Proliferating Fibroblasts and HeLa Cells Co-cultured *In Vitro* Reciprocally Influence Growth Patterns, Protein Expression, Chromatin Features and Cell Survival

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Abstract. *Aim: to identify biological interactions between proliferating fibroblasts and HeLa cells in vitro. Materials and Methods: Fibroblasts were isolated from both normal and tumour human tissues. Coverslip co-cultures of HeLa and fibroblasts in various ratios with medium replacement every 48 h were studied using fixed cell staining with dyes such as Giemsa and silver staining, with immunochemistry for Ki-67 and E-cadherin, with dihydrofolate reductase (DHFR) enzyme reaction, as well as live cell staining for non-specific esterases and lipids. Other techniques included carmine cell labeling, autoradiography and apoptosis assessment. Results: Under conditions of feeding and cell: cell ratios allowing parallel growth of human fibroblasts and HeLa cells, co-cultured for up to 20 days, a series of phenomena occur consecutively: profound affinity between the two cell types and exchange of*

small molecules; encircling of the HeLa colonies by the fibroblasts and enhanced growth of both cell types at their contact areas; expression of carbonic anhydrase in both cell types and high expression of non-specific esterases and cytoplasmic argyrophilia in the surrounding fibroblasts; intense production and secretion of lipid droplets by the surrounding fibroblasts; development of a complex net of argyrophilic projections of the fibroblasts; E-cadherin expression in the HeLa cells; from the 10th day onwards, an increasing detachment of batches of HeLa cells at the peripheries of colonies and appearance of areas with many multi-nucleated and apoptotic HeLa cells, and small HeLa fragments; from the 17th day, appearance of fibroblasts blocked at the G_2-M phase. Co-cultures at approximately 17-20 days display a cell-cell fight with foci of (a) sparse growth of both cell types, (b) overgrowth of the fibroblasts and (c) regrowth of HeLa in small colonies. These results indicate that during their interaction with HeLa cells in vitro, proliferating fibroblasts can be activated against HeLa. This type of activation is not observed if fibroblast proliferation is blocked by contact inhibition of growth at confluency, or by omitting replacement of the nutrient medium. Conclusion: The present observations show that: (a) interaction between proliferating fibroblasts and HeLa cells in vitro drastically influences each other's protein expression, growth pattern, chromatin features and survival; (b) these functions depend on the fibroblast/HeLa ratio, cell topology (cell-cell contact and the architectural pattern developed during co-culture) and frequent medium change, as prerequisites for fibroblast proliferation; (c) this co-culture

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model is useful in the study of the complex processes within the tumour microenvironment, as well as the in vitro reproduction and display of several phenomena conventionally seen in tumour cytological sections, such as desmoplasia, apoptosis, nuclear abnormalities; and (d) overgrown fibroblasts adhering to the boundaries of HeLa colonies produce and secrete lipid droplets.

Before the development of its own environment, at a very initial stage of its existence, a tumour may start from a single cell, which, as a result of an intrinsic carcinogenic process, has undergone specific genome alterations and creates a small colony by successive abnormal and unrestrained divisions (1). This rampant behaviour is challenged by the homeostatic/immune surveillance mechanisms of the organism and, if it is successfully confronted, the malignant cells are eradicated. If not, the small bundle of malignant cells gives rise to a tumour, which *per se* establishes its own microenvironment and "tumour homeostasis". Escape of cancer cells from the homeostatic immune control, one of the "hallmarks of cancer" (2, 3), has long since been suggested as an initial phenomenon in cancer development (4, 5). As these malignant cells mingle with other cell types, such as normal fibroblasts, that grow in parallel with them to form part of the tumour microenvironment (TME), the study of interactions between cancer cells and fibroblasts, as well as the role of fibroblasts in tumour progression have attracted a great interest (6-12).

Within the tumour stroma microenvironment, fibroblast-like cells comprise a large variety of cells with a predominant spindle-shaped *in vitro* morphology (pericytes, endothelial cells, "angiogenic cells", capillary tube cells, myofibroblasts, adipocytes). A particular type of stromal fibroblast, the cancer-associated or tumour-associated fibroblasts (CAFs or TAFs respectively) or myofibroblast within the tumour, has been shown to affect extracellular matrix (ECM) remodeling and activation of various mechanisms for supporting cancer cell growth (11, 13-16), and eventually invasion and metastasis (13, 16, 17-21).

The growth or invasion-supportive activity of CAFs has been associated with altered, induced or suppressed expression of a large variety of genes in both cancer cells and CAFs (17, 21-41). These activities have been described in co-cultures and histological samples containing adjacent cancer cells and stromal fibroblasts. The proportion of CAFs expressing each of these activities may differ in different samples or in different areas of the tumour. Thus, there is reasonable evidence pointing to the existence of multiple subpopulations of stromal fibroblasts (including CAFs) displaying heterogeneity regarding their gene expression and functions (27, 36, 42-49).

The growth of stromal fibroblasts within a tumour and, mostly, at its periphery (42, 50), as well as the creation of a unique ECM extracellular matrix (9, 51), as a response to cancer initiation and growth, has been termed reactive stroma, stromagenesis, fibrovascular stroma, stromal reaction, fibromyxoid reaction, fibrosis, desmoplastic reaction, and the phenomenon 'desmoplasia' (from the Greek for creation of chains) (9, 14, 42, 52-55). Desmoplasia has been suggested as a host defense against neoplasia (6, 46, 52, 56-60), and histologically observed desmoplasia has often been associated with aggressive tumours and poor prognosis (49, 61-64).

Since desmoplasia differs widely by tumour type (49, 64, 65) and since it may have opposing or supporting effects in different areas of the same tumour (39, 48, 66-73), a definitive description of its role in neoplasia has not been given. It remains unclear whether desmoplasia is a homeostatic response to cancer, or is caused by the cancer cells themselves (74). An interplay between desmoplasia, the inflammatory process and cancer has also been the focus of continuing studies indicating the possibility that desmoplasia may represent a post-inflammatory/pre-neoplastic condition (23, 75-77).

In tumour biopsy sections, CAFs can be seen around tumour cell nests, at the tumour periphery (29, 53, 78-81), but mostly at the invasive front (48, 82-84). Therefore, there is adequate evidence to show that heterogeneous distribution of stroma exists in a tumour's microenvironment (29, 65, 68, 85), contributing further to cancer heterogeneity.

The identification of CAFs in most studies has been based mainly on the expression of alpha smooth muscle actin (α-SMA), but also on fibroblast-specific protein 1 (FSP-1), vimentin or desmin (25, 68, 86, 87). A comprehensive description and definition of CAFs has been suggested that should also include the study of several ultrastructural features (88, 89) and gene-expression profiles (37, 90, 91). Thus, the definition of CAFs through a single marker may be inaccurate due to their high heterogeneity within the tumour stroma (53, 68, 70, 82, 92).

Although CAFs do not display somatic mutations, epigenetic changes have been observed, and are thought to regulate part of the gene expression of CAFs (93, 94).

The origin of CAFs remains unclear (45) since there are indications that they may be derived from resident fibroblasts, pericytes, mesenchymal stem cells, endothelial cells, carcinoma cells, epithelial cells, smooth muscle cells, bone marrow-derived cells, fibrocytes or adipocytes (53, 95-99). It should be noted that fibroblasts stimulated by various cytokines produced by cancer cells [such as transforming growth factor α (TGFα), transforming growth factor β (TGFβ), epidermal growth factor (EGF), β epidermal growth factor (EGF), platelet-derived growth factor (PDGF), tumour necrosis factor alpha, Interleukin 1 (IL-1), IL-6, IL-8] are transformed to myofibroblasts (90, 100, 101) located close to cancer cells (101). This effect of cancer cells on fibroblasts has also been suggested to be responsible for the desmoplastic reaction in tumours (74, 101).

