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Fibronectin induces MMP2 expression in human prostate cancer cells

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ABSTRACT

High-grade prostate cancers express high levels of matrix metalloproteinases (MMPs), major enzymes involved in tumor invasion and metastasis. However, the tumor cell lines commonly employed for prostate cancer research express only small amounts of MMPs when cultivated as monolayer cultures, in common culture media. The present study was conducted to ascertain whether culture conditions that include fibronectin can alter MMP2 and MMP9 expression by the human prostatic epithelial cell lines RWPE-1, LNCaP and PC-3. These cells were individually seeded at 2×10^4 cells/cm^2, cultivated until they reached 80% confluence, and then exposed for 4 h to fibronectin, after which the conditioned medium was analyzed by gelatin zymography. Untreated cells were given common medium. Only RWPE-1 cells express detectable amounts of MMP9 when cultivated in common medium, whereas the addition of fibronectin induced high expression levels of pro and active forms of MMP2 in all tested cell lines. Our findings demonstrate that normal and tumor prostate cell lines express MMP2 activity when in contact with extracellular matrix components or blood plasma proteins such as fibronectin. Future studies of transcriptomes and proteomes in prostate cancer research using these cell lines should not neglect these important conclusions.

1. Introduction

Prostate cancer is the most common malignancy in men and the second leading cause of male cancer-related deaths in the Western world [1,2]. Castration-resistant prostate cancer (CRPC) is the most advanced form of prostate cancer exhibiting reduced survival, with reported values varying between 9 and 30 months [2]. In this sense, in vitro culture systems are widely employed in prostate cancer research and, more recently, for transcriptome and proteomic analysis because the cultures reproduce rapidly and readily [3]. Although these monolayer cultures provide rapid and important results, they recreate only a fraction of a complex scenario [4]. The absence of cell-to-extracellular matrix (ECM) interactions can lead to misleading results, especially for prostate cancer, where the cross-signaling between epithelium and stroma is so important [5].

Matrix metalloproteinase's (MMPs) belong to a family of zinc and calcium dependant endopeptidases responsible for the first steps in ECM degradation in both normal development and disease [6,7]. A very invasive and metastatic behavior is a major characteristic of CRPC, and ECM degradation is critical to these processes [2,7]. However, the commonly employed tumoral cell lines on prostate cancer research express small amounts of MMPs when cultivated in monolayer cultures with common culture media [8,9], without ECM components.

Considering that cell-ECM cross-talk, when present in culture systems, leads to behavioral changes in tumor cell biology, such as increased adherence to substrates [10], increased resistance to chemotherapeutic agents [11], and resistance to apoptosis [12], we hypothesized that it could also modulate the expression of MMPs by human prostate cells. Therefore, we conducted the present investigation exposing the classical human prostate cell lines RWPE-1, LNCaP, and PC-3 to fibronectin, a multifunctional extracellular glycoprotein, which is considered of critical relevance for the process of metastasis and cell survival [13]. Not only is fibronectin the most abundant adhesion protein in the blood, but it also interacts with circulating tumor cells [13], and therefore the ECM protein chosen for this investigation.

Furthermore, employing this culture model, we exposed these cells to finasteride, an inhibitor of the enzyme five alpha-reductase, which is widely used in benign prostatic hyperplasia treatment and potentially a chemopreventive agent for prostate cancer [14], to ascertain whether in these conditions finasteride would also modulate the expression of MMP2, as previously reported for the rat ventral prostate [15], where epithelial-stroma interactions occur.

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2. Material and methods

RWPE-1, LNCaP, and PC-3 cell lines were acquired from the American Type Culture Collection (ATCC). The experiments were performed two months after the acquisition of the cell lines, which were cultivated according to ATCC guidelines. These cells were then individually seeded at 2×10^4 cells/cm^2 in 6-well culture plates (TPP™) and the experiments were carried out in triplicate. Upon reaching 80% confluence, cells were washed three times with sterile D-PBS (GIBCO/Invitrogen™), and the recommended culture medium (FBS free) was added as follows: (1) control treatment – 0.1% dimethylsulfoxide (DMSO); (2) fibronectin treatment – 0.1% DMSO plus 25 μg/mL fibronectin (Sigma™); and (3) combined fibronectin/finasteride treatment – 0.1% DMSO plus 25 μg/mL fibronectin (Sigma™) plus 50 μM finasteride (Sigma™). All reagents were dissolved in the culture medium. After 4 h of exposure, the conditioned media (CM) were collected and individually stored at −80 °C.

