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ORIGINAL ARTICLE

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Vitamin D3 modulation of plasminogen activator inhibitor type-1 in human breast carcinomas under organ culture

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Abstract Urokinase plasminogen activator (uPA), its cell-bound receptor (uPAR) and its main inhibitor plasminogen activator type 1 (PAI-1) are present primarily in stromal cells in invasive breast carcinoma. The purpose of this study was to investigate the regulation by 1,25 dihydroxyvitamin-D3 (VD3) of these invasion-associated markers expressed in breast cancer tumors under organ culture, which preserves the interacting network of tumor and stromal cells. Breast carcinoma slices (30 cases), obtained using the Krumdieck tissue slicer, cultured for 48 h in the presence or absence of 100 nM vitamin D3, were embedded in formalin-fixed paraffin. uPA, uPAR, PAI-1 and VD3 receptor (VDR) were analyzed by immunohistochemistry, and their expression, detected in tumor cells and fibroblasts of the specimens, was not statistically changed by culture conditions. The proportion of cases expressing uPA, uPAR and PAI-1 was not affected by VD3 in epithelial cells, but the fraction of cases displaying strong PAI-1 reactivity in fibroblasts was reduced (P=0.016) compared with control slices. Fibroblasts isolated from invasive ductal carcinomas and from normal breast tissues expressed higher VDR mRNA levels than epithelial cells. In cultured tumor fibroblasts, PAI-1 immunostaining and mRNA levels were reduced by VD3-limiting fibroblast contribution to invasion.

Keywords Breast cancer · Calcitriol · Fibroblasts · PAI-1 · Organ culture

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Introduction

The physiologically active form of vitamin D3, 1 alpha,25 dihydroxyvitamin D3 (VD3), not only plays a central role in bone and calcium metabolism, but also has potent antiproliferative effects on several malignant cells, including breast carcinoma cells [27]. VD3 exerts its effects via binding to an intracellular receptor (VDR) present in target tissues. There is accumulating evidence that neoplastic as well as normal breast cells in culture and biopsy specimens express the VDR [15, 17]. The prognosis of breast and other cancers is ultimately determined by the tumor ability to invade and metastasize. Although the modulatory effects of VD3 or its analogous on the invasive process have been shown in a variety of human cancer cell types, relatively little is known regarding the molecular basis for the anti-invasive activity of VD3 in breast carcinomas [21, 29, 32, 37, 41, 46].

Degradation of extracellular matrix barriers is a key step in tumor cell invasion. The plasminogen activator system (PA) plays a key role in cancer progression, presumably via mediating extracellular matrix degradation and tumor cell migration. Urokinase-type plasminogen activator (uPA), a serine protease that catalyzes the conversion of plasminogen to plasmin, which in turn catalyzes the degradation of major extracellular matrix components, is of particular importance. uPA, initially secreted as an enzymatically inactive proenzyme, exerts its proteolytic function after binding to a specific high-affinity cell surface receptor (uPAr). The complexed uPA can be inactivated by the plasminogen activator inhibitor

(PAI-1) [2]. However, there is evidence that PAI-1 promotes tumor invasion by potentiating tumor cell detachment from the matrix [12]. High levels of uPA, uPAr and PAI-1, mostly determined by enzyme-linked immunosorbent assay methods, have been found to be informative markers of poor prognosis in breast cancer [34]. The components of the uPA system are present mainly in stromal cells in invasive breast carcinomas [7, 8, 11, 28, 39, 45], and recent results suggest that strong expression of uPA, uPAr and PAI-1 in fibroblasts, as determined using immunohistochemistry, have the most impact on the clinical behavior of breast cancer [13].

The effects of VD3 on the uPA system have been reported in keratinocytes, osteoblasts, leukemic and breast tumor cells in culture [20, 29, 30, 32]. Our approach to examine a possible regulation of the PA system by VD3 in fibroblasts and epithelial cells of the same tumor was to use organ culture, which preserves the interacting network of tumor cells, stromal fibroblasts, endothelial cells and extracellular matrix, representing, therefore, a good model for this evaluation. It has been shown that surgically obtained human breast cancer tissue can be maintained in culture as intact tissue slices, and previous reports attested to the feasibility of this model for the evaluation of retinoids and estradiol effects in pre-clinical tests [26, 35]. Using immunohistochemistry, we described in the present communication the distribution of VD3 receptor, uPA, PAI-1 and uPAr protein expression in breast tumor slices cultured for 48 h in the presence or absence of VD3.

