Interactions Between Normal Human Fibroblasts and Human Prostate Cancer Cells in a Co-culture System

FORTUNATA IACOPINO, CRISTIANA ANGELUCCI and GIGLIOLA SICA

Institute of Histology and Embryology, School of Medicine, Catholic University of the Sacred Heart, Rome, Italy

Abstract. *Background: Stroma affects the development and the structure of many organs and plays an important role in regulating epithelial malignancies, including those derived from the prostate. Fibroblasts represent the major cell type of the stromal compartment. Aiming at clarifying the relationships between normal fibroblasts and epithelial cancer cells, we utilized a co-culture system, which included both androgen-sensitive (LNCaP) and -insensitive (PC-3, DU-145) prostate cancer cell lines and a human gingival fibroblast cell line (FG). Materials and Methods: The morphological aspects of the cultures were analyzed under an inverted phase-contrast microscope; the proliferation in conditioned media (CM) was assessed by cell counts, and the E-cadherin expression was evaluated by immunocytochemistry. Results: In co-culture, androgen-sensitive LNCaP cells grew in a network on the top of the monolayer formed by FG, while colonies of androgen-insensitive PC-3 and DU-145 cells were surrounded by FG cells. After six days, the LNCaP cell number was apparently lower in the co-cultures than in the plates where they grew alone. Both LNCaP and FG cells underwent morphological changes. After the same period of time, the growth of PC-3 and DU-145 cells overcame the growth of FG cells, which were almost abolished. The CM of FG inhibited the proliferation of LNCaP cells, after three days by 33% ($p<0.01$) and after six days by up to 82% ($p<0.01$), but had no effect on the PC-3 and DU-145 cell growth. The CM of all three prostate cancer cell lines reduced the growth of FG. Growth reduction in DU-145 cells was the most effective (50% inhibition after three days, $p<0.01$, and 55% after six days, $p<0.01$). FG did not express E-cadherin, while strong E-cadherin staining was detected in LNCaP cells. PC-3 cells exhibited E-cadherin nuclear staining, while sporadic membrane expression of the specific protein was observed in DU-145 cells. In co-culture, there seemed to be a reduction in the nuclear E-cadherin reactivity of PC-3 cells. Conclusion: Our data confirm the existence of a dialogue between normal fibroblasts and prostate cancer cells, which results in both a peculiar modality of growth and a regulation of proliferation, probably due to factors secreted in the culture medium. The variation in E-cadherin expression found in PC-3 cells co-cultured with FG merits further investigation.*

Stroma affects the development and the structure of many organs and plays an important role in regulating the emergence and the progression of epithelial malignancies, including those derived from the prostate (1-4). Due to the complexity of the tumour milieu, most observations have been carried out using animal models and cell lines (5, 6). In particular, heterotypic cultures represent a powerful approach for investigating the signalling interactions (7). Fibroblasts are the major cell type of the stromal compartment and synthesize a variety of matrix components and growth factors (4, 8, 9). On the other hand, malignant cells interact with the microenvironment through cell/cell and cell/matrix contacts (1-4, 10, 11) and through the release of soluble factors such as growth factors and interleukins [*e.g.* transforming growth factor-β (TGF-β), vascular endothelial growth factor, insulin-like growth factor-1 (IGF-1), and interleukin-6], which are employed in a paracrine or autocrine communication system (1-4, 12). These factors are involved in the processes of angiogenesis and cancer progression (4, 12-14). Moreover, some factors are known to activate androgen receptors in androgen-sensitive cells in the absence of the androgen (15). However, epithelial cancer cells may also alter stromal cell metabolism, through a modified expression of cell surface proteins, *e.g.* cadherins and integrins (2, 5). Correct localization of the E-cadherin cell/cell adhesion protein to the basolateral plasma membranes of the epithelial cells is essential for the polarization and maintenance of cell integrity and cell function (16). Loss of E-cadherin gene

Correspondence to: Professor Fortunata Iacopino, Istituto di Istologia ed Embriologia, Facoltà di Medicina e Chirurgia, Università Cattolica del Sacro Cuore, Largo Francesco Vito 1, 00168 Roma, Italia. Tel: +39 0630154463, Fax: +39 063053261, e-mail: iaco.forty@rm.unicatt.it

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expression or of its protein is frequently found during tumour progression in most types of epithelial cancer. As the E-cadherin gene is considered an oncosuppressor gene, loss of E-cadherin function is a clinical indicator of poor prognosis and metastasis (11). The loss of E-cadherin expression is frequently associated with the gain of mesenchymal N-cadherin expression, which represents a major hallmark of transformation. This cadherin switch provokes increased cell migration and invasion (11, 17) a phenomenon which occurs in prostate cancers with high Gleason score (18, 19).

Stromal cells, in turn, can release proteases which degrade components of the extracellular matrix, thus promoting invasion (20-22). It should be noted that the characteristics of the tumour stroma are different from those of the normal stroma, and this may be due to some changes occurring in particular in fibroblasts during tumour progression (2, 23-25). Recently, it has been suggested that stromal fibroblasts initially inhibit cancer development until they become activated and start promoting cancer growth (2, 24-26). Among activated fibroblasts, cancer-associated fibroblasts, which express a series of proteins, not expressed by normal fibroblasts, such as α-smooth muscle actin (α-SMA), are involved in tumour invasiveness and metastasis (3, 27).