Terms, such as multipotent mesenchymal stem cells, are extensively used in the literature to declare heterogeneous populations of fibroblast-like cells isolated from various tissues. These cells exert a variety of effects based mainly on their "flexibility" in expressing certain genes, thus facilitating specific final phenomena, including transdifferentiation (102-104). Remarkably, CAFs, myofibroblasts, mesenchymal stem cells and fibroblasts have more similarities than previously recognised (98, 105-109). These include non-tumourigenicity and no or very limited acquisition of chromosomal aberrations (110); immunoregulatory or immunosuppressive properties (75, 111-113); tissue-repair mechanisms, including dermis, tendon and bone cartilage (114, 115); adaptive nature and potential for differentiation (103, 104, 116-118); affinity for cancer cells both *in vitro* and *in vivo* (119, 120-122); and antiproliferative effects (123, 124).

The ratio of CAFs to non-CAFs varies not only by tumour type but also within the same type of tumour in different patients (1, 11, 21, 47, 68, 74, 82, 87, 125-133), therefore justifying the question of the role of non-CAFs in the desmoplastic reaction (46, 51, 72, 131, 133). Only a small fraction of fibroblasts in breast cancer is converted into myofibroblasts (127, 128). In addition, fibroblasts and mesenchymal stromal cells of various origins have considerable *in vitro* heterogeneity regarding morphology (9, 43, 134, 135), response to host-tumour cell-cell interactions (82), phenotype (9, 103, 136, 137), gene-expression profile (7, 9, 27, 46, 135, 138) and immunomodulatory capacity (138, 139). Fibroblasts may remarkably exhibit heterogeneous properties even within the same organ (46, 139-141). Furthermore, stromal fibroblasts from different locations within a tumour may have different properties (39, 48, 68-72, 82, 91). CAFs in endometrial tumour biopsy sections from different patients also exhibit wide variations in the expression of mesenchymal cell markers, growth factors, cell cycle regulators, steroid hormones and angiogenesis factors (78).

Normal fibroblasts *in vitro* are typically senescent spindle-shaped cells exhibiting high mobility, contact inhibition of growth, chromosomal stability in culture (134, 142-145) and absence of malignant cellular phenotypes (15). They have been shown to be prone to alterations of their gene-expression profile during their involvement in embryonic development (53, 146), mammary gland formation and breast morphogenesis (25, 53, 147, 148), endometrial cellular physiological processes (149), prostate development (150) and wound healing and tissue repair (86, 87, 115, 136, 151). During these processes, normal fibroblasts are converted into myofibroblasts (10, 74, 86, 149, 152-154) that exhibit elevated levels of collagen, α-SMA and the ED-A splice variant of fibronectin (25, 87) and of a number of other proteins (150, 155); or transdifferentiate into various final cell types (104, 156). These alterations cease to exist after normal functions have been established or regained and myofibroblasts

disappear by apoptosis (86, 149, 152, 157). Thus, in a normal healthy organism, fibroblasts act as infrastructural elements, as regulators of organ development and as guardian of tissue architecture and homeostasis (74, 123, 150, 152, 158-161). If fibroblasts maintain these fundamental properties within the desmoplastic reaction, it seems reasonable to consider that their presence and growth are involved in the defense mechanism against cancer (46, 51-54, 58, 59, 162). However, there is evidence suggesting that in the tumour microenvironment, at least some of the stromal fibroblasts undertake a supportive role in tumour growth and metastasis (10, 12, 13, 16, 33, 37, 39, 43, 47, 53, 72, 90, 96, 152, 163-178). Notably, α-SMA-positive myofibroblasts were shown to be supportive of tumour invasion and angiogenesis (39, 72, 179), while α-SMA-negative fibroblasts were inhibitory.

Although there are extensive experimental indications of a tumour-suppressing (inhibitory) action of certain fibroblasts (including mesenchymal stem cells) *in vitro* (6, 10, 12, 58, 124, 161, 164, 180-230) or *in vivo* (58-60, 122, 123, 189, 206, 225, 228, 231-243) (excluding fibroblasts with specific gene manipulations), the mechanisms involved and the final outcome of suppressive effects are still obscure.

It should also be mentioned that the regulation of differentiation and proliferation of cancer cells towards more physiological histological patterns, as well as the reversion of the malignant phenotype by normal fibroblasts, has been described in several studies (147, 180, 206, 244-249). Regulatory effects on cancer progression have also been ascribed to stromal endothelial cells (250).

The above discussion underlines the meaning of 'fibroblast plasticity' (92). However, the precise conditions under which fibroblasts, as a highly heterogeneous compartment of a tumour, may become supportive, inhibitive or catastrophic for tumour cell growth remain unknown (6, 7, 9, 19, 42, 92, 206, 227, 251).

It is certain that fibroblasts should be able to replicate in order to accept signals for the modulation of their own gene expression during S phase, or produce signals for the modulation of genes in epithelial cancer cells. Proliferating fibroblasts do not show α-SMA expression and elevated collagen synthesis, while cells expressing α-SMA express high levels of types I and III collagen mRNA (252). Furthermore, a few studies have stressed the importance of feeding (119, 186, 248, 253) as a means of maintaining fibroblasts in a proliferative state, particularly during their co-culture with cancer cells.

Thus, in continuation of our previous experiments (6, 119, 134, 186) and on the same theoretical basis as previously defined (6, 9), the interactions between HeLa cells and proliferating fibroblasts were studied aiming to describe in detail the influence of one cell type on the behaviour, growth pattern and survival of the other, by applying conventional and specific staining [non-specific esterases, lipid, Ki-67, E-cadherin, dihydrofolate reductase (DHFR), silver staining].

Materials and Methods

Cell lines. Eleven finite stromal fibroblastic cell lines (Table I) were used and are referred to as SF or fibroblasts. Of these, seven have previously been described (134, 142) and four (KA-BC, KR-BC, LA-BC and TONS-57) were developed more recently. Fibroblasts were isolated as previously described (134, 142) and were routinely maintained in McCoy's 5A (Flow Laboratories Inc., Rockville, MD, USA) medium supplemented with 10% fetal bovine serum (Flow Laboratories Inc., Rockville, MD, USA), 10^5 IU/L penicillin, 10^5 µg/L streptomycin and 2 mg/L amphotericin B, at 37˚C and 5% CO_2. The same medium was used in all cell co-culture experiments. All cells were free of bacterial, yeast, fungal and mycoplasma contaminations as tested by ^{3}H-thymidine labeling and autoradiography (254, 255), and mycoplasma antibody assay (256). Cells were not sourced from specimens from patients with HIV or hepatitis. Tissue samples were used after hospital's approval and patient's consent.

All SFs used had replication times of 50-56 h, a chromosomal number of 46 and chromosomal abnormalities including aneuploidy were present in fewer than 4% of the examined metaphases with no persistent chromosomal changes. Their plating efficiencies were very low, requiring a minimum of 1×10^3 to 2×10^3 cells/cm^2 to produce a monolayer.

The HeLa cell line used was produced by cell cloning (257) and had a replication time of 16 h, plating efficiency of 97% and chromosome numbers ranging from 52-88 with ~6% hyperpolyploid cells.

Cell-to-cell interactions in vitro. The basic procedures of studying cell-to-cell interactions *in vitro* have been described elsewhere (134, 142, 186). Since the purpose of the present study was to observe cell interactions for as long as possible, HeLa cells were plated at a density of 5 to 6 cells per 1×1 cm^2 glass coverslip contained in a 32 mm diameter plastic petri dish (Lux, Miles Scientific, Naperville, IL, USA). After 2 to 3 days, colonies of 8-16 HeLa cells were produced and, from that time point, the fibroblasts were added suspended in replacement medium at the desired density. The medium was then changed every second day. Under these conditions, co-cultures were studied for up to 20 days. After every 2 days of co-culture, coverslip-attached cells were washed (×3) in physiological saline and fixed in methanol or 5% paraformaldehyde before staining.

