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Myofibroblast and Concurrent ED-B Fibronectin Phenotype in Human Stromal Cells Cultured from Non-Malignant and Malignant Breast Tissue

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Primary cultures of stromal cells from non-malignant and malignant breast tissues contained myofibroblasts based on immunoreactivity to α-smooth muscle (α-sm) actin. The proportions of these cells were variable among cultures from non-malignant origin while consistently high in cultures from carcinomas. High expression of ED-B fibronectin and of type V collagen was observed in myofibroblast-containing cultures. While cells from non-malignant tissues grew relatively steadily, the proliferation of carcinoma-derived cells declined during serial subculturing. In both types of cultures, α-sm actin and ED-B fibronectin expression decreased with increasing passage numbers. Epidermal growth factor (EGF), fibroblast growth factor b (FGFb), transforming growth factor α (TGFα) and platelet-derived growth factor (PDGF) showed consistent mitogenic effects. Addition of FGFb prolonged culture growth and allowed α-sm actin and ED-B fibronectin expression to persist. These results demonstrate similar phenotypic modulations in stromal cells from non-malignant and malignant breast tissues that may reflect a common stromal response to various tissue injuries, including neoplasia.
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INTRODUCTION

SEVERAL LINES of evidence indicate that the stroma may play an important role in cancer development [1, 2]. This role may imply qualitative and quantitative changes in stromal cells, materials and architecture which may either precede or accompany epithelial cell transformation. To better investigate the changes in the stroma which may be implicated in breast cancer, we have recently isolated *in vitro* stromal cells from different sources of breast tissue [3, 4]. A distinct population of fibroblasts defined as myofibroblast [5] has been identified in many primary cultures derived from both non-malignant and malignant breast tissues on the basis of immunoreactivity to α-smooth (α-sm) actin. No such stromal cells or occasionally very few were found in cultures from normal tissues. Additionally, most primary cultures containing myofibroblasts also produced a specific isoform of fibronectin (ED-B fibronectin) which is normally present in fetal and cancerous tissues, but not in normal adult tissues [6, 7].

While only temporarily present in normally healing tissues, myofibroblasts have been shown to persist in chronic pathological tissues as well as in tumour stroma, characterised by excess fibroblast proliferation and extracellular matrix formation [5]. In the breast, extensive development of fibrous tissue is a common feature shared by many non-malignant and malignant lesions [8]. Nevertheless, the proliferation of epithelial cells remains restrained in the former while uncontrolled in the latter. The isolation of myofibroblasts in primary cultures from human breast tissues of both non-malignant and malignant origin prompted us to examine further their biological characteristics during cell culturing in order to see if they show any differences relevant to epithelial cell growth behaviour.

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MATERIALS AND METHODS

Breast tissue biopsies and culture procedures

The 18 biopsies from normal, non-malignant and malignant breast tissues listed in Table 1 were divided into two samples: one used for conventional histopathological diagnosis and the other for cell culture.

For cell culture, the tissue specimens were collected in Dubelcco's modified Eagle's medium (DMEM, Gibco, France) containing 10% fetal calf serum (FCS), sliced into small pieces and dissociated overnight at 37°C with 200 U/ml of collagenase (CLS III, Worthington, U.K.) and 100 U/ml of hyaluronidase (type II, Sigma, La Verpillière, France) as described previously [9]. The resulting enzymatic digest was centrifuged at 600 *g* for 10 min. The cell pellet was then resuspended in 10 ml of fresh enzyme-containing medium and filtrated on Nylon mesh (60 μm) to separate epithelial organoids from the stromal cell population. The filtrate containing most of the stromal cells was transferred into a 25-cm^2 Costar flask and allowed to settle for 2 h to allow these cells to adhere. The supernatant was then removed and replaced by fresh DMEM supplemented with 10% FCS. When the cells formed a confluent layer they were trypsinised (0.05% trypsin plus EDTA solution, Gibco) and serially subcultured at a 1:2 split ratio, so that each passage