Materials and methods

Patients

Samples from 33 primary breast cancers were sequentially obtained from Instituto Brasileiro do Controle do Câncer (IBCC), São Paulo, Brasil. Of the tumors, 30 (90.9%) were histologically infiltrating ductal carcinomas, and the remaining, lobular or medullar carcinomas, were excluded from this study. In addition to conventional gross and histological analyses of the surgical specimen, another fragment from each tumor was immediately dissected to remove residual normal tissue and divided into two pieces. One was immersed in RPMI medium, brought to the laboratory for organ culture and immediately processed. The other sample was frozen and stored in liquid nitrogen until required. The ethics committee of IBCC approved the study proposal.

Northern analysis

Total RNA was extracted and analyzed by Northern blot technique as previously described [40]. The PAI-1 cDNA fragment [1] was provided by Dr. R. Mira y Lopes of Mount Sinai School of Medicine (New York, NY). The VDR probe was a 2.1-kb pGEM, provided by Dr J. Shine, California Biotechnology Inc., Mountain View, CA [16]. The ribosomic RNA 18S probe [3] was obtained from Dr N. Arnhein, Biochemistry Department, State University of New York, Stony Brook, NY.

Organ culture

All procedures were carried out as previously described [26]. Once in the laboratory, a small piece from each specimen, immersed in RPMI medium, was fixed in 10% buffered formalin and used for confirmation of histopathological diagnosis of invasive breast ductal carcinoma. The remaining tissue was cut in consecutive 0.5-mm-thick slices using the Krumdieck tissue slicer (Alabama Research and Development Corporation, Birmingham, AL). Sequential slices were then cultured or fixed in buffered formalin (the pre-culture samples). Slices to be cultured were transferred to siliconized lens paper squares, which were floated on 5 ml organ culture medium in 35-mm dishes. The medium was RPMI supplemented with 10% bovine fetal serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 5 μg/ml insulin. Cultures were maintained at 37°C in a humidified atmosphere of 95% air/5% CO_2, in the presence or absence of 100 nM 1,25(OH)2D3. Normally, three or four slices were included per group. After 48 h of culture, the slices were fixed in buffered formalin for 18 h. After fixation, both pre-culture and post-culture slices were processed in a histoprocessor Autotechnicon Model 2A and embedded in paraffin. Sequential sections of 3 μm were placed in slides previously treated with 3-aminopropyltriethoxy-silane (Sigma, A3648, USA) and submitted to immunohistochemistry.

Immunohistochemical staining

After deparaffinization in xylene and rehydration in alcohol, antigen retrieval was performed with 10 mM citric acid pH 6.0 in a pressure cooker. Endogenous peroxidase activity was blocked with 6% H_2O_2 followed by incubation with the following monoclonal antibodies in 1% bovine serum albumin in phosphate-buffered saline (PBS) pH 7.4, overnight at 5°C: anti-uPA (ref 3689) and PAI-1 (ref 3785) from American Diagnostic, USA and anti-VDR, clone MA1–710 (ref MA1–710) from Affinity Bioreagents, Golden, CO, USA. Anti uPAR (mAbR2) was kindly provided by Dr. Keld Dano (Finsen Institute, Copenhagen, Denmark).

The slides were then incubated for 30 min at 37°C with biotinylated goat anti-mouse/rabbit Ig, followed by incubation for 30 min at 37°C with the streptavidin-biotin peroxidase (Duet, Dako, k0492, USA). Sections were developed by 3,3′ diaminobenzidine tetrahydrochloride (Sigma, D5637, USA), 6% H_2O_2 and dimethyl sulphoxide. Positive and negative controls were included in each run. The samples were observed at light microscope by two observers (SN and VAFA). Each one of the slides was first semi-quantified for the presence of viable cells, and then, the neoplastic epithelial cells and stromal cells were independently semi-quantified for immunohistochemical expression of each antigen as follows: score 0 (negative), less than 10% of reactive cells; score 1, 1–50% of reactive cells; score 2, more than 50% of reactive cells.

