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ORIGINAL ARTICLE

Mutual paracrine effects of colorectal tumour cells and stromal cells: modulation of tumour and stromal cell differentiation and extracellular matrix component production in culture

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Summary

Interactions of tumour and stromal cells influence tumour cell proliferation and differentiation, stromal cell phenotypic transdifferentiation and secretion of extracellular matrix (ECM) components. In this study, we established a monolayer and a three-dimensional cell-to-cell interaction model between canine mammary stromal cells and human colonic carcinoma cell lines (Caco-2 and HT-29) to investigate mutual paracrine effects of tumour cells and stromal cells on (i) tumour cell differentiation, (ii) production of ECM components and (iii) phenotypic transdifferentiation of stromal cells. We showed that when Caco-2 or HT-29 cells are cultured in collagen gels, they form a few small solid cell clusters with no lumina, but when cocultured with stromal cells, the tumour cells formed glandular structures with central lumina. This fibroblast-induced organization and differentiation of Caco-2 cells (not HT-29 cells) appeared to be mediated by transforming growth factor-β (TGF-β). Culturing of stromal cells, Caco-2 cells or HT-29 cells alone in both monolayers and gels resulted in weak tenascin-C expression in stromal cells and HT-29 cells and no expression in the Caco-2 cells. Coculturing of stromal cells with tumour cells resulted in increased tenascin-C expression in the stromal cells and HT-29 cells and induced expression of tenascin-C in the Caco-2 cells. This induction and increased expression of tenascin-C appeared to be mediated by TGF-β. Culturing of stromal cells, Caco-2 cells or HT-29 cells alone on monolayers and in gels resulted in a weak expression of chondroitin sulfate (CS), chondroitin-6-sulfate (C-6-S) and versican in stromal cells and no expression in Caco-2 and HT-29 cells. Coculturing of stromal cells with tumour cells on monolayers and in gels resulted in increased CS, C-6-S and versican expression in stromal cells. This tumour cell-induced expression of CS, C-6-S and versican appeared to be mediated by TGF-β and platelet-derived growth factor (PDGF). Coculturing of Caco-2 and HT-29 and stromal cells promoted the transdifferentiation of stromal cells into myofibroblasts, and this appeared to be mediated by TGF-β. These results suggest that TGF-β and PDGF are part of a paracrine system involved in stromal–epithelial cell interaction important in stromal cell differentiation and ECM component production.

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Activation of the host stromal microenvironment is predicted to be a critical step in epithelial tumour growth and progression. However, the specific mechanisms of stromal activation by tumour cells are not known, and the extent to which the stroma regulates the biology of carcinogenesis is not fully understood. Tumour progression occurs within a microecosystem where cancer cells and stromal cells exchange proteinases and cytokines that promote growth directly through stimulation of proliferation and survival, as well as invasion through local proteolysis of the extracellular matrix (ECM). Evidence is accumulating that in addition to secretion of cytokines, tumour cells also influence stromal cells and vice versa through direct cell-to-cell contact (Zidar *et al*. 2002) and production of ECM components (Donjacour & Cunha 1991).

ECM proteins in the tumours may positively or negatively influence tumour progression. Tenascin-C, a large hexameric glycoprotein, is elevated in colorectal human tumours (Hanamura *et al*. 1997) and canine colorectal tumours (Mukaratirwa *et al*. 2003). The most prominent actions of tenascin-C include antiadhesion effects (Chiquet-Ehrismann *et al*. 1989) and inhibition of cell attachment (Orend & Chiquet-Ehrismann 2000), and these properties favour tumour cell motility and invasion. Chondroitin sulfate proteoglycans (CS-PGs) influence cell growth and differentiation (Caterson *et al*. 1990) and are elevated in malignant tumours (Hauptmann *et al*. 1995; Ricciardelli *et al*. 1997). Versican, a major CS-PG that is known to antagonize cell adhesion to pericellular matrix components (Wight 2002), is also elevated in some tumours (Ricciardelli *et al*. 1998, 2002; Touab *et al*. 2002), suggesting that increased deposition of versican in the stroma may contribute to progression of tumours.

Pathological conditions associated with tissue remodelling and fibrogenesis (wound healing and neoplastic lesions) are characterized by the appearance of stromal cells with ultrastructural features intermediate between those of typical fibroblasts and those of smooth muscle cells. These fibroblasts display phenotypic and functional features of smooth muscle cells and hence are named myofibroblasts Gabbiani *et al*. 1971, 1972; Gabbiani 1981). The function of myofibroblasts is still not clear. Because they are found in fibro-contractive diseases, e.g. in wound healing (Gabbiani 1981) and in stromal reaction to tumours (Schurch *et al*. 1981; Sappino *et al*. 1988; Mukaratirwa *et al*. 2003), it has been proposed that they are responsible for tissue retraction. Through their ability to secrete cytokines, chemokines, prostaglandins, growth factors and ECM components, myofibroblasts are thought to play critical roles in inflammation, growth, repair and neoplasia (Desmouliére *et al*. 1993). In canine colorectal cancer, myofibroblasts were found to be located at the invasion front (Mukaratirwa *et al*. 2003). These observations suggest that cytokines produced by invading tumour cells stimulate myofibroblast transformation. In canine colorectal tumours, there is colocalization of myofibroblasts and tenascin-C (Mukaratirwa *et al*. 2003), suggesting that myofibroblasts are responsible for tenascin secretion, or tenascin secretion and myofibroblast transformation are regulated in the same way or in a coordinated manner.