Giemsa staining. Methanol-fixed cells were stained in freshly-prepared Giemsa solution in a buffer prepared by mixing 3 ml 0.1 M citric acid, 8 ml 0.2 M disodium hydrogen phosphate solutions and 80 ml distilled water and adjusting to pH 6.8. Three milliliters of methanol and 6 ml of Giemsa (Gurr R66; BDH Laboratory Supplies, Poole, UK) were then added and the solution was filtered before use. Cells were stained for 20 min, washed in distilled water, in acetone and xylene twice each, and mounted in Canada balsam or DPX (Sigma-Aldrich Biotechnology, St. Louis, MO, USA). Giemsa staining and cell morphology allowed HeLa cells to be differentiated from fibroblasts. HeLa nuclei were pleomorphic and highly hyperchromatic, staining profoundly dark blue with even darker nucleoli and blue cytoplasm, while fibroblast nuclei stained light red-pink with blue nucleoli and very light blue cytoplasm. HeLa cells had an epithelial polygonal morphology, with a nuclear:cytoplasmic ratio of 1:2 to 1:3 and nuclei with 1 to 5 nucleoli. Fibroblasts had a spindle-like morphology, with a flat ellipsoidal nucleus with 1 to 3 nucleoli. They often developed very long projections, and their cytoplasm spread out on glass or plastic surfaces. The nucleus:cytoplasm ratio ranged from 1:5 to 1:14.

Table I. *Finite human fibroblastic cell lines (SF) used in the present study.*

Cell line designation	Site of specimen derivation
AK	Tracheitis
G-EP	CaM - normal breast epidermis
G-L	CaM - metastatic lymph node
KA-BC	CaM - primary tumour
KR-BC	CaM - primary tumour
LA-BC	CaM - primary tumour
PG-M	Chronic mastitis primary lesion
PL-BC	CaM - primary tumour
SE-L	CaM - metastatic lymph node
TONS-F	Tonsillitis
TONS-57	Tonsillitis

CaM: Breast adenocarcinoma with metastases.

Under the microscope, a light green filter was used to accentuate colour contrast. A Zeiss microscope (Carl Zeiss, Jena, Germany) equipped with a digital camera was used for all observations. Methyl Violet (1% in distilled water; BDH Laboratory Supplies, Poole, UK) was also used as a stain to demarcate boundaries of cell colonies.

Carmine marker. Carmine-labeled HeLa cells were prepared by culturing cells in medium containing 0.2% carmine powder (Fisher Scientific Co., Chicago, IL, USA) for 24 h and by washing twice in serum-free medium (186, 259). Carmine appears as tiny dark particles (1-2 µm) in the HeLa cell cytoplasm and is not transferred from one cell to another.

Silver staining. Methanol-fixed cells were silver-stained using the method of Goodpasture and Bloom (260). Staining was monitored under a microscope.

Autoradiography. Metabolic co-operation and cell–cell communication were examined by monitoring tritiated nucleotide exchange between donor (labeled) and recipient cells. The procedure used has been described elsewhere (261). The donor cells were pre-labeled with ^{3}H-uridine (^{3}H-UdR) [0.1 µCi/ml, specific activity (sp. act.) 21 Ci/mmol]; ^{3}H-thymidine (^{3}H-TdR) (0.1 µCi/ml, sp. act. 25 Ci/mmol) (GE Healthcare, Amersham, UK).

Non-specific esterase (NSE) staining. The method described by Nestor and Bancroft (262) using α-naphthyl acetate (Sigma-Aldrich Biotechnology, St. Louis, MO, USA) as substrate at pH 7.1 was adapted with some modifications. Live cells on coverslips were washed 3 times in physiological saline (PS), placed in a 5cm-diameter petri dish and stained under microscopic monitoring at ×25 for approximately 30 min. Fast red TR (Sigma-Aldrich Biotechnology, St. Louis, MO, USA) was used instead of pararosaniline hydrochloride and counterstaining was not used. Cells were then washed in PS, dehydrated in alcohol, fixed in xylene and mounted in DPX. Acetazolamide (20 µM; Sigma-Aldrich Biotechnology, St. Louis, MO, USA) was used to suppress carbonic anhydrase activity (263-265), which was very profound within 5-10 min if acetazolamide was not used. Areas with NSE activity were stained light red to reddish brown.

Lipid staining. Live cells on coverslips were washed gently in PS, transferred into a 5 cm petri dish with 3 ml PS and 0.2 ml dye solution (Sudan Black B, in 1% alcohol; Sigma-Aldrich Biotechnology, St. Louis, MO, USA) and staining was monitored under the microscope at ×25 for 1 h. Cells were gently washed in water and mounted in glycerin jelly. Large fat and lipid droplets stained light blue-grey were seen by adjusting the focus. Lipid droplets in the cytoplasm were stained grey.

Ki-67. The detection of Ki-67 nuclear proliferation antigen was carried out immunohistochemically as previously described (266).

E-Cadherin immunohistochemical staining. Cell monolayers were fixed in methanol for 5 min at 4°C. After rehydration, endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide for 10 min at room temperature. Subsequently, the cells were exposed to normal serum for 30 min to block non-specific binding and incubated with the antibody to E-cadherin (4A2C7 clone, diluted 1:200; ZYMED Laboratories, San Francisco, CA, USA) for 1 h at room temperature. A biotinylated anti-mouse antibody was then added to cells for 45 min and they were treated with the streptavidin-biotin-peroxidase complex solution (Vectastain Elite ABC Kit; Vector Laboratories, Inc., Burlingame, CA, USA) for 30 min. Finally, cells were incubated with 3,3-diaminobenzidine (DAB), monitored for development of a brown reaction product and then slightly counterstained with Mayer's hematoxylin. MCF-7 and Jurkat cells were used as positive and negative controls, respectively. No specific staining was observed when primary antibody was omitted.

Dihydrofolate reductase (DHFR). Cells on coverslips were fixed in 5% paraformaldehyde and stained for DHFR as described by Huennekens *et al*. (267). Counterstaining with Giemsa for 5 min enabled improved observation of the cells.

Apoptosis assessment. Apoptosis in cell monolayers stained with Giemsa was morphologically verified under microscopy by the co-existence of nuclear fragmentation, nuclear abnormalities, micronuclei, apoptotic bodies, cellular membrane blebbing, cytoplasmic vacuolation, condensed basophilic cytoplasm, and cell shrinkage (268, 269).

Results

1. Description of the finite SF cell lines. Derivation of the cell lines used in the experiments is reported in Table I. Some of them (AK, G-EP, G-L, PG-M, PL-BC, SE-L, TONS-F) were produced and described earlier. SF lines originating from tumour tissues were shown to possess a normal karyotype and an uncontrolled growth pattern *in vitro*, with overlapping and piling up of cells (134, 142). Figure 1 shows the morphology of a sparse (a) and a confluent (b) monolayer of normal skin fibroblast cell line G-EP. Figure 2 shows cell overlapping in a cell monolayer of G-L cells isolated from a metastatic lymph node of a patient with breast cancer. The recently developed SF cell lines, KA-BC, KR-BC, LA-BC, presented the same features as PL-BC.

In SF cell line monolayers produced from malignant tissues, two remarkable features were observed: (i) the presence of small cells with darkly stained nuclear fragments during the first and second *in vitro* passages (Figure 3 a, b), diminishing and disappearing at subsequent passages; and (ii) the very rare observation of cells with fragmented nuclei and cells resembling plasma cells with a clock-face pattern of nuclear chromatin (Figure 4).