Fibroblast short-term culture

Specimens from normal or neoplastic breast tissues freed of adipose tissue were collected in PBS and finely minced. The micro-fragments were suspended in Dulbecco's Modified Eagle medium with 20% of fetal bovine serum and supplemented with ampicillin and streptomycin (100 μg/ml). Fibroblasts spread out from virtually every tissue fragment, and, after confluency, cells were subcultured using trypsin/ethylene diamine tetraacetic acid. Homogeneous cell cultures were obtained after the third replating. Fibroblasts were characterized using standard immunocytochemical procedures with the following antibodies: pan-cytokeratin (AE1/AE3, MxH), α-smooth muscle actin (HHF35, MxHx) both from Dako Corporation, Denmark, USA and anti-vimentin (clone LN-6) from Sigma-Aldrich Corporation, Saint Louis, MO.

Cell culture

Normal breast luminal cells (HB4a) were donated by Dr. M.J. O'Hare (Ludwig Institute for Cancer Research, London UCL Branch, London W1 W 7BS, United Kingdom) [44]. Breast cancer cell lines (MCF-7 and MDA-MB-231) were purchased from American Type Culture Collection. Cells were grown and maintained in RPMI medium plus 10% fetal bovine serum.

Statistical analysis

To probe for statistically significant associations between variables, chi-square analysis was performed, and a P value of less than 0.05 was considered statistically significant.

Results

Specimens of breast cancer and non-neoplastic tissue samples from 30 patients were analyzed for the expression of VDR mRNA by Northern blotting. Representative Northern signals from paired tumors and the adjacent normal breast tissues are displayed in Fig. 1. The VDR mRNA (4.6 kb) was detected in all tissue samples, albeit with a wide spectrum of relative levels. Quantification of Northern signals after normalization with 18S revealed that VDR mRNA expression levels ranged from 0.3 to 3.6, median 2.3. Normal adjacent tissues expressed VDR within similar ranges (median 1.39). Tissue slices, obtained from pre-culture samples or from organ culture as described in the Materials and Methods section, were fixed in 10% buffered formalin and evaluated using immunohistochemistry. VDR, uPA, uPAr and PAI-1 were assessed in the malignant epithelial cells and in tumor-associated fibroblasts within each section.

Results are summarized in Table 1, Table 2 and Table 3 and are presented as proportion of cases of breast cancer explants in each category of reactivity/total numbers of cases. The difference in the number of cases for each of the antibodies resulted from the absence of "viable cells" present in a particular slide, and all the cases where "viable cells" were not spotted were excluded from this study.

Figure 2A illustrates the expression of VDR in one of the control tumor slices subjected to culture. Staining was seen in the cell nuclei and cytoplasm. VDR immunoreactivity was present in the tumor cells in 70% of cases and in fibroblasts in 66% of cases. Slices maintained in culture for 48 h did not show a decline in scores of VDR staining. Treatment with VD3 slightly increased the

Table 1 Immunohistochemical evaluation of vitamin D3 (VD3) receptor (VDR) in epithelial cells and fibroblast (cases in each category/total cases). Breast cancer slices treated or not with VD3 were immunostained with specific antibody against VDR as described in Materials and methods. Results were graded as: 0 (<10% of stained cells), 1 (10–50% of stained cells) and 2 (>50% of stained cells). *n.s.* not significant

Staining score	Preculture	48 h	48 h plus VD3	P (x^2)
Epithelial cells (n=27)				
0	8 (30%)	6 (22%)	4 (15%)	n.s.
1	7 (26%)	8 (30%)	8 (30%)	
2	12 (44%)	13 (48%)	15 (55%)	
Fibroblasts (n=29)				
0	10 (34.5%)	6 (21%)	4 (14%)	n.s.
1	12 (41.5%)	12 (41.5%)	10 (34%)	
2	7 (24%)	11 (37.5%)	15 (52%)	

Table 2 Proportion of tumor cells staining for urokinase plasminogen activator (uPA), its cell-bound receptor (uPAR) and its main inhibitor plasminogen activator type 1 (PAI-1) (cases in each category/total cases). Breast cancer slices treated or not with vitamin D3 (VD3) were immunostained with specific antibodies against uPA, uPAr and PAI-1 as described in Materials and methods. Results were graded as: 0 (<10% of stained cells), 1 (10–50% of stained cells) and 2 (>50% of stained cells). *n.s.* not significant

Staining score	Preculture	48 h	48 h plus VD3	P (x^2)
uPA (n=25)				
0	1 (4%)	1 (4%)	2 (8%)	n.s.
1	12 (48%)	14 (56%)	13 (52%)	
2	12 (48%)	10 (40%)	10 (40%)	
uPAr (n=27)				
0	3 (11%)	2 (7%)	2 (7%)	n.s.
1	17 (63%)	15 (56%)	17 (63%)	
2	7 (26%)	10 (37%)	8 (30%)	
PAI-1 (n=28)				
0	0 (0%)	0 (0%)	0 (0%)	n.s.
1	0 (0%)	2 (7%)	2 (7%)	
2	28 (100%)	26 (93%)	26 (93%)	