The aim of this study was to investigate the *in vitro* paracrine effects of tumour cells on stroma cells and vice versa on (i) tumour cell morphology and differentiation, (ii) production of ECM components [tenascin-C, CS, chondroitin-6-sulfate (C-6-S) and versican] and (iii) phenotypic differentiation of stromal cells to myofibroblasts.

Materials and methods

Cell lines

Caco-2 cells (American Type Cell Collection, HTB37, Rockville, MD, USA) and HT-29 cells (Lesuffleur *et al*. 1998) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Imperial Laboratories, Salisbury, UK) supplemented with heat inactivated 10% fetal calf serum (FCS), 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate and non-essential amino acids (Sigma, St Louis, MO, USA). Both cell lines were incubated at 37 °C in an atmosphere of 5% CO_2 and 95% air. For collection of tumour cell-conditioned medium, subconfluent monolayers of Caco-2 or HT-29 cells were washed two times with phosphate-buffered salt solution (PBS) and once with DMEM without FCS and subsequently cultured in DMEM without FCS for 24 h. The conditioned medium was stored at –70 °C until used in studies on canine stromal cell monolayers.

Isolation of primary stromal cells from normal canine mammary tissue

Primary cultures of stromal cells were isolated from normal non-lactating canine mammary gland using a technique based on that described by van Roozendaal *et al*. (1992) for the isolation of human breast fibroblasts. The resulting stromal cells showed 100% vimentin expression and no cytokeratin or desmin expression. Cells were used between passages 4 and 12. For stromal cell-conditioned medium, subconfluent monolayers of stromal cells were washed two times with PBS and once with DMEM without FCS and subsequently cultured in DMEM without FCS for 24 h. The conditioned medium was stored at –70 °C until used in studies on tumour cell monolayers.

Antibodies

The primary antibodies used were antivimentin diluted at 1:150 (Biogenex, San Ranon, CA, USA), undiluted antidesmin (RD 301 Euro Diagnostica B.V., Arnhem, The Netherlands), anti-α-smooth muscle actin (anti-α-SMA) diluted at 1:1200 (Biogenex), anti-tenascin diluted at 1:50 (Dako, Glostrup, Denmark), anti-CS diluted at 1:100 (Sigma), anti-C-6-S diluted at 1:1000 (Seikagaku, Kogyo, Tokyo, Japan), antiversican 2B1 diluted at 1:1000 (Seikagaku) and anti-TGF-β1 diluted at 25 μ/ml (R&D Systems/Biermann, Bad Nauheim, Germany).

Monolayer cell cultures and cocultures on glass slides

Cells trypsinized and diluted to 2×10^4 cells/ml for stromal cells and 1×10^4 cells/ml for colonic tumour cells (Caco-2 and HT-29) in DMEM with 10% heat-inactivated FCS were seeded on multichambered glass slides. For cocultures, mixtures of stromal cells (2×10^4 cells/ml) and Caco-2 or HT-29 cells (1×10^4 cells/ml) suspended in DMEM with 10% heat--inactivated FCS were seeded on multichambered glass slides. The cells were allowed to attach overnight, then washed with PBS and switched to experimental conditions. For single cultures of Caco-2 and HT-29 cells and cocultures with stromal cells, the cells were grown in DMEM with 10% FCS for 48 h, and then the media were replaced with DMEM without FCS supplemented with growth factors to be tested or stromal cell-conditioned media for 48 h. For single cultures of stromal cells, after 24 h growth in DMEM with 10% FCS, the media were replaced with DMEM without FCS, supplemented with growth factors to be tested, or Caco-2- or HT-29-conditioned media for 48 h. The following growth factors were used: transforming growth factor-β_1 (TGF-β_1; 13 ng/ml) (R&D Systems/Biermann), platelet-derived growth factor (PDGF-AB; 50 ng/ml) (Sigma), epidermal growth factor (EGF; 10 ng/ml) (Sigma), insulin-like growth factor-I (IGF-I; 10 ng/ml) (Sigma) and IGF-II (50 ng/ml) (Gibco, Gaithersburg, MD, USA). Assays were also performed in the presence of a neutralizing antibody against TGF-β_1 (3 ng/ml) (R&D Systems). After 48 h, the slides were washed with PBS before the cells were fixed with ice-cold acetone for 10 min and stored at 4 °C. Each experimental condition was assayed in triplicate, and at least three independent experiments were performed (mean values were used).

Three-dimensional cell cultures and cocultures in collagen gels

Collagen was obtained from Wistar rat-tail tendons under sterile conditions. Tendons were dissolved in 0.1% acetic acid at 4 °C under stirring for 48 h. The solution was centrifuged (100 g, 10 min) to remove undissolved material. Collagen concentration was determined by freeze drying and weighing the residue of a known volume and adjusted to a concentration of 1.55 mg/ml in 0.1% acetic acid. Neutralized collagen was made by adding 1.7 volumes of collagen to 0.4 volumes of a 2:1 mixture of 10-fold concentrated DMEM (Imperial Laboratories) and 0.34 M NaOH.