2. Importance of the HeLa:fibroblast cell ratio and of medium renewal in the growth and behaviour of both cell types. A confluent fibroblast monolayer with contact-inhibited fibroblasts served as a perfect feeder layer for HeLa cells. Even a small inoculum of 1-5 HeLa cells was able to successfully grow on top of the fibroblasts, producing dense colonies of HeLa cells entirely covering and destroying the fibroblast layer with or without replacement of the medium (Figures 5, 6, 7). In contrast, fibroblasts (at any inoculum between 5 and 10^4 cells/ml/1 cm^2) cast over a confluent HeLa cell monolayer did not attach to the HeLa cells. When fibroblasts found any empty space among HeLa cells, they attached and extended, but did not divide. The same effect was observed when a high ratio of HeLa cells:fibroblasts (*e.g.* 20:1; $20×10^3:10^3$ cells) was used. Fibroblasts were able to find space to attach but were very soon contact-inhibited by the HeLa cell population (Figure 8). The same effect was seen when an inoculum of HeLa cells and fibroblasts at ratio of 1:1 with $20×10^3:20×10^3$ cells was used.

When an inoculum ratio of 1:1 with $1×10^3:1×10^3$ to $1×10^4:1×10^4$ HeLa cells:fibroblasts was used, both cell types had space and time to attach and multiply. A high affinity between the two cell types was observed in all modes of co-culture, whether by inoculating HeLa cells and fibroblasts together or separately over a pre-attached non-confluent fibroblast or HeLa cell monolayer, as soon as after 2 days of co-culture. In all these combinations, affinity and absence of overlapping was observed. This phenomenon was observed using the earlier developed fibroblast cell lines (119) and confirmed using all the recently developed cell lines. When medium was not renewed after 2 days of co-culture, fibroblasts stopped replicating, while HeLa cells continued to multiply for three to five days (depending on the initial cell inoculum) before exhausting the medium and lowering the pH below 6.8. The above-described phenomena were observed with all of the finite fibroblast cell lines used in this study.

When an inoculum of 10^5 fibroblasts/ml was cast over a monolayer of small HeLa-cell colonies (6-40 cells in each colony), fibroblasts attached to the entire available glass coverslip surface, but did not overlap HeLa cells, seemingly respecting their territory (Figure 9). However, a rare exception was observed with one cell line, SE-L, in which the fibroblasts showed profound overlapping with HeLa cells (Figure 10).

3. Development of basic procedure for cell-to-cell interactions in vitro. By testing various inocula of fibroblasts, it was found that only inoculation with ≥10^3/cm^2 fibroblasts could provide an actively replicating and motile fibroblast population lasting
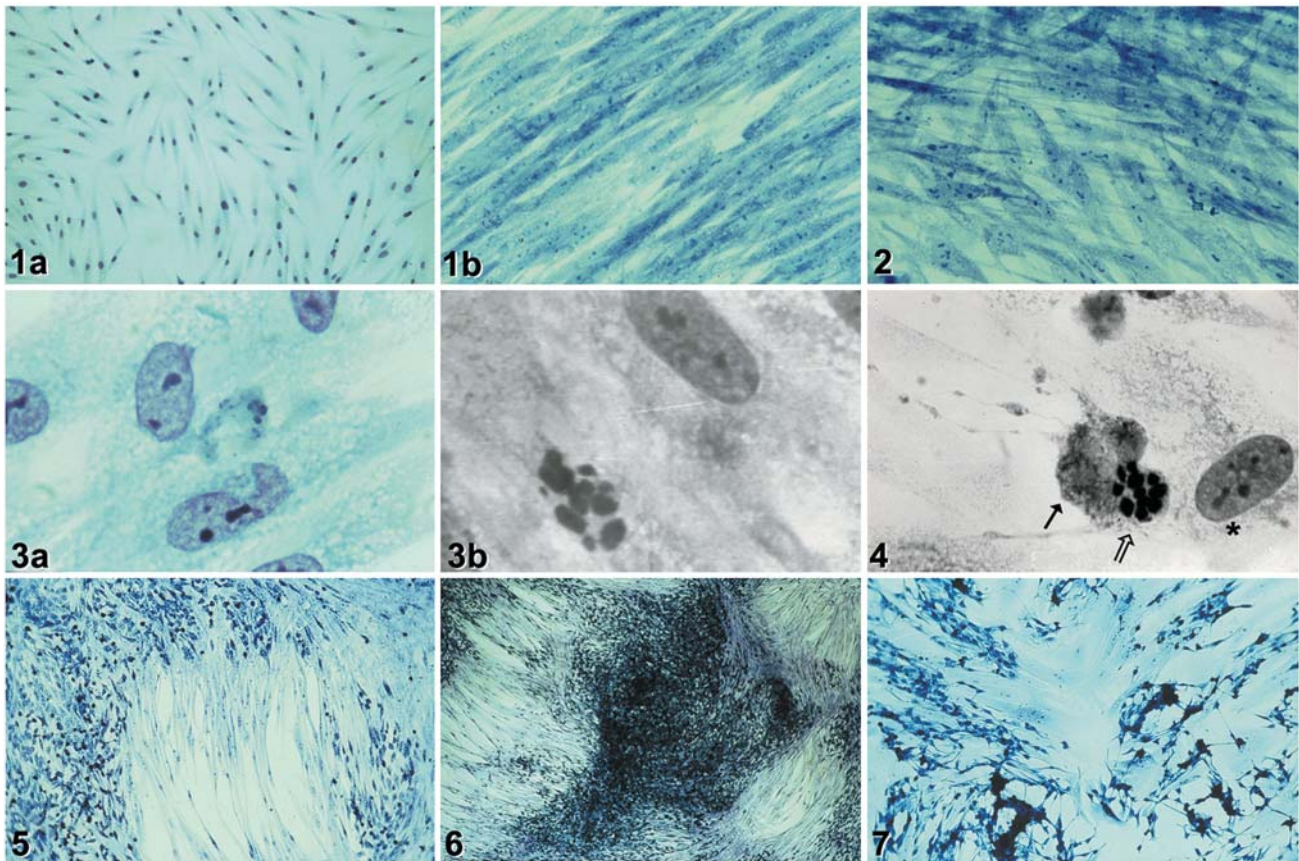
Figure 1. *Normal skin fibroblasts (G-EP) isolated from a patient with breast cancer. Giemsa, a: non confluent monolayer, ×25; b: confluent monolayer, ×80.* Figure 2. *Stromal fibroblasts (G-L) isolated from a metastatic lymph node of a patient with breast cancer. Cells show overlapping. Giemsa, ×80.* Figure 3. *Second passage monolayer of stromal fibroblasts (G-L) isolated from a metastatic lymph node of a patient with breast cancer showing darkly stained nuclear fragments, possibly originating from apoptotic cancer cells. Giemsa, a: ×600; b: ×1000.* Figure 4. *First passage monolayer of stromal fibroblasts (PL-BC) isolated from a primary breast adenocarcinoma, showing darkly stained apoptotic cells (arrow) and a clock face-like cell (double arrow) close to a normal fibroblast (asterisk). Giemsa, ×600.* Figure 5. *Confluent fibroblast (PL-BC) feeder layer in co-culture with HeLa cells. HeLa cells (5×10³ in 1 mL medium) were seeded on a 1×1 cm coverslip with a confluent cell monolayer of PL-BC fibroblasts and incubated for 32 h without medium replacement. Adhesion of HeLa on the top of the fibroblasts is apparent. Giemsa, ×20.* Figure 6. *Same culture as shown in the legend to Figure 5, after 72 h without medium replacement. Overgrowth of HeLa on top of the fibroblasts can be seen. Giemsa, ×15.* Figure 7. *Same culture as shown in the legend to Figure 5, after 144 h without medium replacement. A few remaining fibroblasts with faint cytoplasms and nuclei, and shrunk, obviously starving HeLa cells are visible. Giemsa, ×20.*

longer than 12 days, due to the low plating efficiency of these finite cell lines. Considering the feeding antagonism between HeLa cells and fibroblasts and the faster replication time of HeLa cells, in order to avoid exhaustion of the medium (usually occurring after 2-3 days in HeLa cultures and 4-5 days in fibroblast cultures), it was replaced every 2 days in all co-cultures. The procedures using small HeLa-cell colonies at the start of experiments, as described in the Materials and Methods section, were found to secure the parallel growth of both cell types in co-culture for at least 12-16 days (corresponding to approximately 6-8 fibroblast replications and 18-24 HeLa cell replications) at a HeLa cell:fibroblast ratio of about 1:100, without obstruction of fibroblast growth. Thus, a co-culture

system with equal growth and space advantages for both HeLa cells and fibroblasts was established and used throughout the present experiments. It should be noted that during the whole co-culture period studied, the interactions between HeLa cells and fibroblasts caused remarkable fluctuations in the ratio between the two cell types because of inhibition or acceleration of growth of one or the other cell type in focused areas of the cultures.