Although the dynamic relationships between epithelium and stroma are supported by growing consensus in the literature, further studies are required to fully understand the complex interactions between cancer cells and the tumour microenvironment.

With the aim of adding new information on the relationships between normal fibroblasts and epithelial cancer cells, we performed a study in which the morphological aspects, the effects of conditioned media (CM) and the expression of E-cadherin were analyzed in a co-culture system. This included both androgen-sensitive (LNCaP) and -insensitive (PC-3, DU-145) human prostate cancer cell lines, and a normal human gingival fibroblast cell line named FG.

Materials and Methods

Cells. The human fibroblast cell line (FG) was derived from gingiva as previously reported (28). Cells were used in experiments from passages 4 to 10. LNCaP, PC-3 and DU-145 cells were used between passages 42 and 55, 92 and 104, 105 and 118, respectively. All cell lines were routinely cultured in Dulbecco's modified Eagle's medium (DMEM; Eurobio, Les Ulis, France), supplemented with 5% (v/v) foetal bovine serum (FBS; ICN Biomedicals, Costa Mesa, CA, USA), 10 mM N-2-hydroxyethylpiperazine-*N*'-2-ethane sulfonic acid (HEPES buffer; Eurobio) and antibiotics, and they were seeded at the appropriate density for cell growth.

All cell lines were subcultured weekly. They were maintained in an atmosphere of humidified air:CO_2 (95%:5%) at 37˚C.

Co-culture experiments. FG cells were first plated into 100 mm-plastic Petri dishes at 15,000 or 25,000 cells/ml of culture medium (DMEM supplemented with 5% FBS). After 3 days, aliquots of LNCaP (10,000 or 50,000 cells/ml of culture medium), PC-3, or DU-145 cell suspension (10,000 or 25,000 cells/ ml of culture medium) were seeded onto monolayers of FG cells (heteroculture), and cells were cultured for 3 and 6 days further. For monoculture, cells of each line were seeded alone in 100-mm-plastic Petri dishes.

In another series of experiments, prostate cancer cells were plated before seeding FG cells. In this case, prostate cancer cells were plated at a density of 1,000 and 5,000 cells/ml and after 3 days, fibroblasts were seeded at a density of 10,000 and 25,000 cells/ml.

Morphological observations. LNCaP, PC-3 and DU-145 cells in homotypic or heterotypic cultures were observed and photographed under an inverted phase-contrast microscope at ×100, ×200 and ×400 magnifications. Data collection was performed on the third and sixth day of culture.

Proliferation experiments. Subconfluent cells were trypsinized and plated out at a density of 50,000 (LNCaP) or 25,000 (PC-3 and DU-145) cells/ml of standard culture medium, in 60-mm plastic Petri dishes. Cells were allowed to adhere and 1 (PC-3 cells) or 2 (LNCaP cells) days after plating, the seeding media were changed with CM obtained from FG cells cultured for 3 (A) and 6 (B) days. Similarly, FG cells were seeded at the density of 25,000 cells/ml and after 3 days the medium was replaced with CM derived from prostate cancer cells cultured for 3 (A) and 6 (B) days. The media were renewed on the third day. In all experiments, triplicate cultures were set up and control dishes in which cells were grown in their standard medium were run in parallel.

Cell counts were performed with a haemocytometer after 3 and 6 days of culture.

Immunocytochemistry. In immunocytochemical experiments, cells were seeded in standard medium on sterile circular glass coverslips (Ø 18 mm) placed on the bottom of the tissue culture plates (353043, Multiwell™ 12 well; Becton-Dickinson and Company, Franklin Lakes, NJ, USA). The procedure for cell culture and treatment was the same as described for the co-culture experiments. On the third day, the culture medium was removed from the tissue culture plates and the cells were washed with Phosphate Buffered Saline (PBS; pH 7.6). They were then fixed with methanol and washed with PBS. Subsequently, non-specific binding was blocked with a blocking buffer (BB) containing: 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1% Triton X-100 and 3% normal serum, for 30 min at room temperature. Incubation with a primary antibody (mouse monoclonal antibody to E-cadherin, clone 4A2C7; Zymed Laboratories Inc., South San Francisco, CA, USA), 5 μg/ml in BB, was performed overnight at 4˚C. After rinsing with PBS, cells were incubated with the HRP polymer conjugate (SuperPicture Polymer Detection Kit, Zymed), and after a washing step, 3,3'-diaminobenzidine (Vector, Burlingame, CA, USA) was used as the chromogen. Nuclei were counterstained with Harris' haematoxylin. Negative controls were performed by omitting the primary antibody. E-cadherin-expressing MCF-7 breast cancer cells were used as positive control.