Cells dissolved in DMEM with 10% FCS were added (1×10^5 cells/ml for stromal cells and 5×10^4 cells/ml for Caco-2 and HT-29 cells) to neutralized collagen. For cocultures, mixtures of stromal cells (1×10^5 cells/ml) and Caco-2 and HT-29 cells (5×10^4 cells/ml) were added to neutralized collagen. Two millilitres of cell–collagen suspension was pipetted into each well of 12-well culture plates. Plates were brought to 37 °C to obtain gelation. After gelation, DMEM with 10% FCS was added on top of the gel. After growth for 48 h, the wells were subjected to different experimental conditions. For single cultures of Caco-2 and HT-29 cells, and cocultures, the cells were grown in DMEM with 10% FCS or 1% FCS for 14 days, and the media were replaced every 2–3 days. For single cultures of stromal cells, after 48 h growth in DMEM with 10% FCS, the media were replaced with DMEM with 1% FCS, supplemented with growth factors to be tested, or Caco-2- or HT-29-conditioned media. The following growth factors were used: TGF-β_1 (3 ng/ml), PDGF-AB (50 ng/ml), EGF (10 ng/ml), IGF-I (10 ng/ml) and IGF-II (50 ng/ml). Assays were also performed in the presence of a neutralizing antibody against TGF-β_1 (3 ng/ml). The cells were grown for 14 days, changing the experimental media every 2–3 days, and after 14 days, the gels were fixed in 4% buffered formaldehyde for 24 h. Each experimental condition was assayed in triplicate, and at least three independent experiments were performed (mean values were used).

Immunocytochemistry and immunohistochemistry

Immunolabelling of vimentin, desmin, α-SMA and ECM components (tenascin-C, CS, C-6-S and versican) was performed on cells grown on glass slides and on serial sections cut from

collagen gels. The fixed cells on microscopic slides were washed three times with PBS containing 0.05% Tween 20. Serial sections from paraffin-embedded gels were cut and de-waxed. For labelling of antigens with antiversican and antitenascin-C, deparaffinized sections were pretreated with chondroitinase 0.2 U/ml avidin–biotin peroxidase complex (Seikagaku) in 0.2 M Tris–HCl buffer (pH 8.0) at 37 °C for 1 h and trypsin 0.1% in PBS (0.01 M, pH 7.4) containing $CaCl_2$ 0.1% at 37 °C for 10 min, respectively. Slides and sections were blocked with 1:20 normal horse serum for 20 min. Primary antibody incubations were carried out for 18 h at 4 °C, and secondary antibody incubations were carried out for 30 min at room temperature. After incubation with horse antimouse biotin and avidin–biotin peroxidase complex (Vector, Burlingame, CA, USA), the colour was developed in 3-3′-diaminobenzidine and 0.02% H_2O_2 in Tris buffer pH 7.8. The sections were counter-stained with 10% Mayer's haematoxylin. The intensity of ECM protein expression was evaluated for immunohistochemical reactivity by semiquantitative scoring in four grades (+++, intense; ++, moderate; +, weak and –, negative). Myofibroblasts transdifferentiation was evaluated by counting the number of stromal cells labelled with α-SMA and expressed as a percentage of the total number of cells. Each experimental condition was assayed in triplicate, and at least three independent experiments were performed (mean values were used).

Results

Morphology

When grown in monolayers, canine primary stromal cells presented themselves as large mesenchymal cells with many cytoplasmic extensions. When stromal cells were cocultured with Caco-2 and HT-29 cells on monolayers, the cells segregated after 48 h into distinct populations with stromal cells surrounding nests of Caco-2 cells or HT-29 cells (Figure 1a).

Stromal cells grown inside collagen gel proliferated fast, assuming a spindle shape, sending thin cytoplasmic processes into the collagen. The morphology of Caco-2 and HT-29 cells in collagen gels differed from morphology on monolayers. While on monolayers, Caco-2 and HT-29 cells displayed a typical, cuboidal morphology, in collagen gels, they became rounded and remained quiescent, even at high concentration of 5×10^6 cells/ml. After 14 days, few cells proliferated forming solid aggregates (Figure 1b), while the rest died. When Caco-2 or HT-29 cells were cocultured with stromal cells, or stromal cell-conditioned media, there was no tumour cell death, and both Caco-2 and HT-29 cells formed glandular structures with lumina (Figure 1c). This organization and differentiation of Caco-2 cells, but not HT-29 cells, could be partially induced by TGF-β (3 ng/ml).