4. Tropism and affinity between fibroblasts and HeLa cells, and encirclement of HeLa-cell colonies by fibroblasts. It was observed that fibroblasts showed a remarkable tropism and contact guidance towards HeLa cells. They became very elongated and bent, and finally encircled, but did not intrude
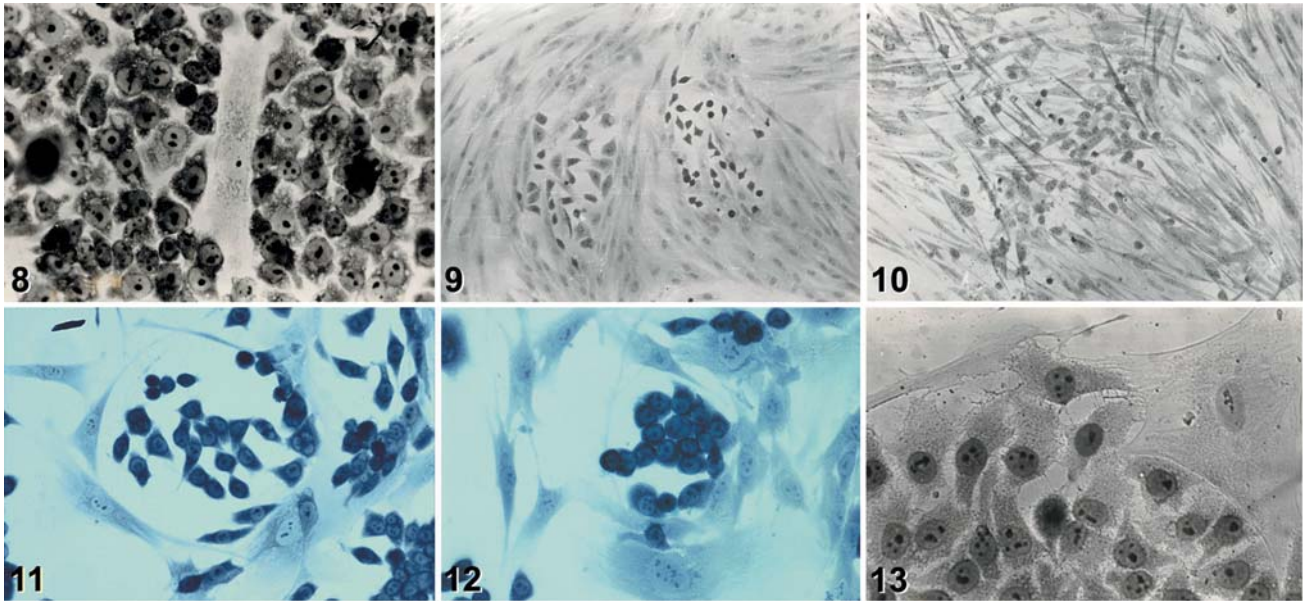
Figure 8. *Monolayer of confluent HeLa cells with a contact-inhibited AK fibroblast. A dilute suspension of fibroblasts (10^3 AK cells in 1 mL medium) was seeded over an almost confluent HeLa monolayer on a 1×1 cm coverslip and cells were cultured for 72 h. In such co-cultures, fibroblasts were only able to attach to empty areas of the coverslip surface among HeLa cells but were unable to multiply. Similar results were obtained with all types of normal and stromal fibroblasts used. Giemsa, ×252.* Figure 9. *G-EP fibroblasts and HeLa cell co-culture. An inoculum of 10^5 G-EP fibroblasts in 1 mL medium was cast over a monolayer of small HeLa colonies (6-40 cells/colony) on a 1×1 cm coverslip and cultured for 72 h, with medium replacement at 50 h. Fibroblasts attached to the available glass surface in this image did not overlap the HeLa cells. Giemsa, ×40.* Figure 10. *SE-L fibroblasts and HeLa co-culture. An inoculum of 10^5 SE-L fibroblasts in 1 mL medium was cast over a monolayer of a small HeLa cell (6-40 cells) colony on a 1×1 cm coverslip and cultured for 72 h, with medium replacement at 50 h. A profound overlapping of SE-L cells on HeLa cells can clearly be seen. This phenomenon was not observed with any of the other fibroblastic cells used. Giemsa, ×40.* Figure 11. *Encirclement of HeLa-cell colonies by fibroblasts. An inoculum of 10^3 KR-BC cells in 1 mL medium was cast over a non-confluent monolayer of large and small HeLa-cell colonies and cultured for 50 h. The tendency of the fibroblasts to encircle the small HeLa colonies is obvious. Giemsa, ×120.* Figure 12. *Same co-culture conditions for 72 h as shown in the legend to* Figure 11. *Giemsa, ×140.* Figure 13. *Edge of a HeLa-cell colony surrounded by G-EP fibroblasts. Same conditions as shown in the legend to* Figure 11. *Fine cytoplasmic connections between HeLa cells and fibroblasts are obvious. Giemsa, ×220.*

into the HeLa-cell colonies (Figures 11, 12, 13). The encirclement of HeLa-cell colonies by the fibroblasts was observed from the second day of co-culture. Provided that growth space was available and the medium was replaced every two days, the fibroblasts appeared to show tropism towards small HeLa-cell colonies and encircled them.

While HeLa-cell colonies started to be surrounded by fibroblasts, the nuclei of the peripheral HeLa cells were characteristically positioned far from the fibroblasts and towards the centre of both small (Figure 14) and large (Figures 13, 15 and also Figures 56, 60) colonies. Control HeLa-cell colonies (without fibroblasts) did not demonstrate this behaviour (Figures 16, 17), at least not so markedly in all peripheral HeLa cells as in the co-cultures with fibroblasts. However, when HeLa cells were cast over a dilute monolayer of fibroblasts, the HeLa cells preferentially attached around the fibroblasts, with their nuclei favorably positioned close to the fibroblasts (Figures 18, 19). In such cultures, a remarkable feature of the affinity between the two types of cells was the

development of long cytoplasmic projections of HeLa cells along the edges of the fibroblasts (Figures 20, 21), as also shown by casting a carmine-relabeled HeLa cell suspension over a sparse fibroblast monolayer (Figure 22).