The fibroblastic nature of FG cells was determined by carrying out the immunocytochemical assay described above and by using fibroblast-(anti-vimentin, V9 clone, Dako, Glostrup, Denmark), epithelial tumor cell-(anti-cytokeratin 18, DC 10 clone, Dako), activated fibroblast-(anti-α-smooth actin, α-SMA, 1A4 clone, Dako), and endothelial cell-(antiCD31, 89C2 clone, Cell Signaling Technology, Beverly, MA, USA) specific markers.

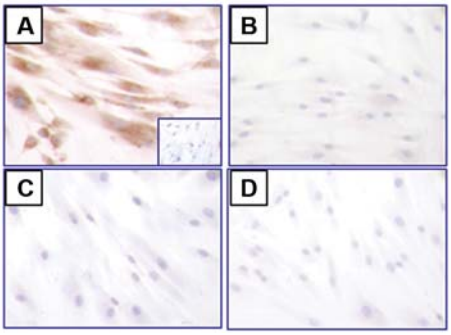

Figure 1. *Immunocytochemical analysis of vimentin (A), α-SMA (B), cytokeratin 18 (C) and CD31 (D) expression in FG cells. The inset shows a negative control. Original magnification ×200.*

Statistical analysis. Comparison between means was performed by the two-tailed Student's *t*-test; $p<0.05$ was considered statistically significant.

Results

Characterization of fibroblasts. The fibroblastic nature of FG cells was evident not only on the basis of their morphology, but also on the basis of homogeneous (≥98%), strong staining for vimentin intermediate filaments. The absence of α-SMA staining in microfilaments indicated that the FG cells were not of myofibroblastic type. FG cells did not express cytokeratin 18 nor CD31, which excluded their epithelial or endothelial origin (Figure 1).

Morphological study. To better illustrate the results obtained in this study, we choose the images with the most appropriate magnifications. Plating density did not influence the cell morphology of fibroblasts or epithelial cells. The images are referred to cells plated at the highest density.

In homotypic culture, androgen-sensitive LNCaP cells had a flattened and elongated morphology, with small cell bodies and multiple long, thin processes. They grew as single cells or in aggregates with a somewhat acinar appearance, which was evident after 6 days of culture (Figures 2 and 3). Androgen-insensitive PC-3 cells grew as colonies taking the form of islands containing polygonal cells mainly arranged in monolayers, with elements in the centre piling-up on top of one another (Figures 2 and 3). DU-145 cell growth was similar to the one of PC-3 cells, but the cells tended to proliferate more rapidly (Figures 2 and 3).

In heterotypic culture, LNCaP cells grew in a network on the top of the monolayers formed by the FG cells (Figure 2). After 6 days, the LNCaP cell number was lower in the co-cultures than in the plates where they grew alone. Both LNCaP and FG cells underwent morphological changes with varying signs of mild-to-severe degenerative alterations and increased cell death (Figure 3). In co-cultures with fibroblasts, PC-3 and DU-145 colonies were surrounded by FG cells (Figure 2). After 6 days, the growth of PC-3 and DU-145 cells overtook the one of FG cells, which in the presence of DU-145 had almost vanished (Figure 3).

In the experiments in which FG cells were seeded after plating of prostate cancer cells, the morphological aspects described above did not change. Fibroblasts extended some processes below LNCaP cells which formed a network above them (Figure 4 A and B). Colonies of PC-3 and DU-145 cells (Figure 4 C and D) were surrounded by fibroblasts.

Cell proliferation. The effects observed in our co-culture systems may be due to factors secreted by the cells in the culture media. For this reason, we collected, filtered and used in some proliferation experiments media derived from FG or prostate cancer cells cultured for 3 and 6 days. CM from FG cells was used for culturing prostate cancer cells, while CM from prostate cancer cells was used for FG cell culture.

CM from FG cells reduced LNCaP cell proliferation after 3 days by about 30% ($p<0.01$) and after 6 days by up to 80% ($p<0.01$), independently of whether the CM was derived from FG cells cultured for 3 or 6 days. No effect on PC-3 and DU-145 cell growth was observed (Figure 5, left panel).

Growth of FG cells was reduced by CM taken from all three neoplastic cell lines. CM derived from LNCaP and PC-3 cells had similar effects. Three-day medium reduced the FG cell proliferation by about 12% only after 6 days, while the CM collected on the sixth day inhibited FG cell growth by 18% after 3 days and by 40% after 6 days of culture. The CM obtained from DU-145 cells, after 6 days of culture, was the most effective, inducing 50% inhibition after 3 days, $p<0.01$, and 55% after 6 days ($p<0.01$; Figure 5, right panel).

E-cadherin expression. E-cadherin was mostly localized at cell/cell contacts among MCF-7 cells; some immunoreactivity was found at the cytoplasmic level. No nuclear staining was observed (Figure 6). FG cells did not express E-cadherin (Figure 6), while strong staining was detected in LNCaP cells at the cytoplasmic and membrane level (Figure 6). PC-3 cells exhibited cytoplasmic, membrane and nuclear staining (Figure 6), while sporadic membrane expression of E-cadherin was observed in DU-145 cells (Figure 6). In heterotypic cultures, no significant variations in E-cadherin expression of FG, LNCaP and DU-145 cells were seen (Figure 6), while a reduction in nuclear E-cadherin immunostaining seemed to occur in PC-3 cells, with a parallel increase of its expression particularly on the cell surface (Figure 6). The staining was mainly located at the areas of contact between epithelial cells. No E-cadherin expression was observed at the level of the cytoplasmic processes that linked PC-3 cells and FG cells (Figure 6).