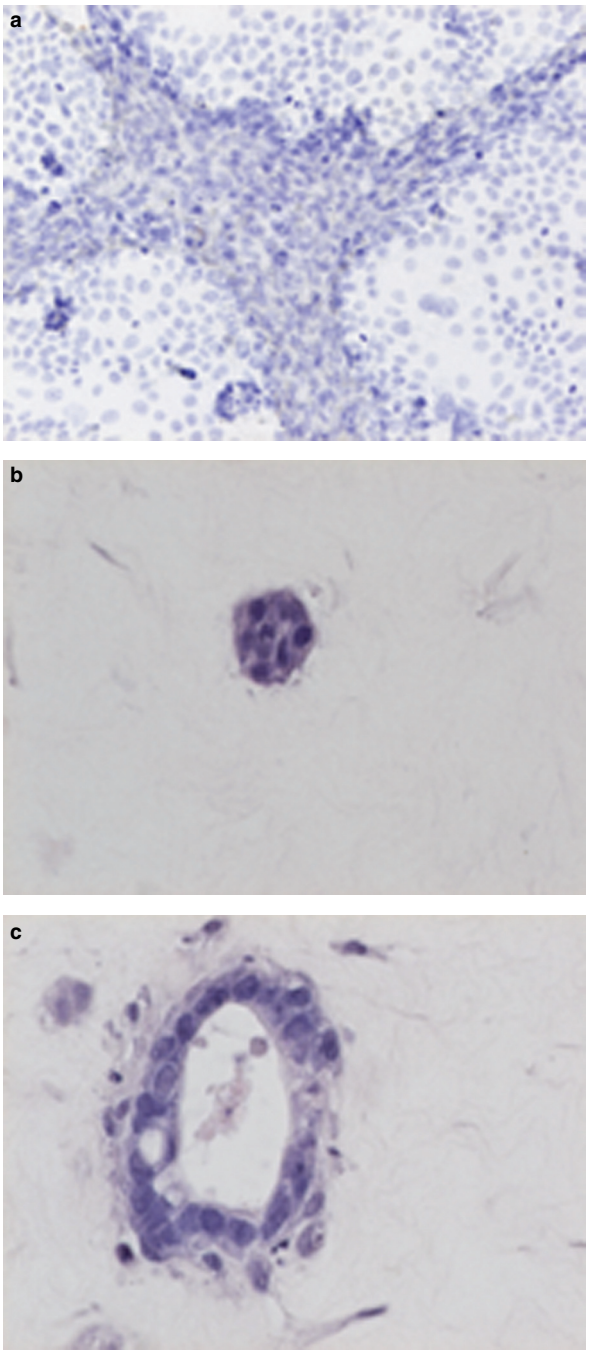

Figure 1 Micrographs showing the morphology of cultured and cocultured tumour and stromal cells. (a) Caco-2 and stromal cell coculture cultured on monolayers; cells segregated after 48 h into distinct populations with stromal cells surrounding Caco-2 cells (haematoxylin ×400). (b) Caco-2 culture in gels; formation of small solid structures after 14 days [haematoxylin and eosin (H&E) ×400]. (c) Caco-2 and stromal cell coculture in gels; formation of glandular structures with lumina (H&E ×400).