Table 1. Origin of breast tissue specimens

Specimen number	Histological diagnosis
Normal breast tissues	
1, 2, 3	Cosmetic surgical reduction mammoplasty free of anomalies
Non-malignant breast tissues	
4, 7, 10, 15, 16	Extensive fibrosis, developing after previous mastectomy for infiltrating carcinoma and followed by radiotherapy
23	Circumscribed fibroadenoma showing moderate fibroblastic and epithelial proliferation
29	Fibrocystic lesion showing discrete intraductal and lobular myoepithelial and epithelial hyperplasia
67A	Fibrocystic lesion adjacent to a carcinoma of grade I
68	Fibrocystic lesion adjacent to a fibroadenoma, showing discrete areas of epithelial hyperplasia
68C	Fibrocystic lesion in the 68 contralateral breast showing discrete areas of epithelial hyperplasia
71	Sclerosing adenosis developing after previous mastectomy for invasive carcinoma and showing no sign of recurrent carcinoma
Malignant breast tissues	
69, 70	Ductal carcinoma of grade II showing an important stromal reaction
75	Residual carcinomatous lesions after previous mastectomy for lobular carcinoma, showing fibrous supportive tissue and discrete areas of epithelial hyperplasia
83	Ductal carcinoma of grade III

represented approximately one population doubling. The subcultivation of the cells was terminated when they failed to reach confluence after a 4-week period, with a medium change once a week.

Immunofluorescence analyses

Stromal cells were prepared for immunostaining analysis as described previously [3]. Mouse monoclonal antibodies (MAb) used for the detection of human cytokeratin 18, vimentin, desmin, as well as fluorescein-labelled anti-mouse immunoglobulin, were purchased from Amersham (Les Ulis, France). Mouse MAb to α-sm actin was obtained from Sigma. Purified goat antibodies against types I, III and V collagen and fluorescein-labelled anti-goat immunoglobulin were obtained from Southern Biotechnology Associates (Clinisciences, Paris, France). Mouse antibodies against human collagen type IV and rabbit antibodies against human laminin were purchased from Sanbio (Tebu, Le Perray, France) and rhodamine-labelled anti-rabbit globulin from Silenus (Eurobio, Paris, France). Mouse MAb IST-4 against all different human fibronectin (FN) isoforms [10] and MAb BC-1 directed to the FN cell-binding domain containing the ED-B alternative splicing sequence [6], were a generous gift of Dr L. Zardi (INRC, Genoa, Italy). The number of positive cells for each MAb was estimated in four high-power fields containing 40–60 cells per field at a ×200 magnification.

Mitogenic assays

Recombinant human epidermal growth factor (EGF), platelet-derived growth factor (PDGF)-BB, tumour necrosis factor (TNF)-α, interleukin (IL)- 1α, IL-2, IL-3, IL-6 and granulocyte–macrophage colony-stimulating factor (GM-CSF) were purchased from Genzyme (Tebu), and human recombinant transforming growth factor (TGF) α from Biomedical Technologies Inc (Clinisciences, U.K.). Human TGF-β and bovine basic fibroblast growth factor (FGFb) were obtained from British Biotechnology Ltd. Oestradiol, progesterone, luteinising hormone releasing hormone (LHRH) and follicle-stimulating hormone (FSH) were purchased from Sigma, and insulin-like growth factor (IGF-I) from Amersham.

For assaying all growth-promoting agents, confluent stromal cell cultures were trypsinised, resuspended in DMEM supplemented with 2% FCS and seeded into 24-well Falcon plates at a concentration of 2×10^4 cells/well. After 24 h, the medium was removed, the cells were refed with fresh medium and a single addition of growth factor or cytokine was made. DMEM without phenol red and supplemented with 2% steroid-free FCS was used for hormone growth-promoting assays. At different times, cells from duplicate wells were harvested by trypsinisation and counted in a Coulter counter. Under these conditions, all cultures entered the plateau growth phase within 4 to 6 days after seeding.

RESULTS

Phenotypes of stromal cells in primary cultures

The morphological appearance of most primary cultures of stromal cells established from non-malignant and malignant breast tissues generally differed from cultures of normal tissues; they developed a hill and valley pattern of growth showing dense aggregates of spindle-shaped cells (Fig. 1a). By comparison, normal tissue cells were arranged in parallel and formed regularly oriented sheets composed of flat elongated cells when reaching confluence (Fig. 1b).

Immunotyping using antibodies to various cytoskeletal and extracellular matrix elements confirmed the fibroblastic nature of stromal cells isolated from three normal breast tissues, 11 non-malignant tissues, including four from benign tumours and seven from benign or irradiated fibrous tissues at sites of previous or concomitant carcinomas, and four malignant tissues. As summarised in Table 2, all primary cultures regardless of their tissue source consistently expressed high levels of vimentin, fibronectin and collagen types I and III. The parallel assessment of cytokeratin 18, laminin and type IV collagen expression, characteristic of epithelial cells, revealed no contaminant cells or occasionally very few (less than 0.001%).