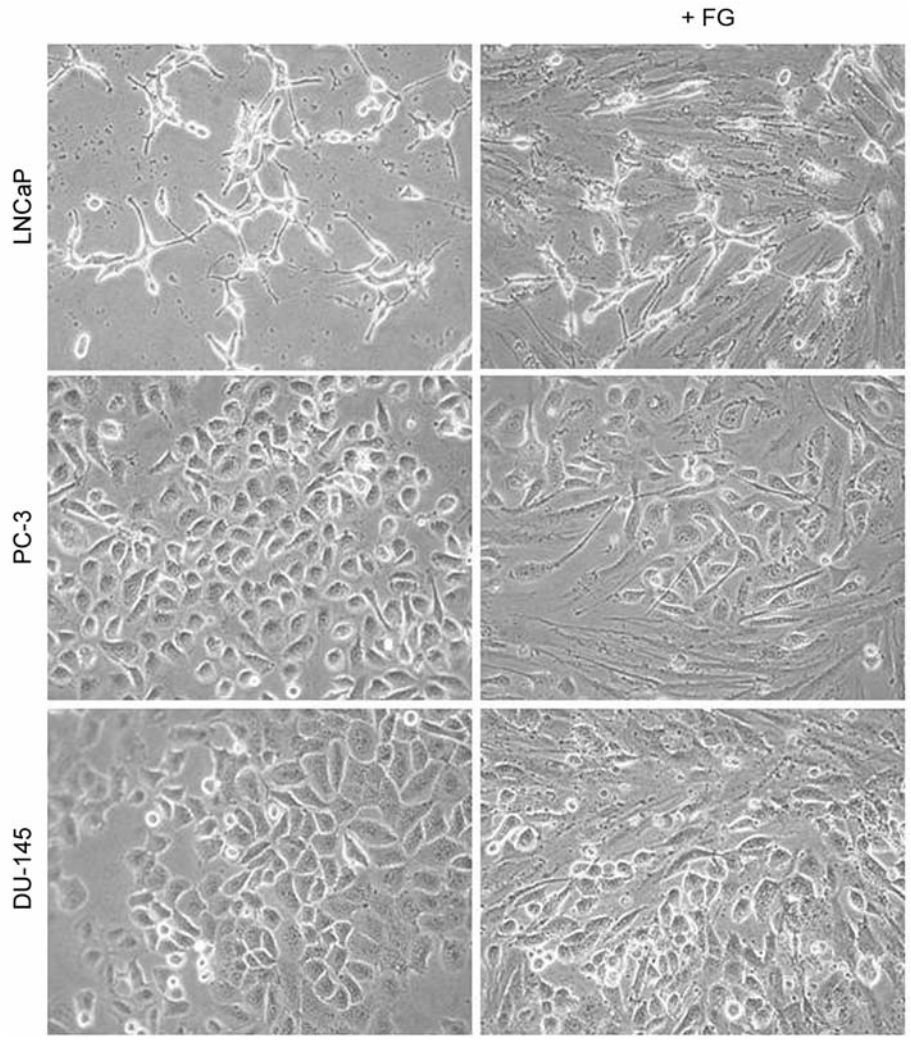

Figure 2. *Homotypic and heterotypic (+FG) cultures of prostate cancer cells examined live, using phase-contrast microscopy at ×200 magnification, after 3 days of culture.*

Discussion

Our results confirm that an interplay exists between prostate cancer cells and normal fibroblasts, in which both secretion of soluble factors and direct intercellular contacts are presumably involved. This dialogue results in a peculiar modality of growth and a distinct regulation of proliferation and protein expression.

From the obtained data, it seems clear that a different behaviour was expressed by the androgen-sensitive and highly differentiated LNCaP cells compared to the PC-3 and DU-145 cells, which are androgen-unresponsive and less differentiated.

Fibroblast monolayers made a good support surface on which LNCaP cells grew. Nevertheless, the proliferation of both LNCaP and FG cells was severely slowed, so that their number was reduced. Moreover, on the sixth day, both LNCaP and FG cells displayed different degrees of morphological alterations leading to cytoplasmic degeneration and cell death.

In previous studies, it has been demonstrated that both rat prostate cancer cell lines (29) and DU-145 cells (30) moved farther on the surface of fibroblasts than on plastic substrata, which indicates that the direct contact of prostate cancer cells with normal cells may facilitate their migration during invasion. Nevertheless, it should be noted that under our conditions, there was not an alignment of prostate cancer cells along the long axis of the underlying fibroblasts, as described in these papers, but we observed that LNCaP cells formed a network on the top of the fibroblasts.