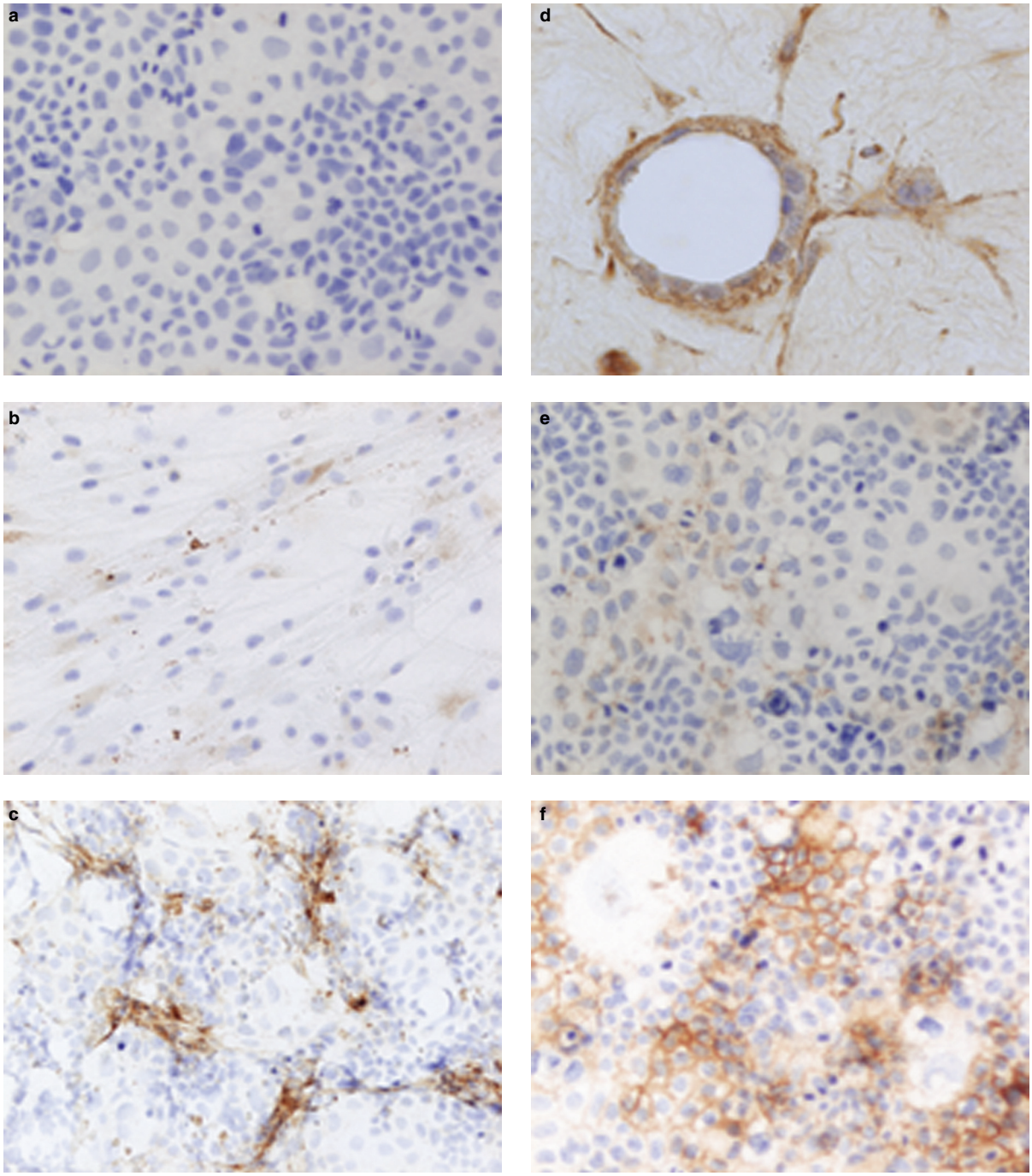

Figure 2 Micrographs showing tenascin-C expression in cultured and cocultured tumour and stromal cells. (a) Caco-2 cell culture on monolayers; no tenascin-C immunoreactivity [avidin–biotin peroxidase complex (ABC), haematoxylin counter-stain]. (b) Stromal cell culture on monolayers; weak tenascin-C immunoreactivity (ABC, haematoxylin counter-stain ×200). (c) Caco-2 and stromal cell coculture on monolayers; strong tenascin immunoreactivity in stromal cells and Caco-2 cells (ABC, haematoxylin counter-stain ×200). (d) Caco-2 and stromal cell coculture in gels; strong immunoreactivity in Caco-2 cells and stromal cells (ABC, haematoxylin counter-stain ×200). (e) Caco-2 cell culture on monolayers, addition of stromal cell-conditioned media; induction of a weak tenascin-C immunoreactivity (ABC, haematoxylin counter-stain ×200). (f) Caco-2 cell culture on monolayers, addition of transforming growth factor (TGF)-β; induction of a strong tenascin-C immunoreactivity (ABC, haematoxylin counter-stain ×200).

Deposition of extracellular matrix component

Tenascin-C. In both monolayers and three-dimensional cell cultures in gels, there was no tenascin-C immunoreactivity in Caco-2 cells (Figure 2a), and there was a weak cytoplasmic reactivity in HT-29 and stromal cells (Figure 2b). Coculturing of Caco-2 or HT-29 and stromal cells on monolayers or gels resulted in a strong tenascin-C immunoreactivity in the stromal cells and granular tenascin immunoreactivity in the tumour cells (Figure 2c,d). Culturing of stromal cells with Caco-2- or HT-29-conditioned media resulted in an increase in weak to moderate tenascin-C immunoreactivity in the stromal cells, and this response was partially blocked by a TGF-β_1-neutralizing antibody. Culturing Caco-2 or HT-29 cells with stromal cell-conditioned media induced a weak tenascin-C expression in Caco-2 cells (Figure 2e) and an increased weak to strong tenascin-C expression in HT-29 cells, and this response was partially blocked by a TGF-β_1-neutralizing antibody. Addition of TGF-β_1 to the media in both monolayers and three-dimensional cell cultures resulted in a strong expression of tenascin-C in stromal cells, induced strong tenascin-C expression in Caco-2 cells (Figure 2f) and an increased weak to strong tenascin-C expression in HT-29 cells. Addition of PDGF-AB, basic fibroblast growth factor (bFGF), IGF-I and IGF-II to cell cultures did not affect tenascin-C expression (Table 1).

Chondroitin sulfate, chondoitin-6-sulfate and versican. In both monolayers and three-dimensional cultures, there was no immunoreactivity of CS, C-6-S and versican in Caco-2 and

Table 1 Effect of coculturing, tumour cell-conditioned media and growth factors on extracellular matrix (ECM) component production on microscopic slides and in three-dimensional gels