With two exceptions (specimens 15 and 23), expression of the α-sm actin isoform which distinguishes between myofibroblasts and fibroblasts, was seen in cultures derived from non-malignant and malginant tissues, while barely detected in cultured normal breast tissues. The typical stress fibre distribution of α-sm actin staining is illustrated in Fig. 1c. However, variable proportions of positive cells (5–>90%) were observed in cultures of non-malignant tissues, while in cultured stromal carcinoma cells these proportions were consistently elevated (50–80%). Expression of muscle desmin was also detected, but only in a few cells (less than 5%) in some cases of cultured non-malignant (specimens 68, 68C and 71) and malignant (specimen 70) tissue

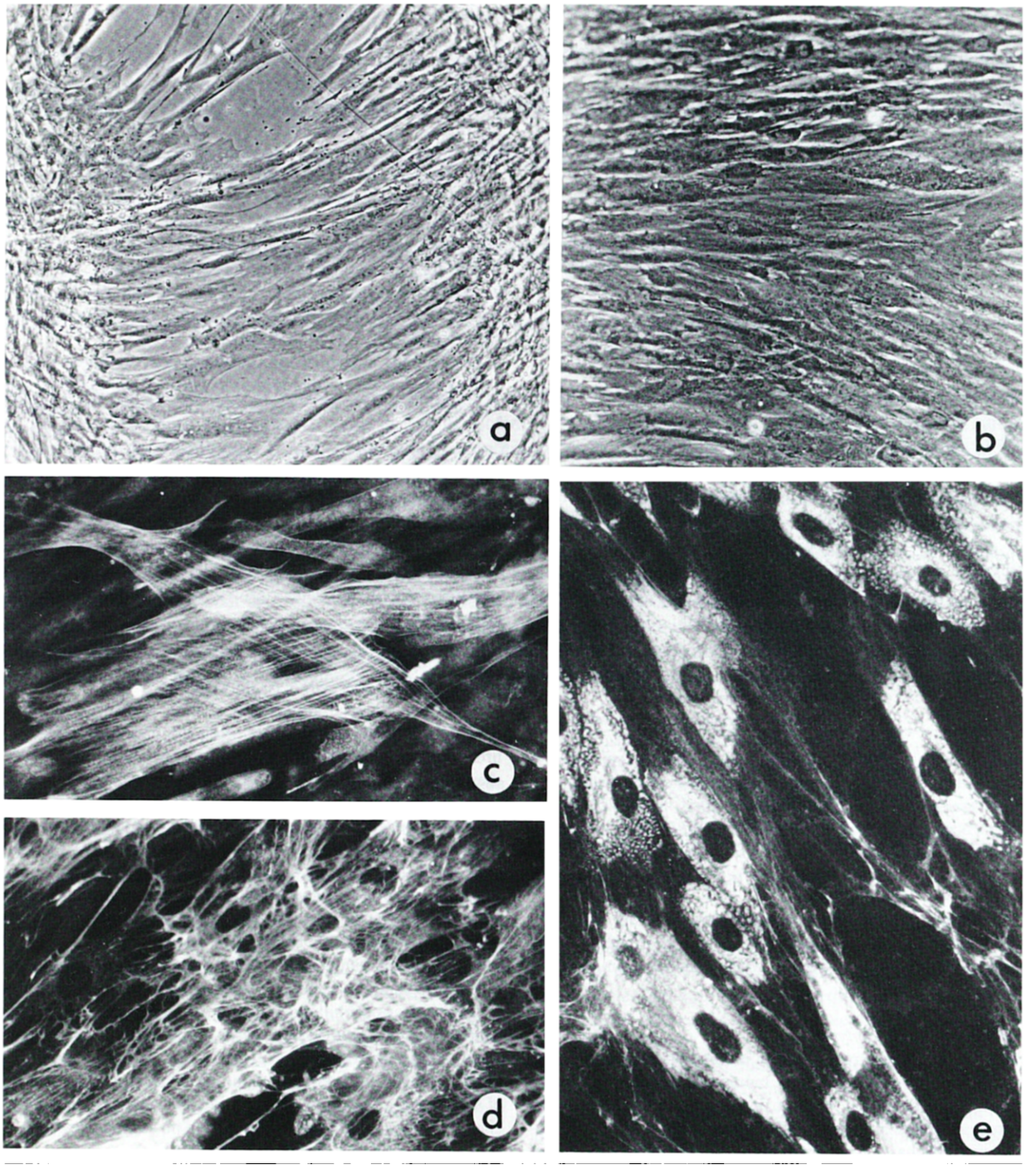

Fig. 1. Morphology of fibroblasts cultured from malignant breast tissue (a, specimen 83) compared with cells cultured from normal breast tissue (b, specimen 3). The same stromal carcinoma cells were stained with MAbs to α-sm actin (c), ED-B fibronectin (d) and collagen V (e). Scale bar = 10 μm.

cells. Concomittant with α-sm actin expression was high expression of the ED-B fibronectin isoform and type V collagen (Fig. 1d, e).