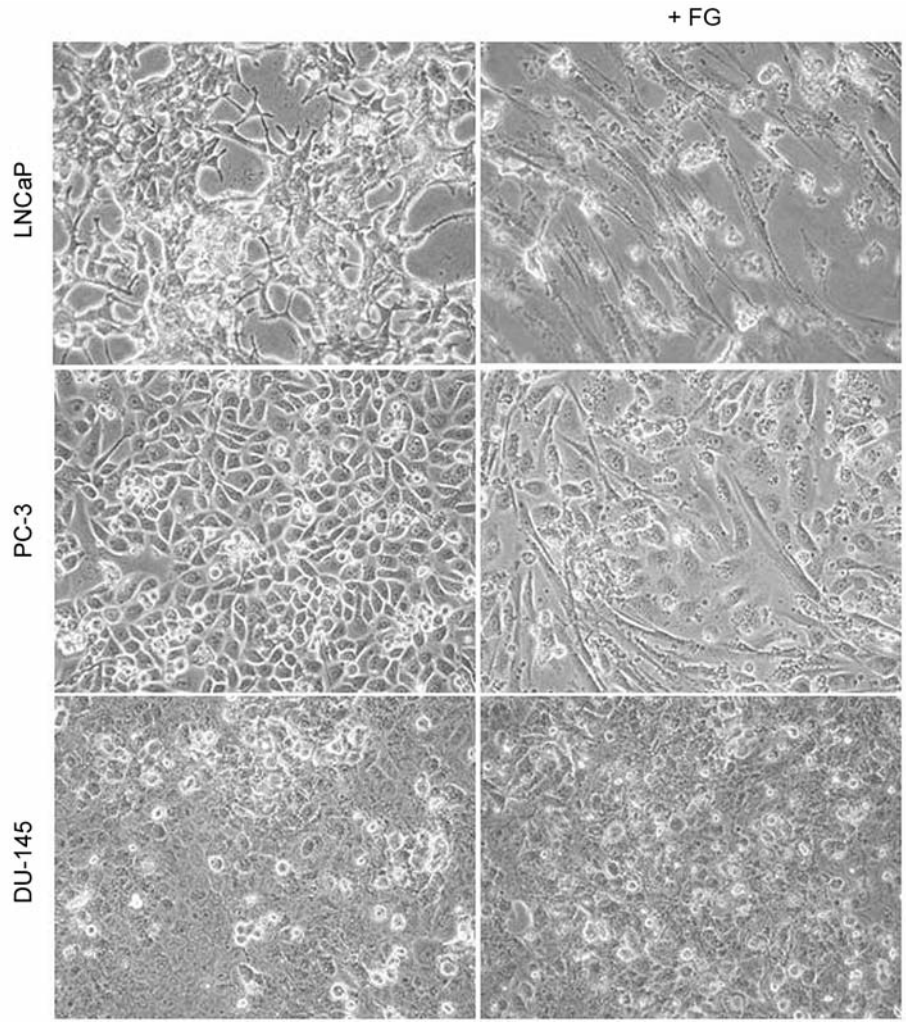

Figure 3. *Homotypic and heterotypic (+FG) cultures of prostate cancer cells examined live, using phase-contrast microscopy at ×200 magnification, after 6 days of culture.*

Regarding androgen-insensitive prostate cancer cells, in early co-cultures, both PC-3 and DU-145 cells were localized in restricted rounded areas surrounded by fibroblasts. After 6 days, the growth rate of prostate cancer cells seemed higher than that of fibroblasts, which had almost disappeared, at least in the presence of DU-145 cells. The arrangement of fibroblasts around islands of tumour cells has been described by other authors in studies of co-cultures of fibroblasts with HeLa cells or breast cancer cells (25, 31, 32). Moreover, Angeli *et al*. showed that fibroblasts attacked and destroyed whole cancer cell colonies (25, 31). Our observations are also consistent with the model proposed by Kalluri *et al*. in breast cancer (9). These authors observed that in ductal carcinoma *in situ*, the lumen contained carcinoma cells. The surrounding tissue was fibrotic and characterized by deposition of fibrillar extracellular matrix and fibroblast accumulation. The ductal epithelial cells were separated from surrounding connective cells by areas of intact basement membrane, but penetrated in a process of degradation and/or reduced synthesis.

We hypothesize that the data we obtained may be due, in part, to the direct contact between prostate cancer cells and fibroblasts, and in part to the factors secreted in the medium, as demonstrated by results obtained in cell proliferation assays in which CM were used. These findings confirm once again the different behaviour of androgen-sensitive cells compared to androgen-insensitive ones. In fact, medium collected from fibroblasts reduced the growth of LNCaP cells, but did not influence the proliferation of the two cell lines which were unresponsive to androgens. As such inhibition only occurred in LNCaP cells, this effect might be considered cell-specific.