		ECM components							
		CS		C-6-S		Tenascin-C		Versican	
Cells	Culture conditions	M	G	M	G	M	G	M	G
HT-29	10% FCS	–	–	–	–	+	+	–	–
	NFCS or 1% FCS	–	–	–	–	+	+	–	–
	S-CM	–	–	–	–	+ ++	++	–	–
	TGF-β	–	–	–	–	+++	+++	–	–
Caco-2	10% FCS	–	–	–	–	–	–	–	–
	NFCS or 1% FCS	–	–	–	–	–	–	–	–
	S-CM	–	–	–	–	++	++	–	–
	TGF-β	–	–	–	–	+++	+++	–	–
Stromal cells	10% FCS	+	+	+	+	+	+	+	+
	NFCS or 1% FCS	+	+	+	+	+	+	+	+
	Caco-2-CM	+++	++	+++	++	+++	++	+++	++
	Caco-2-CM + anti-TGF-β	+	+	+	+	+	+	+	+
	HT-29-CM	++	++	++	++	+	++	++	+++
	HT-29-CM + anti-TGF-β	+	+	+	+	+	+	+	++
	TGF-β	+++	+++	++	++	+++	+++	+++	++
	PDGF	+++	+++	+++	+++	+	+	++ +	+++
	bEGF	+	+	+	+	+	+	+	+
	IGF-I	+	+	+	+	+	+	+	+
	IGF-II	+	+	+	+	+	+	+	+
Caco-2 and stromal cells	NFCS or 1% FCS	+++	+++	+++	+++	+++	+++	+++	++
HT-29 and stromal cells	NFCS or 1% FCS	+++	+++	+++	+++	+++	+++	+++	+++

FCS, fetal calf serum; NFCS, no fetal calf serum; S-CM, stromal cell-conditioned media; Caco-2-CM, Caco-2 cell-conditioned media; HT-29-CM, HT-29-conditioned media; TGF, transforming growth factor; PDGF, platelet-derived growth factor; bEGF, basic epidermal growth factor; IGF, insulin-like growth factor; M, monolayers on plastic; G, three-dimensional gels; +++, strong reaction; ++, moderate reaction; +, weak reaction; –, negative reaction.

