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Uncontrolled Growth of Tumour Stromal Fibroblasts in vitro

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Abstract. The disturbance of the growth control mechanisms in tumour cells in vivo may be manifested as uncontrolled growth of the tumour stroma in vitro. Stromal fibroblast-like finite cell lines produced from benign or malignant human breast tissue specimens exhibited cell overlapping which ranged from multilayers to dense piling up colonies, while cells derived from normal tissues exhibited intense contact inhibition of growth and locomotion, under the same culture conditions. 6 out of 13 lines derived from malignant tissues and 2 out of 5 lines derived from benign lesions exhibited the phenomenon of 'periodic appearance of piling up colonies'.

Introduction

Tumour tissues in culture usually give rise to various types of fibroblast-like cells, referred to as stromal fibroblasts, which overgrow the epithelial cells in the early stages of the culture [8]. Stromal fibroblasts interact with adjacent neoplastic cells in several types of tumours [2, 9, 18, 26, 28]. The varying proportions and the morphological complexity of the tumour stromal elements [10, 32] point to the necessity of further investigation of the properties and the role of the stromal cells in neoplasia. Although much research in immune surveillance, as expressed by extensive lymphocytic infiltrate of the tumour [17], has been performed, to our knowledge, the variety of the stromal elements of breast malignant and benign diseases – including connective tissue, elastic tissue, inflammatory cells, and blood vessels [10, 12, 17, 23, 32] – has not been adequately studied in vitro.

In this paper we describe the growth patterns of 21 finite cell lines produced from malignant, benign, and normal human breast tissues.

Materials and Methods

Patients

Surgical specimens were obtained from 40 female and 2 male patients (table I). No patient had received chemotherapy or radiotherapy prior to surgery. The age of the patients ranged from 18 to 70 years.

Tissue Culture

Specimens were freed of blood, fat, fibrous and necrotic tissue, washed in physiological saline and finely chopped using forceps and scissors. The mash was mixed in a conical flask containing 10 ml of a solution of 0.5% trypsin (Difco), 0.1% EDTA (Sigma) in Mg^{++}-Ca^{++}-free phosphate-buffered saline, pH 7.8, and agitated on a magnetic stirrer for 20 min at 36.8 °C. An equal volume of complete medium was added to inactivate trypsin, and the mixtures were centrifuged at 800 rpm for 5 min. Supernatants were discarded. Each pellet was suspended in 5 ml of complete medium and transferred to plastic 25-cm^2 culture flasks (NUNC). Flasks were incubated at 36.8 °C in an atmosphere of 5% CO_2 and air and examined twice a week. pH was adjusted between 7.3 and 7.5 when needed. When attached cells showed signs of proliferation, the supernatant with the suspended tissue pieces was removed and fresh medium was added. When proliferating cells reached a confluent state, the cell monolayer was trypsinized with a 0.1% trypsin solution in Mg^{++}-Ca^{++}-free phosphate-buffered saline, pH 7.8, and new flasks containing fresh medium were seeded. Routinely, each new flask was seeded with one fourth of the cells of a confluent flask. The medium was changed weekly and the pH adjusted when needed. The cultures were examined weekly for foci and cell growth pattern. McCoy's 5a medium (Flow Laboratories) supplemented with 15% fetal bovine serum (Flow), 10^5 µg/l streptomycin, 10^5 IU/l penicillin, and 2 mg/l amphotericin B was used throughout. In this report, only results concerned with cultures grown in McCoy's 5a medium are presented. All cells were proved to be free of mycoplasma and other contaminations, as shown by ^{3}H-thymidine labelling and autoradiography [31] and occasionally by electron microscopy. Coverslip cultures were prepared on glass coverslips in 3-cm plastic Petri dishes (NUNC) and incubated in a humidified CO_2 incubator. Coverslip cultures were fixed in methanol and stained in Giemsa. Metaphases were routinely prepared by conventional methods every 2–3 months: cells in logarithmic phase of growth were treated with colcemid 0.1 µg/ml for 4–6 h, harvested by trypsinization, subjected to hypotonic treatment with 0.95% sodium citrate pH 7.5 for 45 min, and fixed in methanol-glacial acetic acid (3:1). Air-dried metaphase preparations were stained with Giemsa. At least 50 metaphases were examined in each preparation.