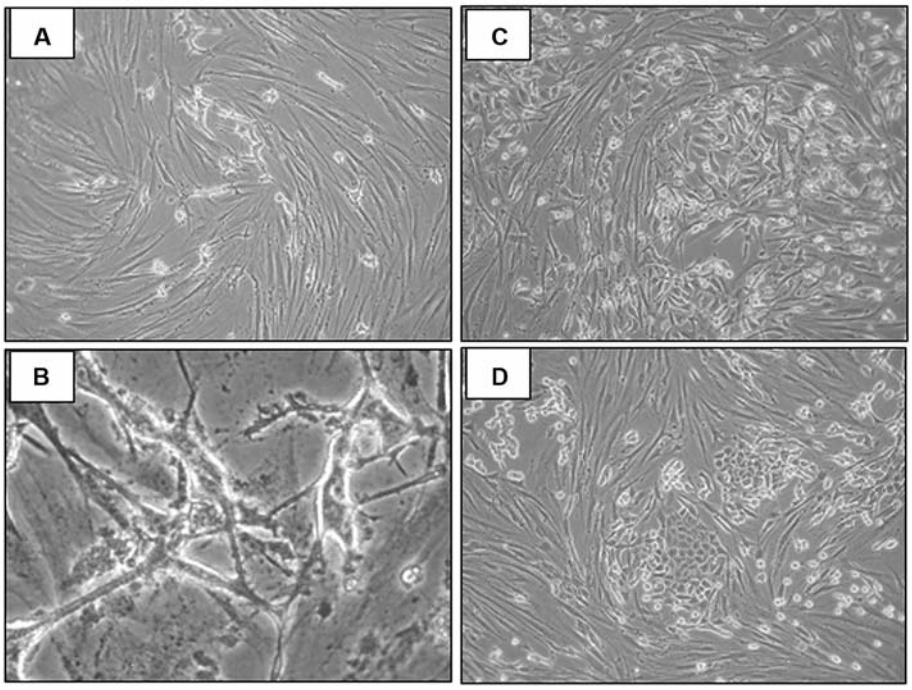

Figure 4. *Heterotypic (+FG) cultures of prostate cancer cells examined live, using phase-contrast microscopy at ×100 magnification (A, C and D), or ×400 (B). Prostate cancer cells were plated at a density of 5,000 cells/ml of culture medium; after 3 days FG cells were seeded at a density of 25,000 cells/ml culture medium. A and B: LNCaP cells and FG; C: PC-3 cells and FG; D: DU-145 cells and FG.*

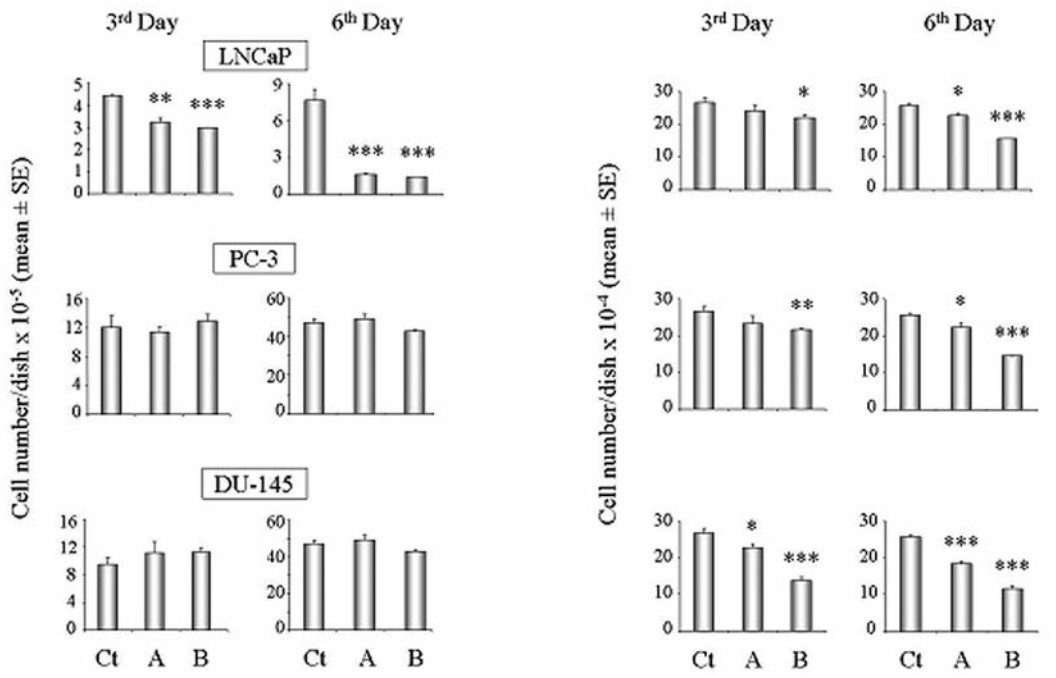

Figure 5. *Left panel: Effect of CM collected after 3 (A) and 6 (B) days from FG cultures on the growth of prostate cancer cell lines. Right panel: Effect of CM collected after 3 (A) and 6 (B) days from prostate cancer cultures on the growth of FG cells. CM collected from LNCaP (top), PC-3 (middle) and DU-145 (below) cells. Cells were cultured in media containing 5% FBS. Each column represents the mean±SE (n=6) of the data obtained from two independent experiments run in triplicate. *p<0.05, **p<0.01 and ***p<0.001. Student's t-test versus control cells (CT, cells cultured in their standard medium).*