Phenotypes of non-malignant and malignant breast stromal cells during cell culturing

Stromal cells from normal and most of the non-malignant breast tissues demonstrated a relative stability during culturing. As can be seen in Fig. 2, a sustained cellular proliferation was obtained which lasted for at least 12–20 passages. Surprisingly, the growth of stromal cells derived from malignant tissues appeared significantly shortened under standard culture conditions (i.e. 5–10 passages depending on the individual specimen). Concomitant with the rapid decline of cellular proliferation was a decrease in the number of cells expressing α-sm actin and in the production of ED-B fibronectin (Table 3,

Table 2. Immunophenotype of primary stromal cell cultures

Stromal cell cultures	*MAb reacting with: Vimentin	α-sm Actin	Fibronectin† FN	ED-B‡	Collagen† I	III	V
Normal breast tissues							
1	+++	−	+++	−	+++	++	−
2	+++	−	+++	−	+++	++	−
3	+++	−	+++	−	nt	nt	nt
Non-malignant breast tissues							
4	+++	+++	+++	+++	+++	++	++
7	+++	+++	+++	+++	+++	++	++
10	+++	+	+++	(+)	+++	++	++
15	+++	−	+++	−	+++	++	nt
16	+++	+++	+++	+++	+++	++	+
23	+++	−	+++	−	+++	+++	−
29	+++	+	+++	(+)	+++	+++	−
67A	+++	+++	+++	+++	+++	+++	+++
68	+++	+++	+++	+++	+++	+++	+++
68C	+++	++	+++	++	+++	+++	+++
71	+++	+++	+++	+++	nt	nt	nt
Malignant breast tissues							
69	+++	++	+++	++	nt	nt	nt
70	+++	++	+++	++	+++	+++	+++
75	+++	++	+++	++	nt	nt	−
83	+++	++	+++	+++	nt	nt	−

*+++ > 90%; ++ 50–80%; + 5–20% of the cells stained; − no or weak staining in a few cells (< 0.01%). †Strong cytoplasmic and extracellular staining. ‡Extracellular network of fibrillar staining, exclusively (see Fig.1): +++ dense; ++ moderate; + sparse; − none. Parentheses indicate weak intensity of staining. nt, not tested.

specimen 69, passage 3). A similar disappearance of α-sm actin and ED-B fibronectin expression was also noted in cultures of stromal cells from non-malignant tissues (specimen 7, passage 17 and specimen 29, passage 6). However, unlike carcinoma cultures, sustained cellular proliferation continued to be observed. Furthermore, the morphological appearance of these proliferating cultures tended to resemble that of normal cultures (data not shown).

Modulation of breast stromal cell proliferation and phenotype by growth factors

A preliminary screening of cellular responsiveness to various factors at early stages in culture (i.e. between passage 3 and 10) revealed that EGF, FGFb, PDGF and TGFα at 10 ng/ml exhibited significant mitogenic effects regardless of whether stromal cells originated from normal, non-malignant or malignant breast tissues (Table 4). While FGFb caused a 2- to 3-fold increase in final saturation densities in nearly all the cultures, the mitogenic potency of the three other factors varied, particularly for cultures from non-malignant and malignant tissues. For example, while EGF stimulated a 2- to 3-fold increase in cell numbers in two out of six cultures from postradiation fibrosis (specimens 4 and 7), in one out of three from fibrocystic diseases (specimen 68C) and in one out of three from carcinomas (specimen 83), it exerted only moderate mitogenic effects in the remaining nine cultures.

Table 3. Disappearance of myofibroblast and ED-B fibronectin phenotype during cell culturing

Stromal cell cultures	Passage number	Expression of: α-sm actin (% of positive cells)	ED-B fibronectin isoform*
7	1	98	+++
	7 to 14	65	+
	17 to 21	<0.01	−
29	1	7.5	(+)
	6	<0.01	−
69	1	50	++
	3	3	−

*+++ dense; ++ moderate; + sparse; − no extracellular fibrillar staining. Parentheses indicate weak intensity of staining.