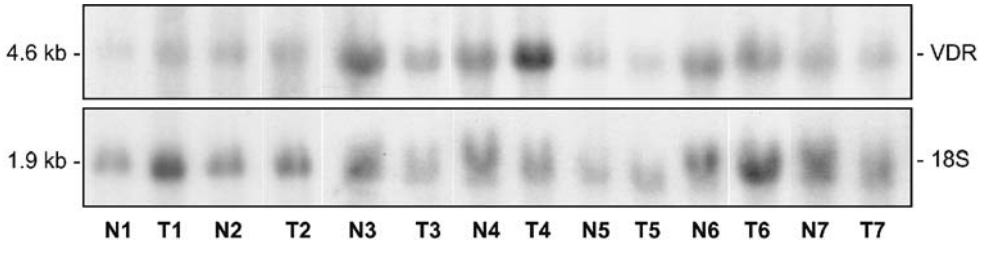

Fig. 1 Expression of vitamin D3 receptor mRNA in breast carcinoma (*T*) and its adjacent normal breast tissue (*N*). Northern blot was performed as described in methods

Table 3 Proportion of fibroblasts staining for urokinase plasminogen activator (uPA), its cell-bound receptor (uPAR) and its main inhibitor plasminogen activator type 1 (PAI-1) (cases in each category/total cases). Breast cancer slices treated or not with vitamin D3 (VD3) were immunostained with specific antibodies against uPA, uPAr and PAI-1 as described in Materials and methods. Results were graded as: 0 (<10% of stained cells), 1 (10–50% of stained cells) and 2 (>50% of stained cells). *n.s.* not significant

Staining score	Hours	48 h	48 h plus VD3	P (x^2)
uPA				
(*n*=25)				
0	0 (0%)	1 (4%)	3 (12%)	n.s.
1	12 (48%)	13 (52%)	11 (44%)	
2	13 (52%)	11 (44%)	11 (44%)	
uPAr				
(*n*=27)				
1	3 (11%)	4 (15%)	3 (11%)	n.s
2	4 (15%)	7 (25%)	7 (25%)	
3	20 (74%)	16 (59%)	17 (63%)	
PAI-1				
(*n*=28)				
0	1 (4%)	3 (11%)	14 (50%)	0.016
1	5 (18%)	8 (29%)	8 (29%)	
2	22 (78%)	17 (60%)	6 (21%)	

number of tumor cells and fibroblasts classified as presenting a highly positive VDR score (Table 1).

Figure 2B shows that uPA is localized in the cancer cells and, in general, a predominantly cytoplasmic staining was found, sometimes localized in the nucleus. uPA is also evident in fibroblasts (Fig. 2C). Of the cases, 96% had expression of uPA in their tumor cells, and, in 100% of cases, it was present in fibroblasts. After a 48-h culture period, in the absence or presence of VD3, no significant differences were seen in the distribution of positivity in either stromal or epithelial cancer cells. Of the cases, 89% revealed immunoreactivity for uPAr in fibroblasts and in epithelial cells. In these cells, a cytoplasm-associated immunostaining was observed (Fig. 2D). VD3 treatment did not affect uPAr expression, independently of cell type (Table 2 and Table 3).

PAI-1 expression was present in cancer cells in cytoplasm and eventually in nuclei in 100% of the specimens (Fig. 3A), and this proportion remained constant throughout the 48-h culture period or after VD treatment. Positive staining was found in the fibroblasts of 96% of the specimens (Fig. 3B). Significantly fewer samples expressed strongly stained PAI-1 (i.e., score 2) in fibroblasts upon VD3 treatment (60% versus 21%, P=0.016) compared with control slices after 48 h in culture (Table 3).

PAI-1 mRNA expression was studied in control slices in culture. PAI-1 mRNA (3.3 kb and 2.4 kb) expression was detected in all cases analyzed (Fig. 4).