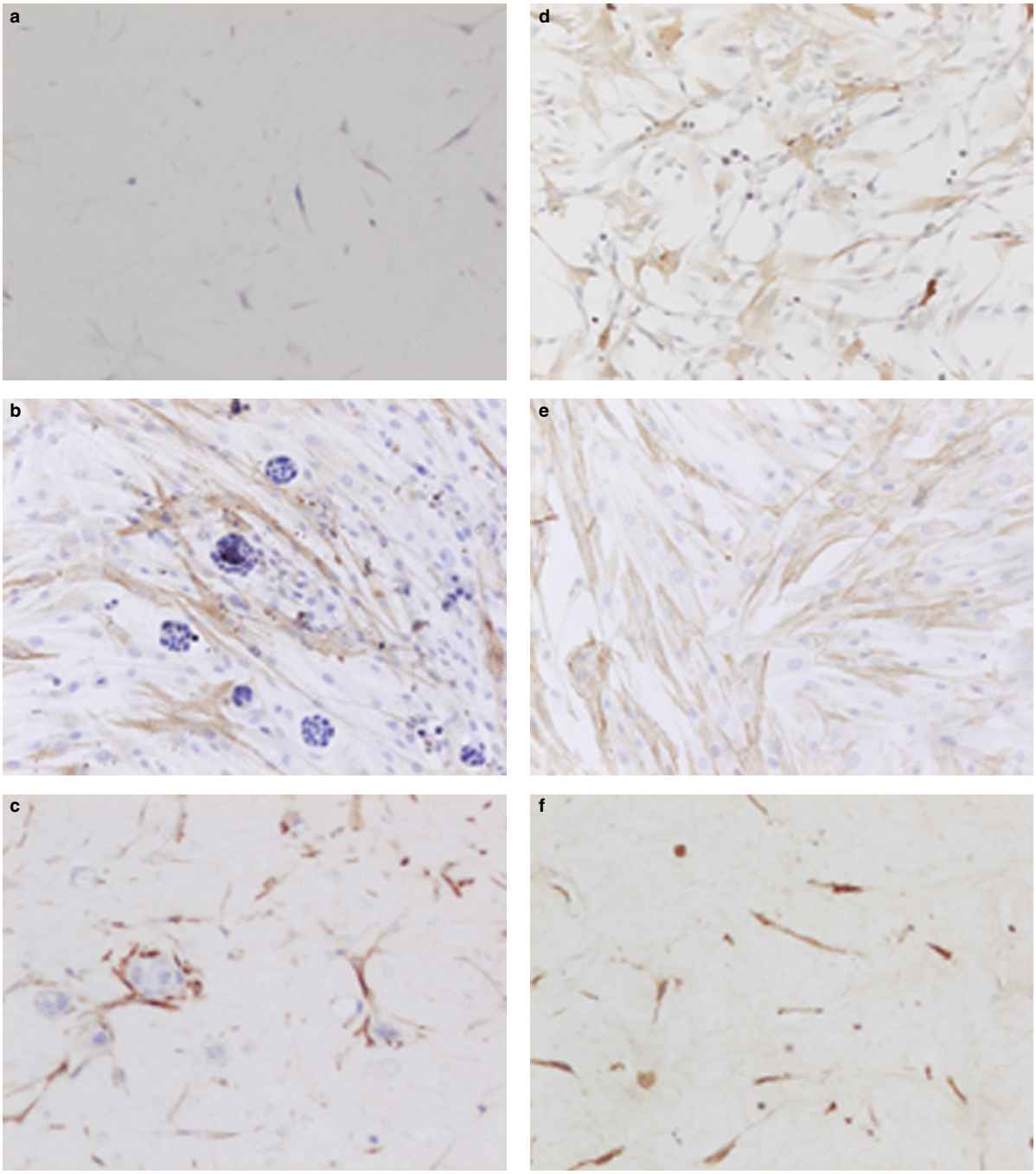

Figure 3 Micrographs showing α-smooth muscle actin (α-SMA) expression in cultured and cocultured tumour and stromal cells. (a) Stromal cells cultured in gels in medium with 1% fetal calf serum; less than 5% of the cells are positive for α-SMA (ABC, haematoxylin counter-stain ×200). (b) Coculture of stromal cells with HT-29 cells on monolayers; more than 90% of the cells expressing α-SMA (ABC, haematoxylin counter-stain ×200). (c) Coculture of stromal cells with Caco-2 in gels; more than 90% of the cells expressing α-SMA (ABC, haematoxylin counter-stain ×200). (d) Stromal cells cultured on monolayers with HT-29-conditioned media in monolayers. Increase in the number of stromal cells expressing α-SMA (ABC, haematoxylin counter-stain ×200). (e) Stromal cells cultured on monolayers, addition of TGF-β; more than 90% of the cells expressing α-SMA (ABC, haematoxylin counter-stain ×200). (f) Stromal cells cultured in gels, addition of TGF-β; more than 90% of the cells expressing α-SMA (ABC, haematoxylin counter-stain ×200).

Table 2 Effect of coculturing, tumour cell-conditioned media and growth factors on myofibroblast differentiation of stromal cells in collagen gels

	% of stromal cells positive for α-smooth muscle actin	
Culture conditions	**Monolayers**	**Gels**
10% FCS	20 ± 2.6	24 ± 1.0
NFCS or 1% FCS	6 ± 0.2	15 ± 0.8
Coculture with Caco-2 cells	96 ± 4.2	91 ± 3.1
Coculture with HT-29 cells	94 ± 3.8	90 ± 3.2
Caco-2-CM	53 ± 1.7	74 ± 1.3
HT-29-CM	60 ± 0.8	70 ± 1.3
TGF-β	90 ± 0.6	100 ± 0.8
PDGF	1 ± 0.1	1 ± 0.1
bEGF	5 ± 0.5	15 ± 0.8
IGF-I	5 ± 0.4	15 ± 0.6
IGF-II	5 ± 0.3	15 ± 0.5

FCS, fetal calf serum; NFCS, no fetal calf serum; S-CM, stromal cell-conditioned media; Caco-2-CM, Caco-2 cell-conditioned media; HT-29-CM, HT-29-conditioned media; TGF, transforming growth factor; PDGF, platelet-derived growth factor; bEGF, basic epidermal growth factor; IGF, insulin-like growth factor. Each experimental condition was assayed in triplicate, and three independent experiments were performed and the mean values are shown above.

HT-29 cells. There was a weak CS and C-6-S immunoreactivity and no versican immunoreactivity in stromal cells. Coculturing of Caco-2 or HT-29 cells and stromal cells on monolayer and gels resulted in strong expression of CS, C-6-S and versican in and around stromal cells. Culturing of stromal cells with Caco-2- or HT-29-conditioned media resulted in a strong CS, C-6-S and versican immunoreactivity. Addition of TGF-β_1 or PDGF-AB to the media resulted in a strong immunolabelling of CS, C-6-S and versican in stromal cells. Addition of stromal cell-conditioned media or TGF-β_1, PDGF-AB, bFGF or IGF-II to tumour cells did not induce CS, C-6-S and versican expression in Caco-2 or HT-29 cells (Table 1).

Myofibroblast transdifferentiation

When stromal cells were grown in monolayer cell cultures with media containing 10% FCS for 48 h, about 20% of the cells were positive for α-SMA. Growing the stromal cells in serum-free media for 48 h resulted in a decrease in the number of cells (5%) expressing α-SMA, suggesting that FCS contains a factor which induces myofibroblast transdifferentiation. In three-dimensional cell cultures, growing stromal cells in media with 10% FCS for 14 days resulted in about 25% of the cells expressing α-SMA, and culturing of the stromal cells in media containing 1% FCS resulted in a decrease in the number of cells (>5%) expressing α-SMA (Figure 3a). Coculturing stromal cells with Caco-2 or HT-29 cells on monolayers or in gels resulted in about 90% of the cells expressing α-SMA (Figures 3b,c). The cytoskeleton in the stromal cell was much more brightly stained, and many individual actin filaments were discernable. Culturing stromal cells with Caco-2- or HT-29-conditioned media in monolayers (Figure 3d) and on gels resulted in an increase in the number of stromal cells expressing α-SMA. As expected, treating the stromal cells with TGF-β on monolayers (Figure 3e) and in gels (Figure 3f) resulted in about 90% of the cells expressing α-SMA. Treating the cells with PDGF in both monolayers and on gels resulted in a decrease in the numbers of cells expressing α-SMA. Addition of EGF, IGF-I and IGF-II did not affect α-SMA expression (Table 2).