During the parallel growth of the HeLa-cell colonies and the encircling fibroblasts, HeLa cells started piling up in the central areas of the colonies, but also grew actively at the peripheries of the colonies, thus forcing the fibroblasts to become more elongated while being pushed back, without intermixing or overlapping. At this stage, two distinct types of HeLa-cell colonies were observed: (a) colonies with densely growing cells and piling up of cells in the centre, which were encircled by a dense fibroblastic bow, and (b) colonies with densely or loosely growing cells and piling up of cells in the centre but which were not amply encircled by fibroblasts. This phenomenon was previously described using a finite skin fibroblastic cell line (G-EP) (186) and confirmed in the present study for all fibroblastic lines used. Interestingly, this phenomenon shows that even in a single HeLa clone, a variation in colony morphology, as
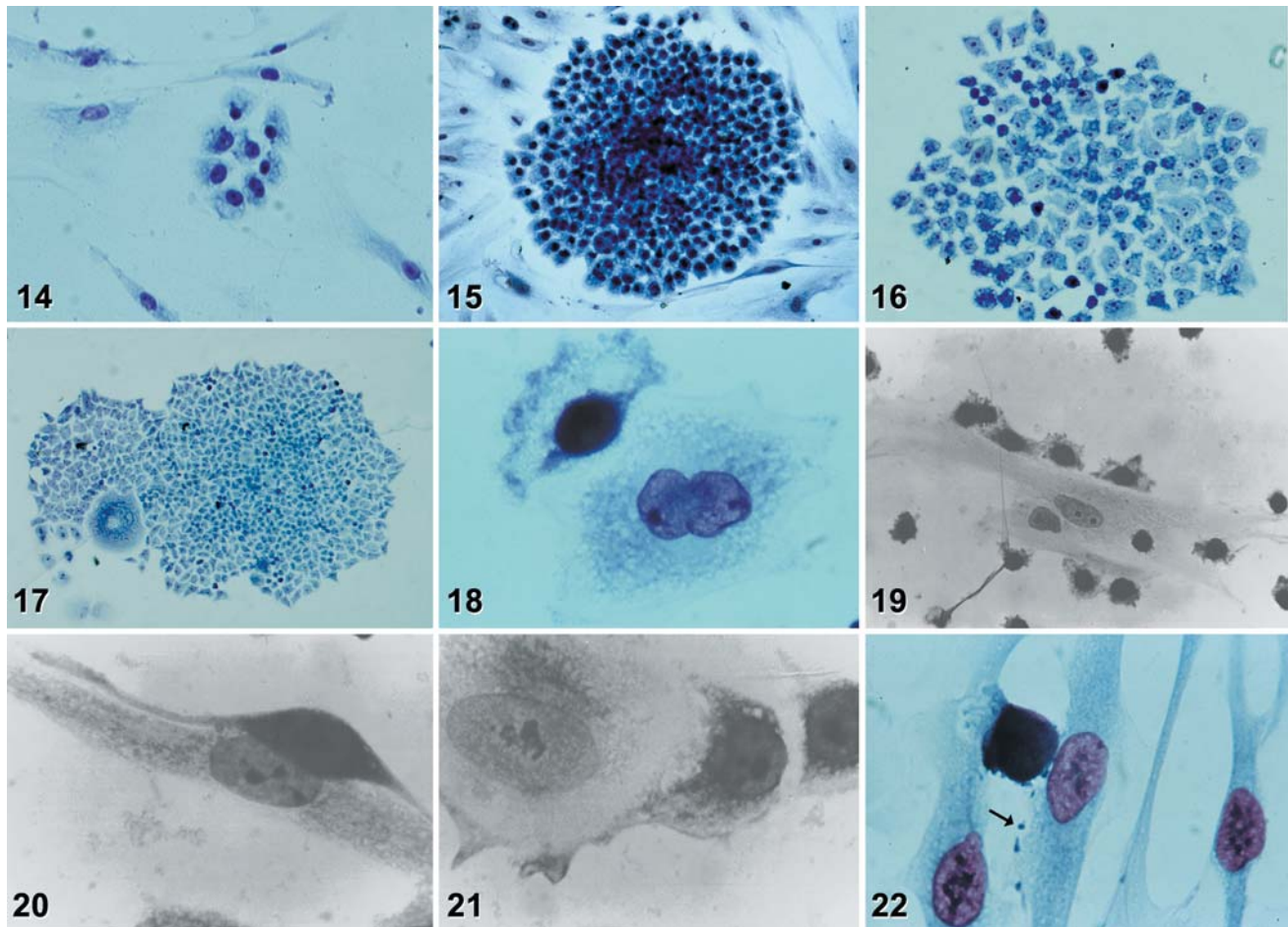
Figure 14. *Small HeLa-cell colony surrounded by fibroblasts, showing movement of nuclei toward the centre of the colony. An inoculum of 10^3 KR-BC cells in 1 mL medium was cast over a monolayer of sparse HeLa colonies with 4-10 cells and cultured for 24 h. Giemsa, ×140.* Figure 15. *Large HeLa-cell colony surrounded by fibroblasts, showing that all cells at the outer edges of the colonies move their nuclei towards the centre of the colony. An inoculum of 10^5 KR-BC cells in 1 mL medium was cast over a monolayer of large HeLa colonies and cultured for 24 h. Giemsa, ×100. The same phenomenon is also obvious in Figure 13.* Figure 16. *A control HeLa-cell colony. Methyl violet, ×100.* Figure 17. *A large control HeLa-cell colony, not showing nuclear movement of peripheral HeLa cells, with a giant multinucleated cell. Methyl violet, ×40.* Figure 18. *A HeLa cell (left) just attaching to a fibroblast. A dilute suspension of 10^3 HeLa cells in 1 mL medium was cast over a monolayer of dilute AK fibroblasts and cultured for 2 h. Note that the attaching HeLa cell locates its nucleus close to the fibroblast. Giemsa, ×1000.* Figure 19. *HeLa cells attaching to a AK fibroblast. Same conditions as shown in the legend to Figure 18. The affinity between the two types of cells is profound and the movement of the HeLa cell nuclei towards the fibroblasts is apparent after 4 h of co-culture. Giemsa, ×220.* Figure 20. *AK fibroblasts and HeLa co-cultures as shown in the legend to Figure 18. After 6 h of co-culture, the affinity between the two cells is obvious. ×1000.* Figure 21. *Same condition of AK fibroblasts and HeLa co-cultures as shown in the legend to Figure 18, but after 12 h of co-culture. ×1000.* Figure 22. *G-EP fibroblasts and HeLa cells co-cultured for 2 h, as in the legend to Figure 18. HeLa cells were pre-labeled with carmine particles which are apparent in the HeLa-cell cytoplasm as well as on the cytoplasmic projection of the HeLa cell at the edge of the fibroblast (arrow). Giemsa, ×600.*

previously shown (257), may develop, along with a specific response from the fibroblasts.

5. Overgrowth of fibroblasts around HeLa-cell colonies. From the fourth to the eighth day of co-culture, the encircling fibroblasts progressively outgrew and created very dense areas with frequently observed multilayers and crossing growth pattern, indicating uncontrolled growth with loss of contact inhibition, as well as loss of parallel pattern of cell growth (Figures 23, 24).

This phenomenon was evident for normal skin fibroblasts (G-EP), tonsillitis fibroblasts and all cancer-derived SF.