As Northern blot analysis does not distinguish among functionally different cell types of the tumor, in a final set of studies, fibroblasts isolated from one area near an invasive ductal breast carcinoma (T) and from normal breast tissue (N) were treated with VD3. Both fibroblast populations were characterized as myofibroblasts and expressed higher VDR mRNA levels (approximately twofold more than the values expressed by the breast epithelial cells analyzed concomitantly: a normal human

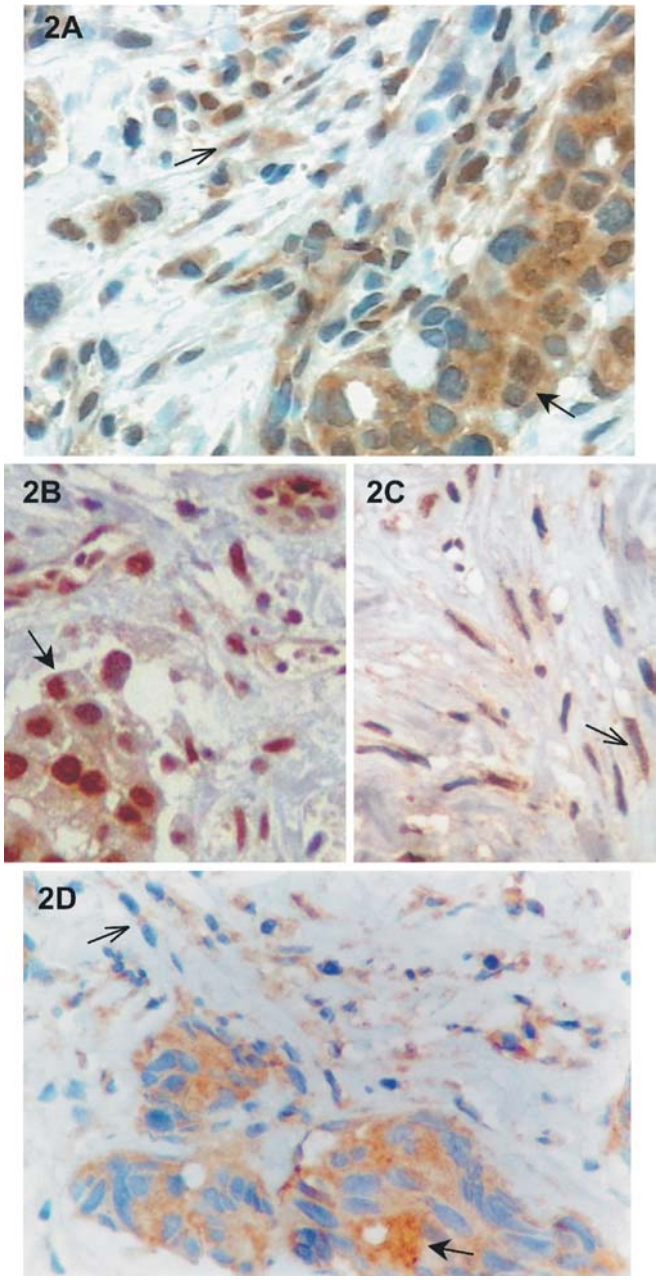

Fig. 2 A Nuclear and cytoplasmic vitamin D receptor immunoexpression indicated by *arrows* in breast tumor cells and fibroblasts. Original magnification ×400. **B** Urokinase plasminogen activator (uPA) immunoexpression located in neoplastic cells (*arrow*). Original magnification ×400. **C** uPA immunoexpression in fibroblasts (*arrow*). Original magnification ×600. **D** The cytoplasmic immunoexpression of the cell-bound receptor of urokinase plasminogen activator (uPAR) was seen in breast tumor cells (*arrow*) and fibroblasts (*arrow*). Original magnification ×600