Table I. Yield of successful cultures from breast tissue specimens

Origin of tissue[1]	Total number of specimens	Number of cultures proliferating in vitro longer than 5 months
Normal breast epidermis	4	3
Normal breast tissue	3	0
Chronic mastitis	16	5[2]
Primary infiltrating ductal carcinoma	2	1
Infiltrating ductal carcinoma with metastases in lymph nodes	13	8
Metastatic lymph nodes from patients with infiltrating ductal carcinoma	3	3
Nipple melanoma	1	1
Total	42	21

[1] Identified by histological examination; none of the specimens used was of sarcomatous nature.

[2] Two of these specimens were derived from male patients; all other specimens were derived from female patients.

Results

Of the 42 surgical specimens cultured, 21 (50%) were successful (table I), yielding cell populations that formed confluent monolayers after 3–6 weeks in culture, and proliferated in vitro for longer than 7 months. Unsuccessful cultures exhibited very slow growth, which usually terminated after the first passage, and inability to form a confluent monolayer. The number of subcultures and the months of serial cultivation varied for each culture (table II). The in vitro maintenance period was approximately estimated to be the time elapsed from the first appearance of cell outgrowth to the last subculture before cessation of growth. Growth of all cells described in this study was extremely density dependent, and we had no success in establishing subcultures from single cells. There was no correlation so far between the yield of successful cultures and age or pathological condition of the patient.

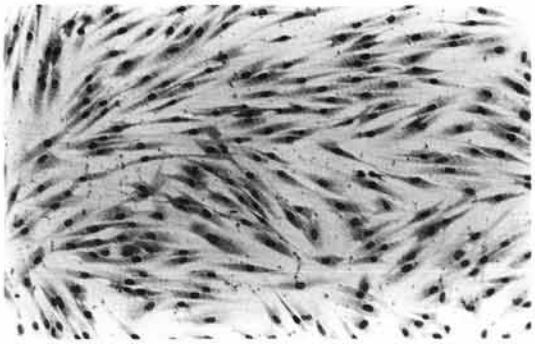
Figures 1–8 show the variety of cell and nucleus shape and cell orientation of eight different finite cell lines derived from breast

Table II. History of finite stromal cell cultures derived from normal, benign, and malignant breast tissues

Cell culture designation	Case	Site of specimen derivation	Months in serial cultivation	Number of 1:4 subcultivations	Morphology of in vitro colonies
AL-BC	CaM	primary tumour	8	27	PAPU
AND-BC	CaM	primary tumour	7	14	ML
AND-TH	CaM	nipple	20	42	CPU
CHA-EP	CaM	normal breast epidermis	18	67	DPCM
CHA-L	CaM	metastatic lymph node	7	26	ML
DA-ME	NM	primary tumour	22	42	CPU
EL-M	M	primary lesion	9	27	CPU
G-BC	CaM	primary tumour	9	17	ML
G-EP	CaM	normal breast epidermis	23	84	DPCM
G-L	CaM	metastatic lymph node	14	52	PAPU
GA-NB	N	normal breast epidermis	10	32	DPCM
KA-BC	CaM	primary tumour	9	20	PAPU
KR-BC	Ca	primary tumour	10	17	PAPU
LE-M	M	primary lesion	10	26	ML
MI-M	M	primary lesion	12	43	ML
PAP-M[1]	M	primary lesion	18	52	PAPU
PG-M[1]	M	primary lesion	22	88	PAPU
PL-BC	CaM	primary tumour	8	23	ML
SE-L	CaM	metastatic lymph node	25	91	PAPU
ST-BC	CaM	primary tumour	9	34	ML
SX-F	CaM	primary tumour	10	9	PAPU

CaM = Infiltrating ductal carcinoma with metastases in lymph nodes; Ca = infiltrating ductal carcinoma without metastases; M = chronic mastitis; NM = nipple melanoma; N = normal healthy donor; PAPU = periodically appearing piling up colonies; ML = multilayers; CPU = continuously present piling up colonies; DPCM = dense parallel cell monolayers.