If at this stage the medium was not replaced for 4 days, a HeLa cell layer would appear outside the dense fibroblast layer (Figure 25) and if medium was then renewed, fibroblasts again started to form a second encircling dense bow, as previously described (186). This phenomenon shows (a) the importance of medium replacement since fibroblasts need nutrients for growth and for maintenance of their
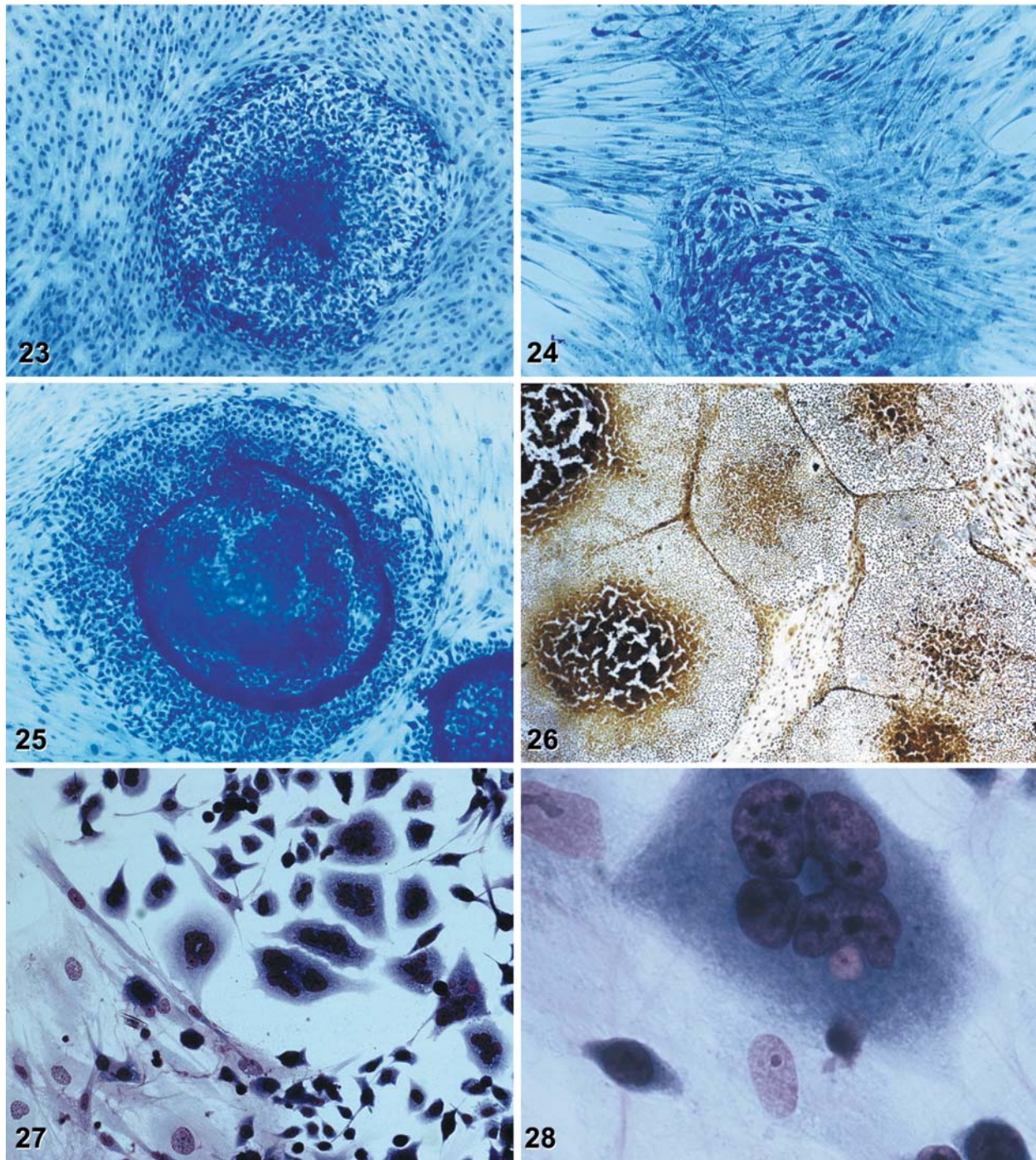
Figure 23. *Overgrowth of fibroblasts around HeLa-cell colonies. G-EP cells (10^5 in 1 mL medium) were cast over a monolayer of dilute HeLa-cell colonies (6-10 cells/colony) on a 1×1 cm coverslip and cultured for 7 days with medium replacement every 2 days. Giemsa, ×50.* Figure 24. *Under the same conditions as described in the legend to* Figure 23. *PL-BC fibroblasts were cast over HeLa-cell colonies. Overgrowth of the fibroblasts with criss-cross growth and multilayers can be seen around HeLa-cell colonies. Giemsa, ×60.* Figure 25. *Formation of a HeLa cell layer outside the dense G-EP fibroblast layer surrounding a HeLa-cell colony following four days without renewal of the medium. Co-culture conditions were similar to those given in the legend to Figure 23, with a total co-culture period of 11 days. Giemsa, ×50.* Figure 26. *Polygonal pattern of co-growth of KR-BC fibroblasts with HeLa on the 10th day of co-culture under same conditions as given in the legend to Figure 23. The same polygonal pattern was obtained with all cells used in this study. Silver staining, ×20. Figures of this polygonal pattern are also shown in Figures 31, 37 and 41.* Figure 27. *A focus of abundant multinucleated HeLa cells can be seen located at the boundary of a HeLa-cell colony surrounded by LA-BC fibroblasts, on the 15th day of co-culture. Giemsa, ×120.* Figure 28. *A multinucleated HeLa cell which also contains a small light-pink nucleus, possibly of fibroblastic origin. LA-BC fibroblasts and HeLa, 11 days, Giemsa, ×600.*

capacity to confine HeLa-cell colonies, and (b) the capacity of HeLa cells to pass the dense layer of fibroblasts. When the medium was regularly replaced every 2 days, this phenomenon was not observed.

The pattern of co-growth after approximately the seventh day of co-culture was characterized by the development of a polygonal morphology of HeLa-cell colonies, surrounded by densely growing fibroblasts (Figures 26, 41). The width of the fibroblast area around the HeLa-cell colonies seemed to depend on the initial cell-to-cell ratio and cell number, the degree of pushing back by the expanding HeLa-cell colonies and the strength of fibroblast proliferation.

6. Appearance of areas with abundant multinucleated HeLa cells. The frequency of multinucleated cells in HeLa cell monolayers was very low. Usually, 1-2 multinucleated cells were seen in a HeLa-cell colony with approximately 300 cells (Figure 17). However, in the co-cultures of HeLa and fibroblasts, areas with abundant multinucleated HeLa cells, always at the edges of the colonies, were frequently observed (Figure 27) (see also Figures 8, 9 in reference 186). Most of these cells contained nuclear abnormalities and micronuclei, indicating previous abnormal mitoses. Rarely, some of these multinucleated HeLa cells contained a nucleus of possibly fibroblast origin, as judged from the distinctly different colors of the nuclei of HeLa cells and those of fibroblasts when stained with Giemsa (Figure 28). Whether or not this was a result of cell fusion was not further investigated. Multinucleated HeLa cells soon became apoptotic with nuclear fragmentation and profound cytoplasmic membrane blebbing as described below (section 8; apoptosis) (Figures 63, 64).

7. Proliferation and metabolic activities in HeLa cell-fibroblast co-cultures. The following techniques and markers indicating proliferative and metabolic activities were applied in HeLa cells, fibroblasts and their co-cultures: Ki-67 proliferation antigen, NSE, E-cadherin, silver staining, DHFR and lipid staining.

7a. Ki-67. HeLa cells positively stained for Ki-67, with a high intensity in the nuclei, indicating cell proliferative capacity. HeLa-cell colonies staining for Ki-67 showed an increased activity in the foci of cells in central areas. The periphery of the HeLa colonies did not share the same increased activity, even in areas where two HeLa-cell colonies adjoined (Figure 29). Fibroblasts did not exhibit positive Ki-67 staining even in areas of dense growth. However, in co-culture with HeLa cells, fibroblasts induced intense Ki-67 staining in HeLa nuclei at the edges of the HeLa-cell colonies which were in contact with the dense encircling fibroblast areas (Figures 30, 31). The latter exhibited a pink cytoplasmic color (Figure 32), which became very intense after 10 days of co-culture, still without any staining of the nuclei (Figure 33).

7b. NSE live staining. HeLa cells expressed NSE activity, especially in the area surrounding the nucleus; however, when acetazolamide, an inhibitor of carbonic anhydrase (CA) was used, this activity almost disappeared. This shows that the activity in HeLa is attributed to CA and not to NSE. CA was amply expressed around the nucleus and in numerous cytoplasmic granules in both cell types (Figure 34), as shown utilizing the live cell staining technique described in the Materials and Methods section. The CA-granules in cytoplasmic projections of fibroblasts in contact with HeLa cells were markedly larger (Figure 35, live cell staining; Figure 36, paraformaldehyde-fixed cells). CA in co-culture was abundantly expressed, especially in the encircling fibroblast bows and the foci of HeLa cell piling up, with intense diffusion in paraformaldehyde-fixed preparations (Figure 36). However, when acetazolamide was used, the true NSE activity was revealed to be red only on the dense fibroblast areas surrounding the HeLa-cell colonies and the HeLa colony piling up in the centre seen with live-cell staining for $2^{1/2}$ h (Figures 37, 38). When staining was continued for 4 h, higher activity also appeared in HeLa cells (Figure 39). Staining of paraformaldehyde-fixed cells for NSE gave inferior results, with blurred appearance of the stained areas. Using live-cell NSE staining, NSE activity first appeared at 5 min on the nuclear membrane of the fibroblasts and then at 15 min in the cytoplasm as spherical stained droplets.

