-b*), fibroblasts released a large proportion of MV in the CM (Fig. 1*C*) after TMV treatment. The difference of CX3CL1-dependent PC3 migration seems related to the number of fibroblast MV bearing CX3CL1, interacting with CX3CR1 on PC3 cells to a greater extent than with TMV. This is confirmed by the absence of PC3 basal migration in the presence of anti-CX3CL1, and by the lack of effect of TMV on their own (Fig. 5*A-d*). This suggests CX3CR1/CX3CL1 involvement in cancer cell migration toward CM of TMV-treated fibroblasts, together with other soluble factor(s). This is in agreement with a study where fractalkine increased integrin avidity to their ligands on monocytic $CX3CR1^+$ cells (36).

Consistent with a model of chemokine receptor heterodimerization (37), transactivation of growth factor receptor by chemokines has been reported to induce prostate cancer cell invasion (38). Moreover, the membrane-bound form of fractalkine, vehicled by lipid vesicles, was observed to increase natural killer cell activation, compared with the soluble form (39). Due to MV potency as vehicles of selectively sorted biological signals, it is tempting to speculate on their differential effects elicited in target cells possibly resulting from receptor cooperation.

Altogether our results support the evidence that fibroblasts and MV are key actors within tumor environment to sustain and/or promote cancer pathogenesis.

In conclusion, we postulate that human TMV are important mediators of intercellular crosstalk between cancer cells and normal fibroblasts. The present results emphasize the role of MV in cancer progression and in mechanisms attributable to membrane-associated mediators in addition to truly soluble factors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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