[1] Derived from male patients.



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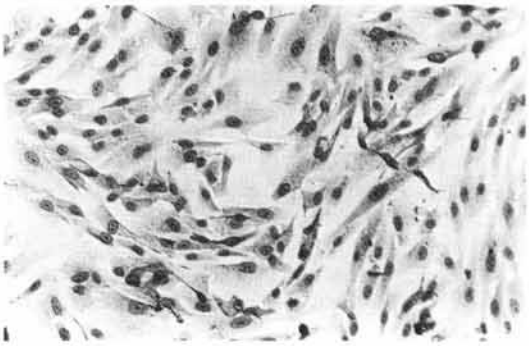

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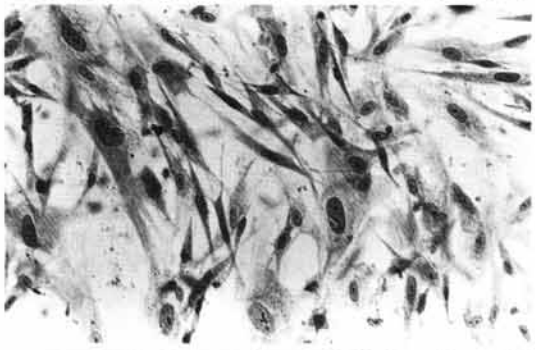

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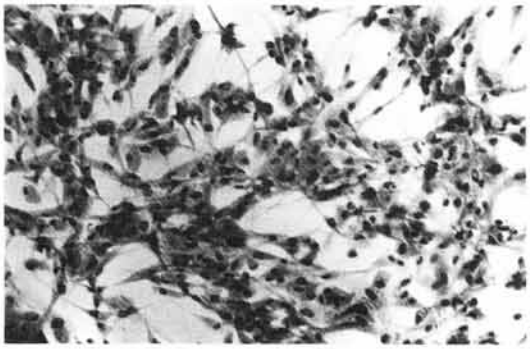

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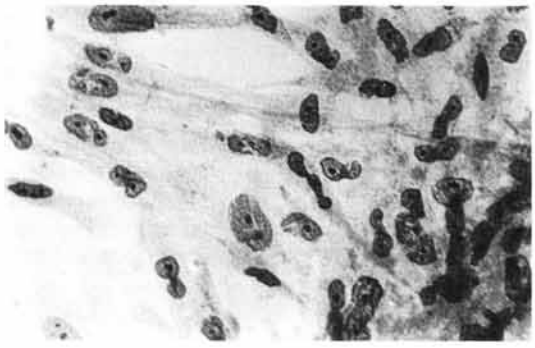

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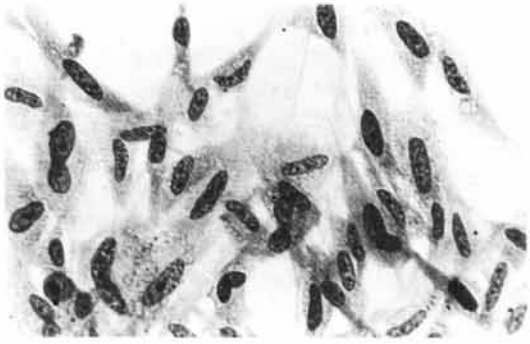

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tissues. Figures 9–12 show the morphology of colonies (foci) observed, in four lines. The morphological criteria suggest that all cultures described are derived from mesenchymal tissues.

Four main growth patterns were observed during maintenance in vitro for periods varying from 7 to 25 months (table II):

(1) Confluent cell monolayer of parallel, long, spindlelike, typical fibroblastic cells (fig. 1). At confluency, these cells appeared closely packed, exhibiting strict contact inhibition of growth and locomotion and absence of cell overlapping. This growth pattern was unique to all three lines derived from normal epidermis (table II). These lines showed all the characteristics described by *Hayflick* [13] and *Hayflick and Moorhead* [14].