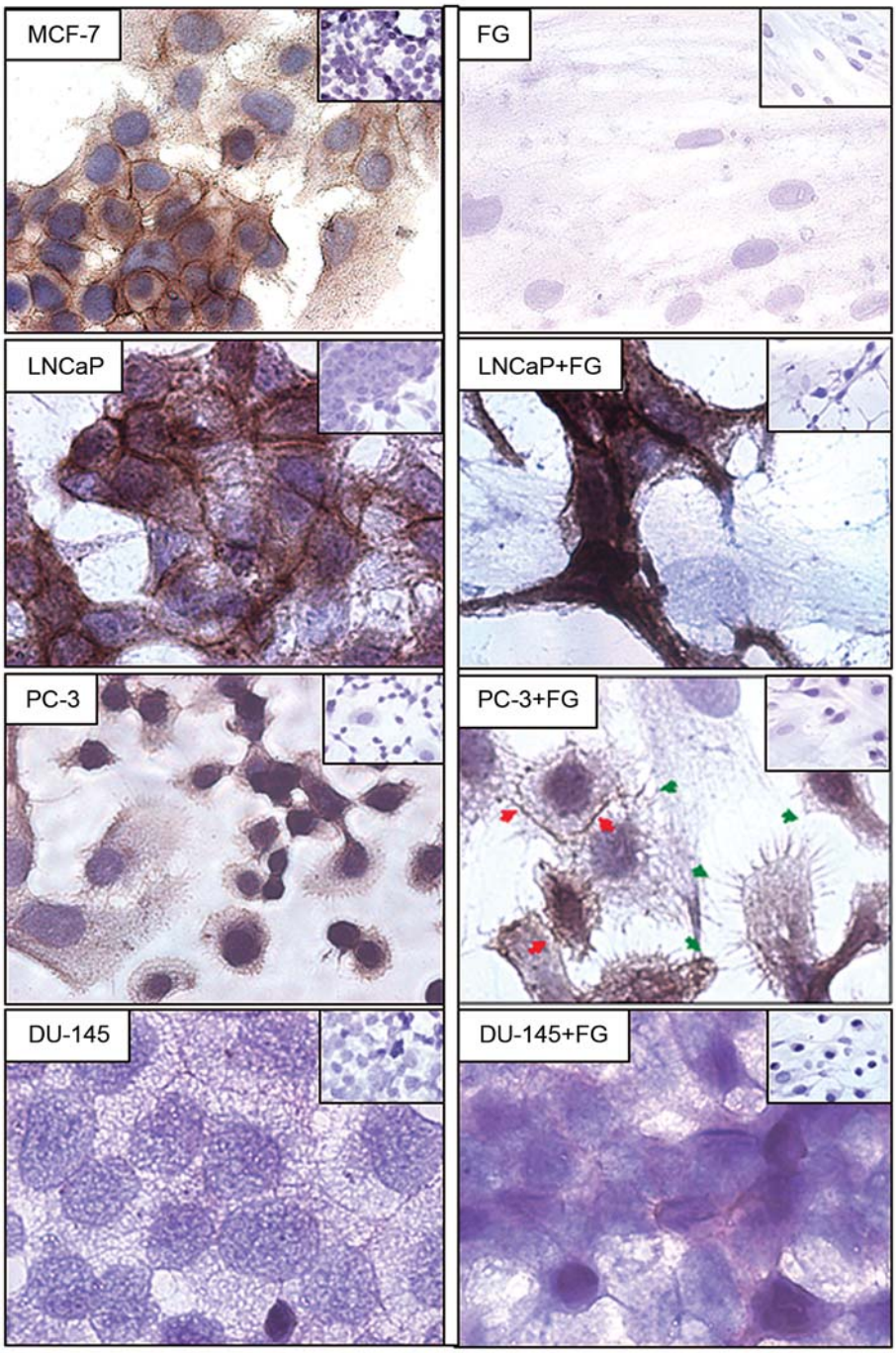

Figure 6. *Immunocytochemical analysis of E-cadherin expression of prostate cancer cells in homotypic and heterotypic cultures. Red arrows indicate contacts between epithelial cells and green arrows contacts between epithelial cells and fibroblasts. Original magnification ×630. The insets show a negative control. MCF-7 breast cancer cell line (positive control) and FG cells are also shown, original magnification ×400.*

Our results are in agreement with data reported by Degeorges *et al*. and Placencio *et al*. (33, 34). The latter suggested that the reduced LNCaP cell proliferation in stromal CM transfer experiments could be due to the increased levels of TGF-β. Interestingly, an increase of the expression of this growth factor may be involved in the lack of inhibition we observed both in DU-145 and PC-3 cell lines. In fact, Kawada *et al*. reported that TGF-β1 increased the expression of IGF-1 and IGF-binding protein-3 which stimulate DU-145 cell growth (35).

On the contrary, a stimulatory activity of stromal cells on both prostate cancer cell lines and cells derived from normal and cancerous prostate samples has been shown by others (36-38). However, Paland *et al*. observed that normal fibroblasts inhibited the growth of immortalized epithelial cells but promoted the growth of metastatic PC-3 cells, while cancer-associated fibroblasts promoted the growth of immortalized epithelial cells but not the growth of PC-3 cells (39).