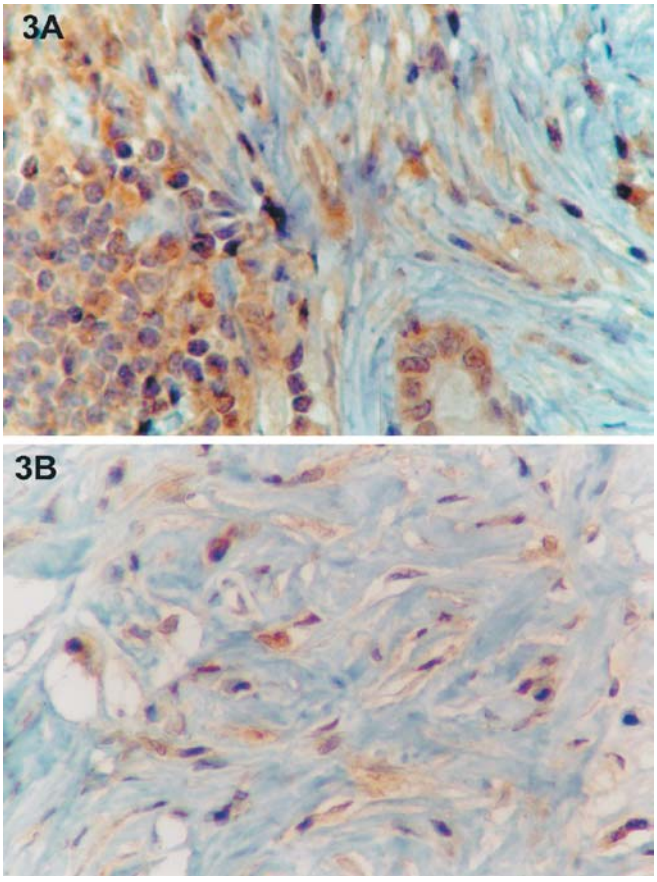

Fig. 3 Plasminogen activator type-1 immunoexpression. Strong immunostaining is depicted in breast carcinoma cells (**A**) and in fibroblasts (**B**). Original magnification ×600

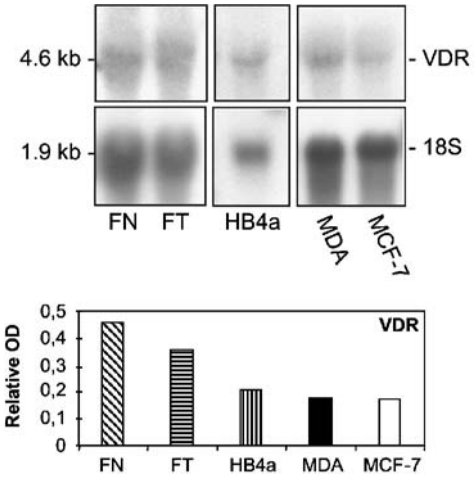

Fig. 5 Expression of vitamin D3 receptor mRNA in human mammary fibroblasts obtained from normal breast tissue (*FN*); tumor-associated fibroblasts (*FT*), normal human mammary luminal (*HB4a*) and breast carcinoma cell lines (*MDA-MB-231* and *MCF-7*). RNA total were extracted, and submitted to Northern blot as described in methods. Band intensities in autoradiograms were quantified by densitometric scanning and data expressed as the ratio of the specific mRNA to 18S ribosomal RNA

epithelial cell (HB4a) and two breast cancer cell lines (MDA-MB-231 and MCF-7) (Fig. 5). Peritumoral fibroblasts in culture responded to the addition of VD3 to the medium, showing a 34% reduction in PAI-1 mRNA expression measured after 48 h of treatment (Fig. 6). In normal fibroblasts, a decrease of 15% was observed. In contrast, in normal human mammary luminal cells and breast cancer cells, PAI-1 mRNA levels were not modified by VD3. All of the tumor fibroblasts in culture were reactive to the anti-PAI-1 antibody. After VD3 treatment, fibroblasts were stained less intensively, showing decreased PAI-1 expression as compared with control tumor fibroblasts (Fig. 7).

Discussion

Our results showed that organ culture of human carcinoma tissues maintained for 48 h preserved the expression profile of VDR, PAI-1, uPA and uPAr similar to that existing in vivo, thus, suggesting cell viability. Each component of the PA system was found both in fibroblasts and epithelial cells, in keeping with several