The CM were concentrated using Centriprep® 10,000 MWCO (Millipore™) tubes and submitted to protein quantification at a NanoDrop 2000 (Thermo Scientific™) spectrophotometer. Equal amounts of protein from CM (15 μg) were analyzed by gelatin zymography on 8% polyacrylamide gels co-polymerized with 0.1% gelatin (Merck™) as substrate. Purified human MMP2 (20 ng) and MMP9 (30 pg) (Calbiochem™) were also loaded as positive controls. After electrophoresis, the gels were rinsed twice in 2.5% Triton X-100 and incubated 24 h in an activation buffer (50 mM Tris–HCl, 5 mM $CaCl_2$ and 0.02% $ZnCl_2$). Gels were stained with Coomassie brilliant blue R-250 and de-stained with 20% methanol and 10% acetic acid in distilled water until the clear bands had been visualized. The activities of the MMP2 and MMP9 bands were quantified using the ImageJ™ software for each sample (as triplicates). The integrated optical density (IOD) was measured and the data were analyzed with INSTAT™ software using the two tail Student's t test ($p < 0.05$) to compare the different treatments. Values were calculated as the mean ± SD of the totality of IODs for the pro- and active forms of the MMP2 and MMP9 enzymes. Finally, a fold-change graphic was made by dividing the means of the values for the treated cells by the mean of the values for the untreated cells.

3. Results

When cultivated in common culture media, all cell lines produced very low detectable levels of MMP2 activity (Fig. 1A). Fibronectin exposure significantly upregulated MMP2 activity in all three cell lines, with a 25.5-fold-change in RWPE-1 cells, a 22.6-fold-change in the LNCaP cells and a 24.0-fold-change in PC-3 cells compared with controls (Fig. 1B). However, when these cell lines were co-exposed to fibronectin and finasteride, the activity of MMP2 was significantly lower than those treated only with fibronectin; reduced at least by 33% (Fig. 1B).

Curiously, the LNCaP cells primarily expressed pro-MMP2 whereas PC-3 expressed active-MMP2 (Fig. 1A), a phenomenon most likely related to the greater invasive behavior of PC-3. In contrast, only RWPE-1 cells expressed low but detectable amounts of MMP9, mostly in its pro-form (Fig. 1A). Fibronectin treatment induced partial activation of MMP9, whereas finasteride reduced this activation (Fig. 1A) ($p > 0.05$).

4. Discussion

In this study, we evaluated the expression of gelatinases A and B in the prostate cells lines RWPE-1, LNCaP and PC-3, which are widely employed in prostate cancer research, using two different culture conditions. In our initial experiments, we demonstrated