TGFβ (up to 5 ng/ml) was poorly active, stimulating or inhibiting the proliferation of stromal cells by approximately 30%. TNFα (up to 100 U/ml) and IL-1 (up to 10 U/ml) had stimulatory effects of about 80 and 40%, respectively, but only in two unrelated cultures, one derived from normal breast tissue (specimen 1) and one from postradiation fibrosis (specimen 4). The following cytokines and hormones, tested as indicated, remained consistently inactive: IL-2 (up to 1000 U/ml), IL-3 (up to 50 U/ml), IL-6 (up to 10 U/ml), GM-CSF (up to 50 U/ml), IGF-I (up to 100 U/ml), FSH and LHRH (up to 2.5 μg/ml), oestradiol and progesterone (up to 1 μmol/ml, data not shown).

FGFb was added to cultures derived from benign (specimen 29) and malignant (specimen 69) tissues, which showed a reduced cell proliferation during serial culturing in order to sustain cell growth. This factor was chosen because of its more consistent mitogenic potency, compared to other growth factors. As illustrated in Fig.3, the continuous addition of 10 ng/ml of FGFb to the standard culture medium resulted in a marked

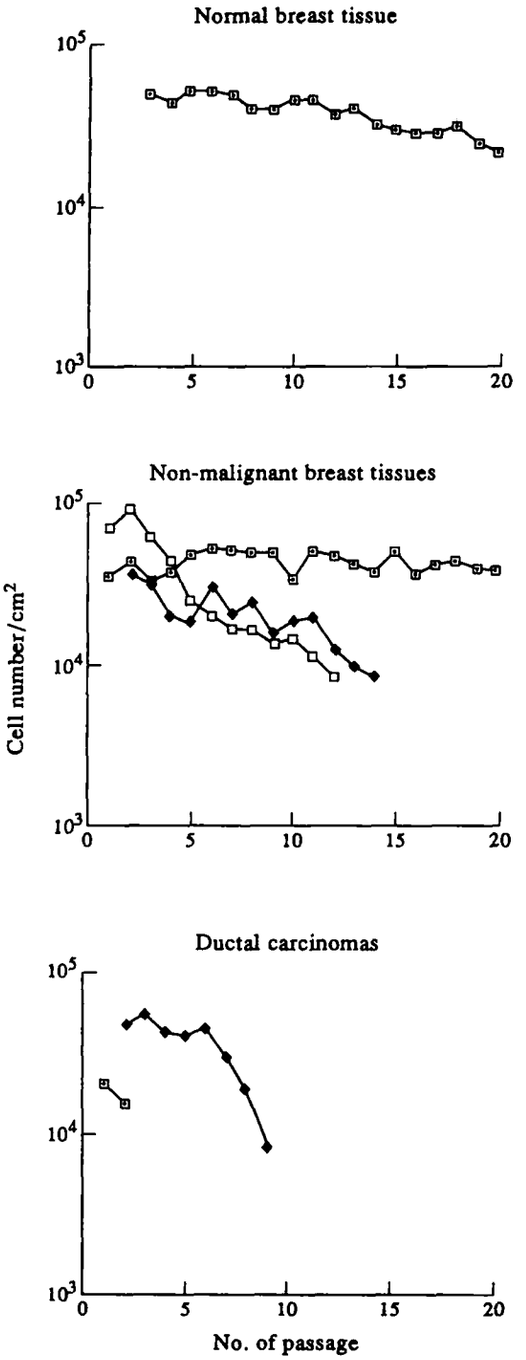

Fig. 2. Long-term proliferation of human breast fibroblasts derived from reduction mammoplasty (specimen 3), non-malignant tissues (●, specimen 7; ◆, specimen 23; □, specimen 29) and ductal carcinomas (●, specimen 69; ◆, specimen 83). At each passage level maximal cell densities were determined once the cultures had reached confluence.