Discussion

Signals transmitted from stromal cells to tumour cells or *vice versa* can occur through direct cell–cell contact, in the form of growth factors or through secretion of ECM molecules, and this constitute the basis of reciprocal mesenchymal–epithelial interaction. In this study, we used two models of stromal–tumour cell coculture systems to investigate the regulation of cell differentiation and ECM component production.

Our study using a three-dimensional coculture system revealed that stromal cells reduce tumour cell death and promote cell differentiation. Previous experiments (Olumi *et al.* 1998) have shown that death of cultured tumour cells is five-fold less when they are cocultured with normal human prostatic fibroblasts in comparison to when cultured alone, and proliferation of the tumour cells was similar when cocultured

with normal prostatic fibroblasts or when grown alone. These data suggest that stromal cells promote tumour progression through attenuation of cell death rather than increased proliferation. The ability of normal fibroblasts to induce differentiation of tumour cell lines such as of a human colon carcinoma cell line HT-29 (Halttunen *et al*. 1996) has been demonstrated in our study as well. Furthermore, our results showed that this stromal-induced organization of and differentiation Caco-2 cells is partially mediated by TGF-β.

In previous studies where HT-29 cells were grown in a three-dimensional culture system with human embryonic lung fibroblasts or skin fibroblasts (Halttunen *et al*. 1996), the HT-29 failed to form glandular structures. This is in contrast to the present study, where canine mammary stromal cells induced HT-29 cell differentiation. This observed difference may result from variations in the establishment of reciprocal interactions between epithelial cells and mesenchymal cells.

Of interest in our investigation was the regulation of the synthesis of the ECM proteins: tenascin-C, CS-PGs and versican, because these proteins are expressed in large quantities in a variety of malignant tumours and have prognostic value in some tumours (Koukoulis *et al*. 1991; Hauptmann *et al*. 1995; Ricciardelli *et al*. 1997, 1998, 2002; Karvinen *et al*. 2003). In this experiment, both epithelial cells and stromal cells were able to produce tenascin-C. This accorded with previous experiments (Ishihara *et al*. 1995; Vollmer *et al*. 1997). *In vivo*, the quantitative and qualitative contribution to tenascin-C accumulation by epithelial tumour cells and stromal cells remains to be investigated. In this study, stromal cells or stromal cell-conditioned media were able to induce tenascin-C secretion by tumour cells and *vice versa,* suggesting that paracrine interaction between tumour and stromal cell is important for tenascin-C synthesis. This suggests that the modulation of stromal cells by tumour cells and *vice versa* to secrete tenascin-C was due to some soluble factors in the conditioned media. Culturing of stromal or tumour cells with various growth factors showed that TGF-β was responsible for the increase and induction of tenascin-C secretion. This is in accordance with previous reports (Vollmer *et al*. 1997; Wilson *et al*. 1999; Tuxhorn *et al*. 2002).

In this study, coculturing of stromal cells and tumour cells increased the production of CS, C-6-S and versican by stromal cells. Their increased production was mediated by TGF-β and PDGF. PDGF and TGF-β have been shown to increase the net synthesis of versican by stimulating transcription and translation and increase the glycosaminoglycan chain length (Schonherr *et al*. 1991).

Myofibroblasts are the main stromal cells in human (Sappino *et al*. 1989) and canine (Mukaratirwa *et al*. 2003) colorectal tumours and are responsible for the synthesis of ECM proteins (Powell *et al*. 1999). We therefore investigated whether coculturing of stromal cells and tumour cells has an effect on the phenotypic transdifferentiation of stromal cells to myofibroblasts. We showed that coculturing of stromal cells with tumour cells resulted in transdifferentiation of stromal cells to myofibroblasts (Figure 3) and that TGF-β enhanced and PDGF reduced stromal transdifferentiation to myofibroblasts. Similar results have been observed in a variety of stromal cells from rats (Desmouléire *et al*. 1993), rabbits (Nakamura *et al*. 2002) and humans (Valenti *et al*. 2001; Kinner *et al*. 2002). Myofibroblasts may stimulate tumour progression by stimulating the growth of a cancer cell population (Atula *et al*. 1997; Nakamura *et al*. 1997), sustaining blood vessel formation and lymphangiogenesis (Orimo *et al*. 2001), attenuating cancer cell death (Olumi *et al*. 1998) and stimulating invasion and metastasis by activating proteolysis (Basset *et al*. 1990; Singer *et al*. 1997).

Combining our results on phenotypic modulation of stromal cells by tumour cells and the modulation of ECM components' secretion, we conclude that TGF-β is part of a paracrine system regulating stromal–epithelial cell interaction. The first step in this pathway might be induction of myofibroblast transdifferentiation of stromal cells. The second step might be the induction of myofibroblasts to secrete ECM proteins, which in turn regulate tumour cell differentiation, proliferation and invasion. The cellular mechanisms of how TGF-β mediates myofibroblast transdifferetiation and ECM production are as yet undefined. The partial blockage of myofibroblast differentiation and ECM production by anti-TGF antibodies in our study suggest that TGF-β pathways involved are not direct and the processes involved appear to be regulated by a complex system with other molecules involved, or there are other cytokines involved in these processes.

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