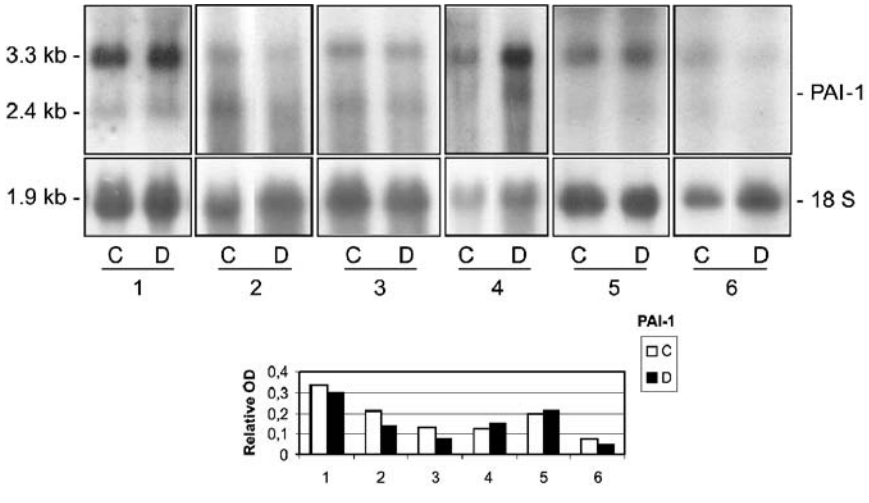

Fig. 4 Expression of plasminogen activator type-1 mRNA in breast carcinoma slices in culture. Total RNA was extracted and submitted to Northern blot as described in methods. Band intensities in autoradiograms were quantified by densitometric scanning and correspondent data expressed as the ratio of the specific mRNA to 18S ribosomal RNA. **C** Control, **D** Vitamin D3 treated

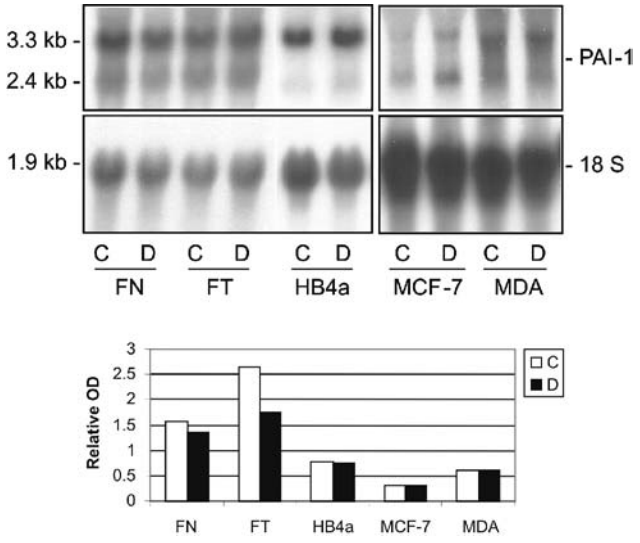

Fig. 6 Expression of plasminogen activator type-1 mRNA in human mammary fibroblasts obtained from normal breast tissue (*FN*); tumor-associated fibroblasts (*FT*) and normal human mammary luminal (*HB4a*), mammary carcinoma cell lines (*MDA MB-231* and *MCF-7*). Cells lines were exposed or not to vitamin D3 (VD3) and total RNA was extracted, and submitted to Northern blot analysis as described in Materials and methods (two independent assays were performed and mean values were presented). Band intensities in autoradiograms were quantified by densitometric scanning and data expressed as the ratio of the specific mRNA to 18S ribosomal RNA. **C** Control. **D** VD3 treated