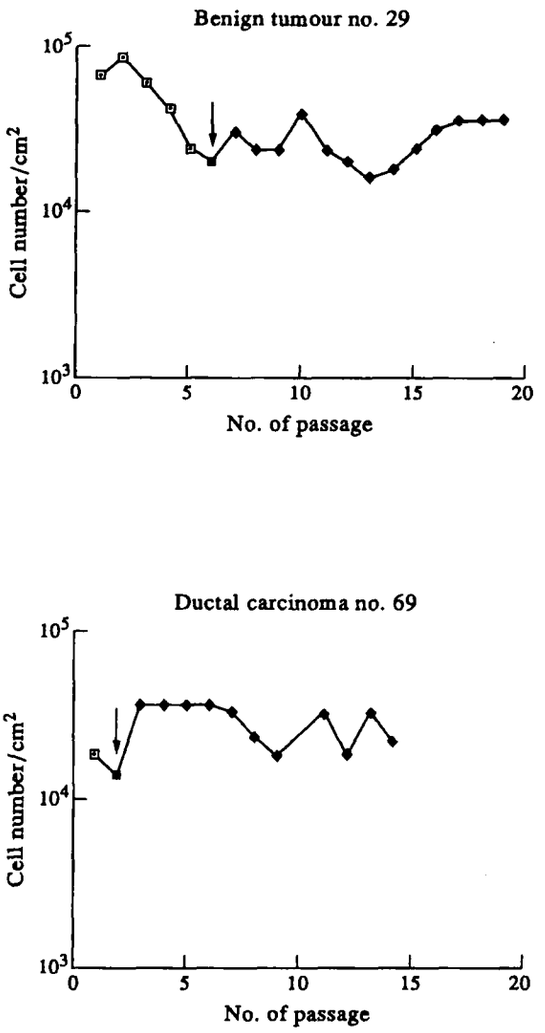

Fig. 3. Increased proliferative activity of human fibroblasts from benign and malignant breast tissues upon continuous treatment with FGFb. Addition (↓) of 10 ng/ml FGFb to poorly proliferative cultures (●) was made at the first day of cell subculturing for 12 to 14 passages (◆), after which experiments were terminated.

increase in the proliferative activity of the two cultures and significantly prolonged their growth *in vitro*. Additionally, a striking change in the cellular morphology also occurred in association with an increased expression of α-sm actin and ED-B fibronectin which persisted for many passages (Fig.4). Interestingly, similar results were obtained in a third culture derived from a benign tumour (specimen 23). These cells showed an increased proliferative activity and their morphology altered during culturing; they also acquired an α-sm actin and concurrent ED-B fibronectin phenotype while these two characters were absent in the original cell population (refer to Table 2).

DISCUSSION

The stroma of various types of cancer has been shown to harbour a subpopulation of fibroblastic cells, namely myofibroblasts, which exhibit some features of smooth muscle cell differentiation upon untrastructural examination, as well as expression of α-sm actin [11–14]. In the breast, however, myofibroblasts have also been demonstrated in some cases of non-malignant lesions [13]. The data presented herein of cultured breast stromal cells confirm the occurence of myofibroblasts in non-malignant and malignant tissues on the basis of α-sm actin expression. The proportions of myofibroblasts in cultures of non-malignant tissues were variable whereas in cultured carcinomas they were consistently high. As particularly true for this study, several types of histological changes (i.e. fibrosis, cyst, epithelial hyperplasia) often coexist in non-malignant breast lesions [8] and are found to be associated with

Table 4. Proliferative responses of stromal breast fibroblasts to growth factors

Stromal cell cultures (passage number)	Response to: (10ng/ml) EGF	FGFb	PDGF	TGFα
Normal breast tissues				
1 (p 9)	3.29±0.28	3.10±0.17	1.97±0.18	3.05±0.28
2 (p10)	2.18±0.18	2.93±0.16	nt	nt
3 (p 8)	1.97±0.17	2.30±0.13	1.86±0.17	1.85±0.17
Non-malignant breast tissues				
4 (p 5)	1.83±0.15	2.82±0.16	1.71±0.16	1.60±0.15
7 (p 7)	2.88±0.24	3.05±0.17	1.65±0.15	2.34±0.21
10 (p 7)	1.76±0.15	2.06±0.11	1.62±0.15	1.53±0.14
15 (p 5)	1.57±0.13	2.04±0.11	1.62±0.15	1.55±0.14
16 (p 9)	1.50±0.19	1.87±0.10	1.58±0.15	1.40±0.13
23 (p10)	1.65±0.14	1.92±0.11	1.29±0.12	1.67±0.15
29 (p 6)	1.15±0.90	2.22±0.12	nt	nt
67A (p 4)	1.63±0.14	1.68±0.90	1.72±0.16	1.35±0.12
68 (p 3)	1.65±0.14	2.94±0.16	2.29±0.21	1.86±0.17
68C (p 3)	2.02±0.17	3.78±0.21	3.25±0.30	2.21±0.20
Malignant breast tissues				
69 (p 3)	1.56±0.13	1.96±0.11	2.10±0.20	1.42±0.13
75 (p 4)	1.36±0.11	1.97±0.11	1.67±0.16	1.26±0.12
83 (p 5)	2.69±0.23	2.79±0.16	1.70±0.16	2.36±0.22

The proliferative response is expressed as increases in the final cell densities of cultures treated with 10 ng/ml of growth factors relative to untreated control cultures. Data are means of at least two separate experiments ± S.E.M. nt, not tested.

variable amounts of myofibroblasts [13]. Thus, more or fewer myofibroblasts may grow in culture depending on their proportions in the original tissue.