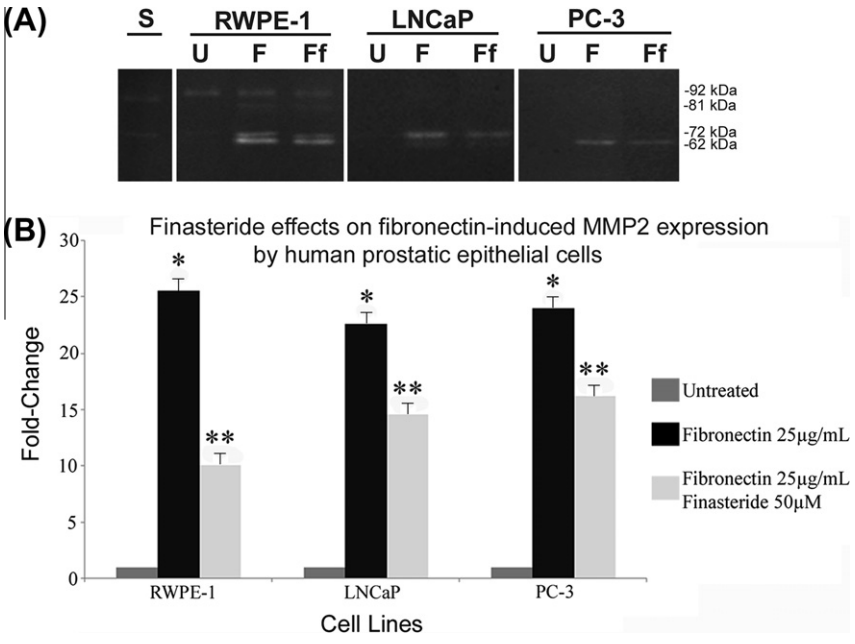

Fig. 1. (A) Representative zymography of the gelatinolytic activity observed in the conditioned medium from untreated cells (U), fibronectin-treated cells (F), and fibronectin plus finasteride treated cells (Ff). Lane S corresponds to the reference standard of human active-MMP9 and pro-MMP2 enzymes. Gelatinolytic bands of 92, 81, 72, and 62 kDa were observed, which correspond to pro-MMP9, active-MMP9, pro-MMP2, and active-MMP2, respectively. In normal culture conditions only RWPE-1 cells express MMP9 activity. Fibronectin treatment induced high MMP2 activity in all tested cell lines. Finasteride-treatment significantly downregulated this induction. Note that LNCaP expresses mainly pro-MMP2, whereas PC-3 expresses active-MMP2 after fibronectin treatment. (B) Densitometry analysis of the gelatinolytic bands from the conditioned media from untreated, fibronectin treated and fibronectin/finasteride treated cells (RWPE-1, LNCaP and PC-3). The data are expressed as the fold-change of the means ± SD of MMP2 total band IOD between the fibronectin and combined fibronectin/finasteride treatments over the untreated cells, which were given the value of 1. Fibronectin treatment significantly increased MMP2 activity in the three cell lines, whereas finasteride treatment significantly reduced total MMP2 gelatinolytic activity. *Statistically significant difference from untreated cells with $p < 0.001$. **Statistically significant difference from fibronectin treated cells with $p < 0.05$.

that when these cells were cultivated as monolayers, using regular culture medium, all of them secreted very low amounts of MMPs in their CM, in accordance to previous results [8,9]. Interestingly, only the non-tumoral cell line RWPE-1 expressed detectable levels of MMP9 under these culture conditions. However, when the cell cultures were grown with fibronectin in the culture medium, all the cell lines tested exhibited significantly higher MMP2 levels in their CM. Although this effect was observed for MMP2 in all tested cell lines, this was not observed for MMP9. Furthermore, our results demonstrated that the co-treatment of these cells, with fibronectin and finasteride, resulted in the same pattern of downregulation of MMP2 activity after finasteride treatment, as observed in the rat ventral prostate [15], and now reproduced in an in vitro model of human prostate cancer.

Although Das et al. [16] reported higher expression of MMPs-2 and 9 upon exposure of human breast cancer cells to fibronectin, and Meng et al. [17] reported increased invasion of lung cancer cells through MMP9 activity after fibronectin treatment, this is the first report of enhanced activity of MMPs in prostate cancer cell lines after fibronectin exposure. Our findings highlight the importance of cell-ECM interactions in the modulation of these endopeptidases. Similar results were obtained by Pentyala et al. [18], who showed that the interaction of LNCaP cells with the extracellular matrix plays a dominant role in uPA and uPAR gene expression, another protease system involved in ECM degradation [19].

Fibronectin has been shown to support cell proliferation and invasion, which are major events for metastasis, and to protect tumoral cells from the cytotoxic action of natural killer cells [13]. Now we have demonstrated that fibronectin can also upregulate the expression of MMPs that are directly involved in prostate cancer aggressiveness. Interestingly, our findings demonstrated that fibronectin could only induce MMP2 activity, but not MMP9, in the tested prostate cell lines. Although MMP2 has been historically considered a constitutive gene due to the absence of well-characterized regulatory elements in the MMP2 promoter region [20], recent reports on MMP2 promoter analyses have demonstrated numerous regulatory elements that could modulate MMP2 expression [21]. The mechanism by which fibronectin induces MMP2 expression has been described in MCF-7 human breast carcinoma cells and A549 human lung cancer cells, and involves fibronectin–integrin $\alpha5\beta1$ interaction and/or FAK/PI-3K/ERK signaling pathways activation [16,17], but this mechanism remains to be confirmed for prostate cancer cells, in future studies.

Taken together, all these reports highlight the importance of the cell-ECM interactions for studying transcriptomes and proteomes from both normal and tumoral cells, at in vitro systems [3–5,8–10,13,16–18]. In this sense, future investigators should be aware that RWPE-1, LNCaP and PC-3 cells, and most likely other tumor cells, require cell-ECM interaction in order to express detectable levels of MMPs on their CM.

In conclusion, our results confirm that: (1) fibronectin exposure upregulates the activity of MMP2 in all tested human prostate cell lines up to a 25-fold-increase and (2) by employing a human cell culture model with cell-ECM interaction, it was possible to reproduce previous results of an in vivo study where finasteride downregulated the activity of MMP2 in the rat ventral prostate.

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