Similarly, contradictory data have been reported in the literature about the action of CM harvested from prostate cancer cells on fibroblast proliferation. Some researchers demonstrated promotion, while others reported an inhibition of fibroblast growth (38, 40, 41).

It should be noted that culture conditions and cells used in different studies are very variable and this makes it rather difficult to compare the different results obtained by different researchers. We would like to stress that our fibroblast line is not composed of myofibroblasts or senescent cells as demonstrated by morphological aspects, growth rate (data not shown) and immunocytochemical marker expression. In fact, these cells have an elongated shape, they grow quickly (reaching subconfluence after 3 days in culture) and only express vimentin. Immunocytochemistry did not reveal any expression of E-cadherin in fibroblasts, as expected (42). It is well known that this adhesion molecule is a transmembrane calcium-dependent glycoprotein, involved in homotypic contact and its loss characterizes the epithelial/mesenchymal transition in various kinds of epithelial cancer, including prostate cancer (43). The lack of E-cadherin expression in stroma has been also confirmed by more recent *ex vivo* studies (43, 44).

E-cadherin expression was found in all three prostate cancer lines studied here, even at different levels, and was related to the degree of cell differentiation. Immunocytochemical data are in agreement with our previous findings obtained by western blotting, which showed a strong expression of this adhesion molecule in both LNCaP and PC-3 cells. Nevertheless, this technique did not reveal E-cadherin expression in DU-145 cells (45), which is in agreement with the sporadic and low protein expression in this cell line, shown by immunocytochemistry. Interestingly, in LNCaP as well as in DU-145 cells, the signal was largely confined on the membrane, while in PC-3 cells, it was located in the nucleus, the cytoplasm and the membrane. These findings are in agreement with those reported in literature (46-48), although Lang *et al*. did not detect E-cadherin expression in PC-3 cells (49). The presence of E-cadherin in the nucleus, as observed in PC-3 cells, with loss of the characteristic membrane staining pattern of the cells, has been described in cases of pancreatic neuroendocrine tumours, Merkel cell carcinomas, clear cell renal cell carcinomas, esophageal squamous carcinomas and synovial sarcomas (50-53). One could speculate that the nuclear localization may represent a mutated or improperly processed protein that fails to be transported to the cell membrane, or a protein which has been transported back into the nucleus, evoking a profoundly altered function of this cell-to-cell adhesion molecule in tumours.

In support of this hypothesis, Salahshor *et al*. have suggested that the cytoplasmic domain of E-cadherin can be cleaved or disrupted, leading to the release of E-cadherin from the adhaerens junctions into the cytosol. The intact cytoplasmic domain fragment can be transported into the nucleus, where it might induce signalling and invasion likely through activation of the non-canonical Wnt signalling pathway coupled to the AP-1 promoter and STAT1/STAT2 transcription activities (50).

The reduction in the E-cadherin staining of the nucleus of PC-3 cells associated with the enhancement of its expression at the membrane, observed in the co-culture system, is interesting. In fact, it may suggest a restoration of a more appropriate function of E-cadherin in androgen-insensitive cells. Yates *et al*. showed that co-culturing DU-145 and PC-3 cells with rat hepatocytes resulted in increased expression of E-cadherin and cytokeratin 18, a marker of mature epithelial cells, and reduced the epidermal growth factor receptor expression. These authors hypothesized that the observed events argue for a reversion of mesenchymal phenotype to a more differentiated phenotype (54). On the other hand, our group has previously demonstrated that interferon-β, a differentiating agent, induced an increase in E-cadherin expression concomitant with reduced expression of N-cadherin and the oncogene c-MET in PC-3 cells (45).

Interestingly, the distribution of E-cadherin shown in Figure 6 (PC-3 + FG) is similar to the one observed by Omelchenko *et al*. in co-cultures of canine kidney cells and fibroblasts, and by Chambers *et al*. in co-cultures of prostate BHP-1 cells and stromal cells. The former authors suggested that heterocellular contacts could direct cell migration or stabilize the position of different types of cells into separate territories; this initial cellular distribution could then be stabilized by elaboration of extracellular matrix structure (*e.g*. basal lamina) (55). The latter authors found an increase in E-cadherin/actin co-localisation on the basolateral membrane of BHP-1 cells when cultured with stromal cells (56).

In conclusion, our study emphasises the importance of crosstalk between normal fibroblasts and epithelium. This dialogue seems to depend on the level of differentiation of the prostate epithelium. In fact, fibroblasts had a different behaviour if co-cultured with androgen-sensitive or androgen-insensitive prostate cancer cells. Our results attest to an inhibitory role of normal fibroblasts in prostate cancer. Fibroblasts and the factors that they produce are involved both in inhibiting the proliferation of androgen-sensitive cells and in regulating the expression of some proteins in androgen-insensitive cells.

The next step of our work will be the evaluation of the interactions between fibroblasts derived from tumour stroma and the cell lines which were used in the present study.

The principles that govern heterotypic cell/cell interactions in cancer are complex, but a better understanding of them may facilitate new therapeutic approaches, by targeting the tumour/stroma interface with the aim of improving survival of patients with prostate cancer, as reported in the literature (57, 58).

Acknowledgements

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