(2) Monolayer with multilayered areas consisting of cells with long processes and a significant degree of cell overlapping at confluency (fig. 2–8).

(3) Periodic appearance (every 3–5 subcultures, corresponding to 40 days) of dense piling up colonies. Piling up colonies were characterized by fast upward growth and a failure to spread out on the available horizontal substrate.

(4) Continuous development of piling up colonies among multilayers (fig. 10, 11).

The development of piling up colonies and multilayers was neither a result of seeding of cell aggregates nor a result of retraction of monolayer areas. Since all cultures were examined following cell adhesion and thereafter very frequently (every 24 h), it was certain that piling up colonies or multilayers were developing as a cell division and growth phenomenon. In addition, it was observed that piling up colonies were increasing in size, since cells preferred to growth on piles and not to emigrate on the available empty surface (e.g., fig. 9–12) [7, fig. 1].

Cell monolayers produced from specimens of primary or metastatic tumours and chronic mastitis lesions exhibited growth patterns with varying degrees of cell overlapping which ranged from multilayer development to the formation of dense piling up colonies. Such lines never showed a constant dense parallel confluent cell monolayer growth pattern. None of the malignant or benign tissue categories studied so far gave fibroblast-like cells showing a unique cellular morphology or growth pattern characteristic of the patient's pathological state.

In six lines (AND-TH, G-BC, G-L, KA-BC, PG-M, and SE-L) numerous 'mini cells' were observed in coverslip cultures at early generations. These peculiar 'mini cells' consisted of a very small darkly stained nucleus surrounded by little basophilic cytoplasm

Fig. 1. Growth pattern of G-EP cells derived from normal breast epidermis. Giemsa. × 50.

Fig. 2. Growth pattern of AL-BC cells derived from a primary infiltrating ductal carcinoma. Giemsa. × 50.

Fig. 3. Growth pattern of KA-BC cells derived from a primary infiltrating ductal carcinoma. Note the intense cytoplasmic basophilia. Giemsa. × 80.

Fig. 4. Growth pattern of MI-M cells derived from a chronic mastitis lesion. Giemsa. × 80.

Fig. 5. Growth pattern of G-BC cells derived from a primary infiltrating ductal carcinoma. Giemsa. × 100.

Fig. 6. Growth pattern of CHA-L cells derived from a lymph node metastasis from an infiltrating ductal carcinoma and maintained in vitro for 7 months. Giemsa. × 80.

Fig. 7. Nuclear abnormalities and cell overlapping in SE-L cells derived from a lymph node metastasis from an infiltrating ductal carcinoma. Giemsa. × 160.

Fig. 8. Elongated nuclei and cell overlapping in PG-M cells derived from a chronic mastitis lesion. Giemsa. × 160.