It is noteworthy that nearly homogeneous myofibroblastic cell populations were obtained from postradiation fibrosis, benign fibrocystic tumour and sclerosing adenosis. All of these cases involved pronounced development of fibrous tissue. Some, however, were obtained from tissues which were sites of previous or concomitant carcinomas. Although a relatively high frequency of myofibroblasts has been seen *in vivo* in certain irradiated fibrous tissues [15] and similar benign tumours [13] apparently devoid of breast tumour history, it is possible that previous or adjacent carcinomas may contribute to the expansion of this fibroblastic cell population.

The extracellular matrix of breast stromal cells in primary cultures contained collagen types I and III, normally produced by fibroblasts. Additionally, production of type V collagen was also noted. This latter form, which has been found in relatively high amounts in some breast carcinomas [16], seems to relate to the presence of myofibroblasts which are particularly predominant in this type of tissue.

Elevated levels of ED-B fibronectin expression were also observed in cultures from non-malignant and malignant tissues while they were barely detected in cultures from normal breast tissues. The newly identified fibronectin isoforms appear to be regulated in a tissue- or cell-type-specific manner [17, 18]. In malignant tissues from the breast and other organs, a preferential accumulation of ED-B isoform has been recently demonstrated but without establishing which particular cell type was producing it [6, 7]. In cultured stromal cells from either non-malignant or malignant sources, expression of ED fibronectin and α-sm actin were consistently associated, suggesting that this may be a general feature of the myofibroblastic cell population. It would be particularly interesting to determine whether myofibroblasts present in non-pathological conditions also produce this type of fibronectin isoform.

Unlike carcinoma cultures, the proliferative activity of stromal cells from non-malignant breast tissues persisted longer during cell subculturing. The differences in culture survival may reflect differences in growth rates between carcinoma myofibroblasts and those from non-malignant tissues. As previously reported [19], cultured carcinoma myofibroblasts grow much slower compared to normal fibroblasts. However, in cultures of non-malignant tissue cells long-term growth activity was observed when no more myofibroblasts were present in the cultures, as judged by the absence of α-sm actin and concurrent ED fibronectin expression. It may be that myofibroblasts were replaced by more rapidly dividing fibroblasts pre-existing in the original tissues. Alternatively, myofibroblasts might undergo dedifferentiation, and thereby may resemble normal dividing fibroblasts. It is interesting that the reverse process has been described in cultured normal breast fibroblasts; these were shown to express α-sm actin in increasing proportions after several days in culture [20]. This expression, however, seems to vary according to serum concentration and initial seeding density. Furthermore, varying proportions of α-sm actin-expressing cells have been consistently obtained in cultured fibroblasts from different tissue sources. Thus, it would appear that α-sm actin expression in fibroblasts is readily modulated during various experimental and pathological conditions.

Little is known about the precise nature of factors modulating growth and differentiation in myofibroblasts. From a wide spectrum of peptide factors, cytokines and hormones tested, only four consistently demonstrated mitogenic activites. These were EGF, FGFb, PDGF and TGFα. However, none of these factors appeared to have any selective effects; they all produced