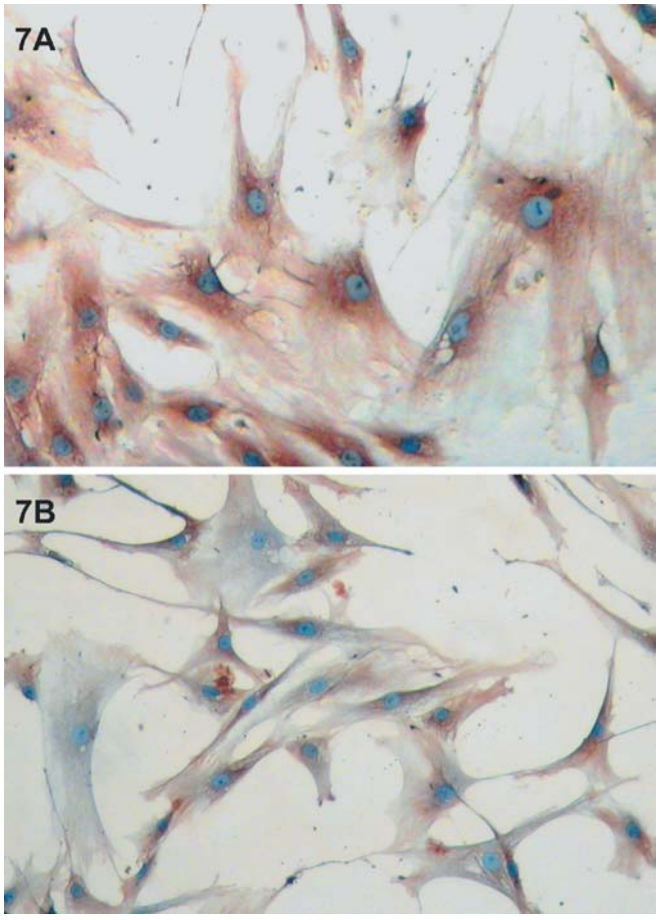

Fig. 7 Cytoplasmic plasminogen activator type-1 immunoexpression in human mammary fibroblasts obtained from tumor-associated fibroblasts. Cultures were maintained at 37°C in a humidified atmosphere of 95% air 5% CO_2, in absence (**A**) or in presence of 100 nM vitamin D (**B**) for 48 h, and then the cells were formalin fixed for 18 h. The immunocytochemical procedure was performed as described in Materials and methods

published studies [7, 8, 11, 28, 39, 45]. Although uPA and PAI-1 have been described as localized in the cytoplasm of cancer cells, we found immunostaining of uPA and PAI-1 to also be present in the nucleus in some samples, as previously published by Jankun et al. [24], using the same antibodies. Those authors suggested that rapid removal of uPA/uPAR/PAI-1 complexes by endocytosis exposes protein to acid proteases of lysosomes, producing different protein fragments, which are able to move to the nucleus. We examined other tissues for uPA so as to ascertain if the tissue preparation caused the unexpected localization of uPA and PAI-1 proteins, but normal tissues never displayed a nuclear localization, thus, suggesting that the nuclear staining of uPA was not due to faulty specimen preparation.

uPAR is attached to the cell membrane by a glycosylphosphatidylinositol anchor, and its expression should be found at the cellular membrane. As demonstrated in our results, some studies also reported a cytoplasmic staining [7, 11, 13]. One possible reason is that the antibody uPAR2 recognizes the cytoplasmic domain or uPAR present in the cytosol. Other possibilities already suggested by Dublin et al. [13] included either inhibition of antibody binding by uPAR binding to its ligand or that the epitope recognized by the antibody at the membrane was hidden by conformational changes in the uPAR molecule [23, 36, 42].

According to Friedrich et al. [18], no visible differences in the expression of VDR at the mRNA level were found compared with normal breast tissue. VDR nuclear staining localization was previously demonstrated in breast cancer [6, 17, 18, 19]. Treatment of slices with vitamin D induced a slight increase in VDR protein expression, due, perhaps, to the fact that VDR half-life was prolonged by vitamin D [33]. Upregulation of VDR immunoreactivity in fibroblasts was previously reported [9].

Vitamin D3 treatment reduced uPA activity in MDA-MB-231 breast carcinoma cells in cultured keratinocytes and in fibroblasts [30, 31]. We did not detect a similar decrease in abundance of uPA protein in our slices in culture. It is possible that uPA protein levels determined using immunohistochemistry might not have been equivalent to PA activity.

In the present study, we have demonstrated a decreased PAI-1 protein content in fibroblasts in either tumor slices or in primary cultured fibroblasts obtained from breast tumors when exposed to VD3. In accordance with our results, it has been previously demonstrated that

VD3 decreases PAI-1 mRNA in osteoblasts [20], and, in cultured keratinocytes, PAI-1 secretion and deposition was similarly inhibited [30]. However, we have not observed PAI-1 modulation in epithelial breast cancer cells, suggesting that VD3 displays divergent effects, which might be cell specific. It is possible to speculate that the differential responsiveness of the epithelial and stromal cells to $1,25(OH)_2D_3$ observed in the present work may reflect the VDR content of each cell or take place at a post-receptor site. A VDR was not demonstrated in the promoter of PAI-1 gene, suggesting that VD3 may not directly affect PAI-1 transcription, but may instead act through alternative pathways, which counteract induction effects by growth factors already described in breast tumor fibroblasts [43].

Our observation of PAI-1 reduction by VD3 treatment is of potential therapeutic interest, given the relevant role proposed for PAI-1 in fibroblasts in promoting cell invasion [4, 13]. Studies in rodent models reported the importance of vitamin D to prevent breast cancer metastasis in vivo and promising results of treatment with vitamin D of metastatic androgen-independent prostate cancer patients were published [5, 14, 22]. The possibility that vitamin D may have attenuated the contribution of breast tumor associated-fibroblasts to invasion adds to the body of evidence described in recent reviews that vitamin D analogues, exerting few calcemic side effects, may be effective in the treatment of breast cancer [10, 25, 38].

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