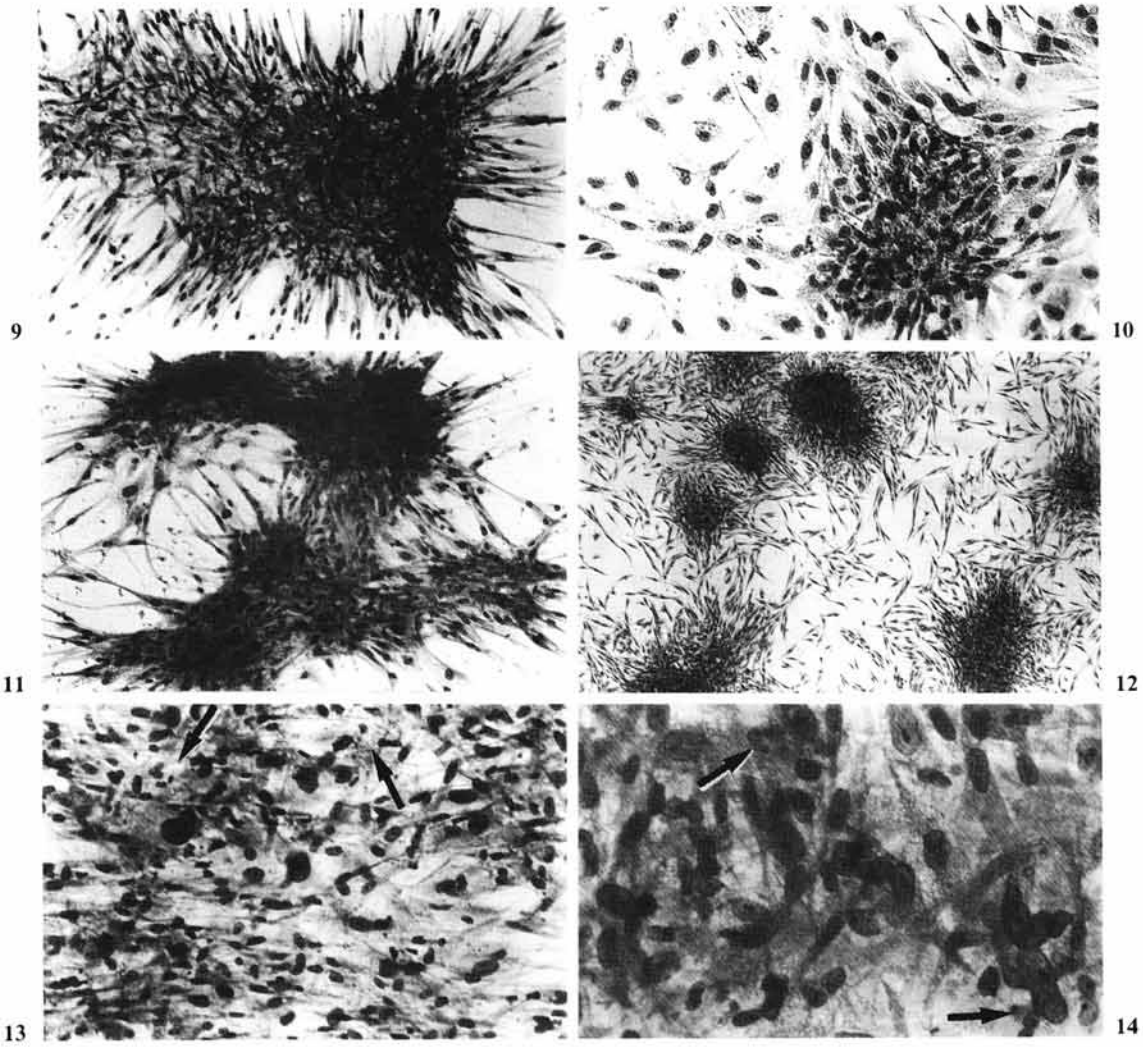

Fig. 9. Periodically appearing piling up colony morphology of PG-M cells derived from a chronic mastitis lesion. Giemsa. × 50.

Fig. 10. Continuously present piling up colony morphology of DA-ME cells derived from a nipple melanoma. Giemsa. × 130.

Fig. 11. Continuously present piling up colony morphology of EL-M cells derived from a chronic mastitis lesion. Giemsa. × 50.

Fig. 12. Periodically appearing piling up colony morphology of SX-F cells derived from a primary infiltrating ductal carcinoma. Giemsa. × 35.

Fig. 13. Multilayer of AND-BC cells after their second transfer, exhibiting 'mini cells' (arrows). Giemsa. × 100.

Fig. 14. Multilayer of G-L cells after their first transfer exhibiting 'mini cells' (arrows). Giemsa. × 160.

(fig. 13, 14). They disappeared completely in later generations.

The following were considered as signs of degeneration: decreased viability, inability to adhere onto the glass, cessation of mitoses, and accumulation of granular debris [13, 14].

None of the cell cultures described exhibited viral particles under the electron microscope [*Baltatzis-Voloudakis* et al., unpublished results].