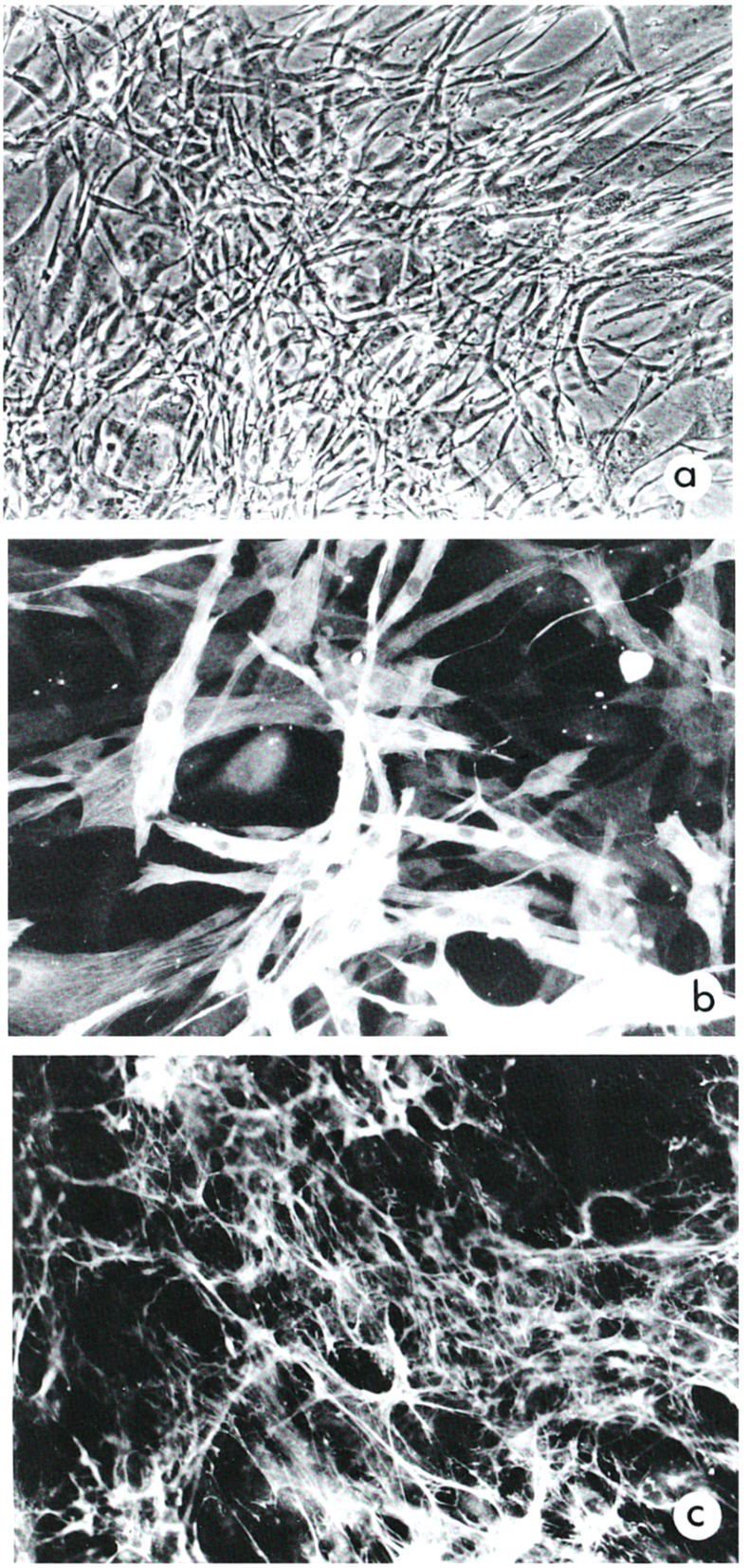

Fig. 4. Morphology (a) of human fibroblasts from benign breast tumour (specimen 29) after 14 passages with FGFb and parallel immunostaining with MAbs to α-sm actin (b) and ED-B fibronectin (c). Scale = 10 μm.

mitogenic effects whatever the tissue source of the cells. Although the cellular growth factor responses were qualitatively consistent, there was some variability in the magnitude of the response among cultures of the same type, and among cultures from different pathological backgrounds. This may reflect inter-individual variations regarding the potency of separate growth factors [22].

Interestingly, one of those mitogenic factors, FGFb, was found to not only prolong growth in culture but also to restore, and even induce, α-sm actin and concurrent ED-B fibronectin expression in stromal cells. While the mitogenic activity of FGFb in fibroblasts and many non-fibroblastic cell types is well documented, so far its contribution to the modulation of myofibroblast differentiation and concurrent ED-B fibronectin expression has not been reported. According to a recent study, FGFb is associated with the myoepithelial cell population of the breast [23]. It seems, however, unlikely that the modulation of myofibroblastic and ED-B fibronectin phenotypes depends only on factors released by myoepithelial cells, since these phenotypes are quite prominent in breast carcinomas without myoepithelial cells. This would suggest that other factors may influence myofibroblast differentiation. High concentrations of serum, as well as conditioned media from carcinoma cell cultures, have been shown to induce α-sm actin expression in normal breast fibroblasts [20, 24]. Consistent with these findings, preliminary studies have indicated that factors such as TGFβ and GM-CSF, although not mitogenic for breast stromal cells, were able to induce α-sm actin expression in cultures of normal breast fibroblasts initially devoid of this marker (data not shown). It is noteworthy that some of these factors which are known to be implicated in normal tissue repair and inflammatory processes are also produced by certain tumour epithelial cells [25].

From the results described here, it would appear that cultured stromal cells from different sources of non-malignant and malignant breast tissues are similar in terms of myofibroblast characteristics, ED-B fibronectin expression and proliferative/differentiative responsiveness to growth factors. This may reflect a common stromal cell's reaction to different tissue injuries rather than specificity for malignancy. In this respect, similarities in certain morphological characteristics have also been reported in cultured myofibroblasts from differently injured tissues [19]. Further detailed characterisation of stromal cells from normal tissues and from neoplastic conditions is needed for a better understanding of how phenotypic modulations of stromal fibroblasts contribute to cancer development.

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