The mode chromosome number of all lines described was within the normal diploid range during their in vitro life. Aneuploid cells (less than 5%) showed only a slight nonpersistent deviation from the normal mode. The slight deviations from the normal mode could not be considered as indicative of the presence of a clone of malignant cells because (1) no persistent chromosomal abnormality was noticed and (2) deviations from the normal diploid mode were within the expected frequencies for cultures of human fibroblasts. Several lines exhibiting periodically appearing piling up colonies showed varying low frequencies of cells containing double minute chromosomes [7].

Discussion

The stromal origin of all the finite lines described in this study is supported by the following three facts: (1) all cells were of fibroblastic morphology; (2) none of the specimens was of sarcomatous nature, and (3) all lines exhibited a normal diploid chromosome number.

Our description of stromal breast tissue cells in vitro shows a large variety of growth patterns in different individuals. Growth patterns characterized by various degrees of contact control loss or cell overlapping were developed only in cultures derived from malignant or benign breast lesions. This indicates that the disturbance of the control mechanisms involved in tumour cell growth in vivo may be manifested as uncontrolled growth of the tumour stroma in vitro. No correlation between high (periodically or continuously appearing piling up colonies) or low (multilayers) degrees of cell overlapping in vitro with malignant or benign breast disease (chronic mastitis), respectively, could be established. This might be expected, if we suppose that there is a relationship between breast benign and malignant disease, such as simultaneous occurrence, or if we consider chronic mastitis as a preneoplastic state [15, 24].

Normal fibroblasts in culture form parallel arrays, are contact inhibited, do not show nuclear abnormalities, have a short-time lifespan, and generally represent the expression of cell aging in vitro [13, 14, 22, 30]. Spontaneous deviations from these features have never been reported for fibroblast-like cells derived from normal human tissues, except under the influence of specific agents [25] or hereditary parameters [19]. However, fibroblast-like cell populations with properties similar to those presented here have been isolated from human sarcomas [11, 29]. Cell overlapping, formation of intense cell piles, cell aggregations, and increased cytoplasmic basophilia have been associated with transformation in many cell systems either under the influence of mutagenic parameters or spontaneously and have often been suggestive of malignant growth [3, 4, 19, 27]. Compared to untransformed parentals, transformed fibroblast-like cells usually have a shorter replication time and are capable of infinite growth in vitro. These characteris-

tics, however, do not apply to the cell cultures described here. Furthermore, persistent chromosomal alterations, a feature of transformed cells, were not shown for our cells which maintained their diploid mode throughout their in vitro life. Therefore, the properties of the cells described in the present study do not parallel with the properties of tumour cells. The evolvement and outgrowth of clones exhibiting varying degrees of cell overlapping from stromal elements cannot be excluded, though this possibility seems to be higher in cells produced from malignant or benign tissues.

Recently, *Seemayer* et al. [28] and *Tremblay* [32], using detailed electron microscopy results, postulated that stromal elements in breast carcinoma may represent a reaction of the immune system against the tumour. The significant role played by the connective tissue has been stressed in morphological studies for several tumour types [2, 9, 18, 26, 28, 32]. It should also be mentioned here that in cases of complete tumour regression, constantly accompanied by heavy lymphocytic infiltrate (tumour stromal reaction – immune surveillance) [17], tumour cells disintegrate and the tumour is eventually replaced by fibrous tissue [5, 17].

We believe that tumour stroma derived from mesenchymal tissues is influenced by adjacent neoplastic growth and/or by the immune response of the organism to the neoplastic growth. The parallel increase of stromal elements with that of tumour growth [32] should enhance the interest in the study of the relationship and interactions between malignant and non-malignant cells within a tumour. In vitro studies of interactions between the various cell types found in a tumour [1, 6, 16, 20, 21, 33] will greatly assist the clarification of the role of each cell type in the tumorigenesis and antineoplastic processes. Our recent results have shown that human fibroblasts, under certain conditions, may cause extensive disintegration of human lymphoblastoid cells leading to the formation of 'mini cells' [6].

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