7c. Silver staining. Using silver staining, the cytoplasm of HeLa cells was stained light yellow and the nuclei dark brown with black nucleoli. Fibroblast nuclei were stained brown to dark brown with black nucleoli and the cytoplasm was stained brown (Figure 40). By monitoring the staining under the microscope, after the same duration of staining (about 30 s), HeLa cytoplasm was yellow, while that of fibroblasts was brown. Longer staining resulted in very dark uninterpretable staining.

From the fourth day of co-culture, progressively increasing argyrophilia (silver staining) (Figures 26, 41) and basophilia (Giemsa staining) (Figures 23, 24) were observed in the dense layer of fibroblasts surrounding the HeLa-cell colonies (Figure 41). Eventually, from the sixth day of co-culture, the fibroblasts developed an anarchic growth pattern, with overlapping cells and unusually long dark brown argyrophilic cytoplasmic projections (Figures 42, 43). These observations indicate that in the regions of dense growth, the fibroblasts underwent a further phenotypic change (after their dense growth), characterized by higher amounts of argyrophilic (basic) proteins and an intense tendency to elongate and overlap each other. Alongside this phenomenon, gaps started to appear in the periphery of the HeLa-cell colonies, clearly due to detachment of HeLa cells (Figure 43 a, b; described in Results Section 8).
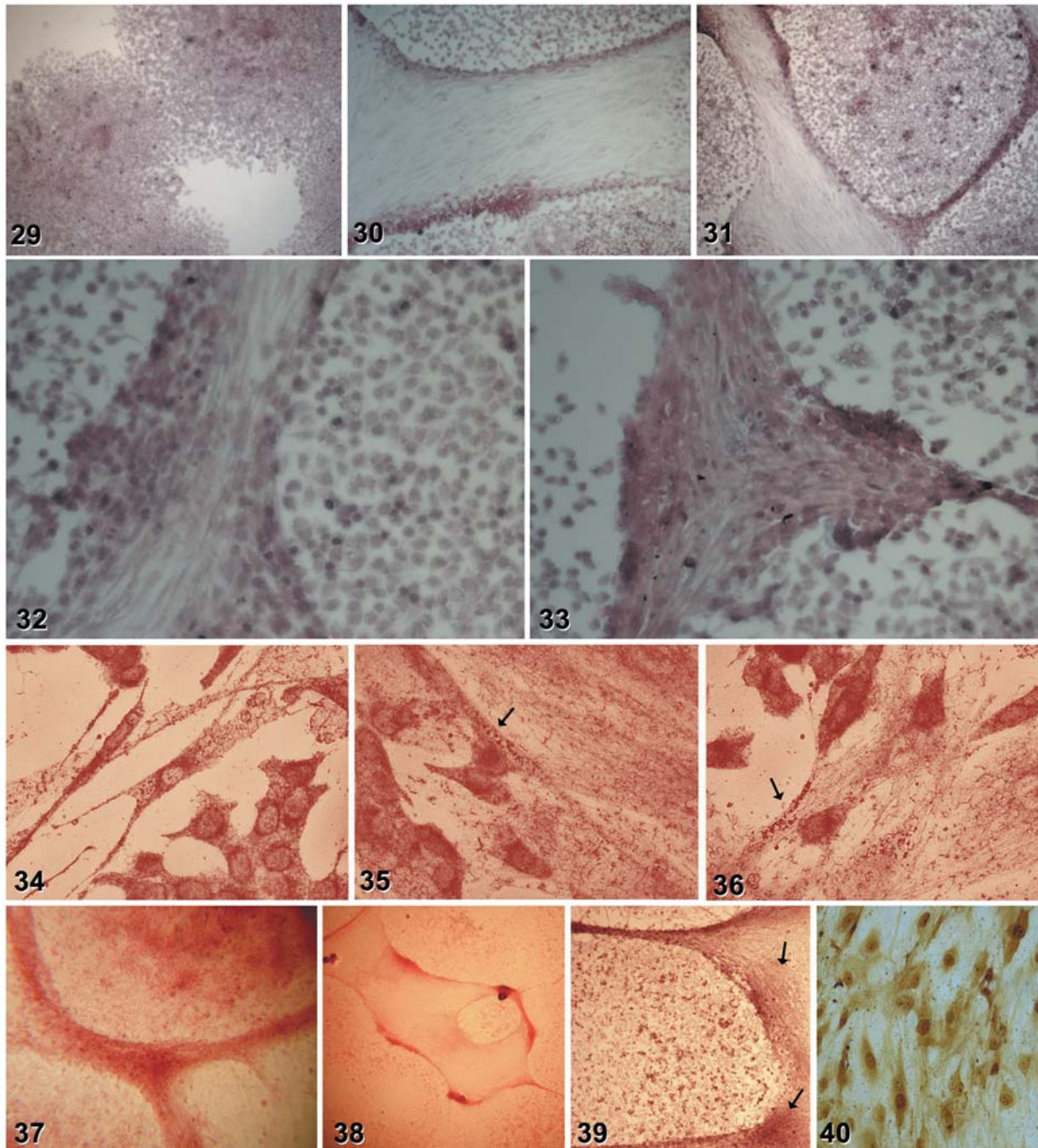
Figure 29. *HeLa-cell colony boundaries do not show increased staining of Ki-67 even in areas where two HeLa-cell colonies adjoin. ×40.* Figures 30, 31, 32, 33. *Ki-67 activity in co-cultures of HeLa cells with LA-BC cells after 6 days (Figure 30, ×40), 8 days (Figure 31, ×40, Figure 32, ×100) and 10 days (Figure 33, ×100). Intense pink cytoplasmic staining of the fibroblasts after 10 days can be seen.* Figure 34. *Carbonic anhydrase expression (CA) in numerous cytoplasmic granules in both fibroblasts and HeLa cells. KR-BC fibroblasts and HeLa cells co-cultured for 11 days. Live staining. ×252.* Figure 35. *Intense CA expression (arrow) in fibroblast cytoplasmic projections in contact with HeLa cells. KR-BC fibroblasts and HeLa co-cultures for 11 days. Live staining. ×252.* Figure 36. *CA reaction in KR-BC fibroblasts and HeLa co-cultures for 11 days. Esterase staining of paraformaldehyde-fixed coverslips. Intense CA staining in both HeLa and fibroblasts is obvious. The arrow indicates intense expression in fibroblastic cytoplasmic projections in contact with HeLa cells. ×252.* Figure 37. *Non-specific esterase (NSE) staining of LA-BC fibroblasts and HeLa cells, co-cultured for 13 days, using acetazolamide (CA inhibitor) and the live staining technique. NSE activity is located in the areas of fibroblasts surrounding the HeLa-cell colonies as well as in the piling up at the centre of the HeLa colonies. Staining time: 2 1/2 h. ×30.* Figure 38. *As in Figure 37, live NSE staining with acetazolamide. Staining time: 2 1/2 h. ×10.* Figure 39. *As in Figure 37, live NSE staining with acetazolamide. Staining time: 4 h. Intense NSE activity can be seen in the areas of dense fibroblastic growth (arrows). ×25.* Figure 40. *Silver staining of a monolayer of KR-BC cells. ×120.*
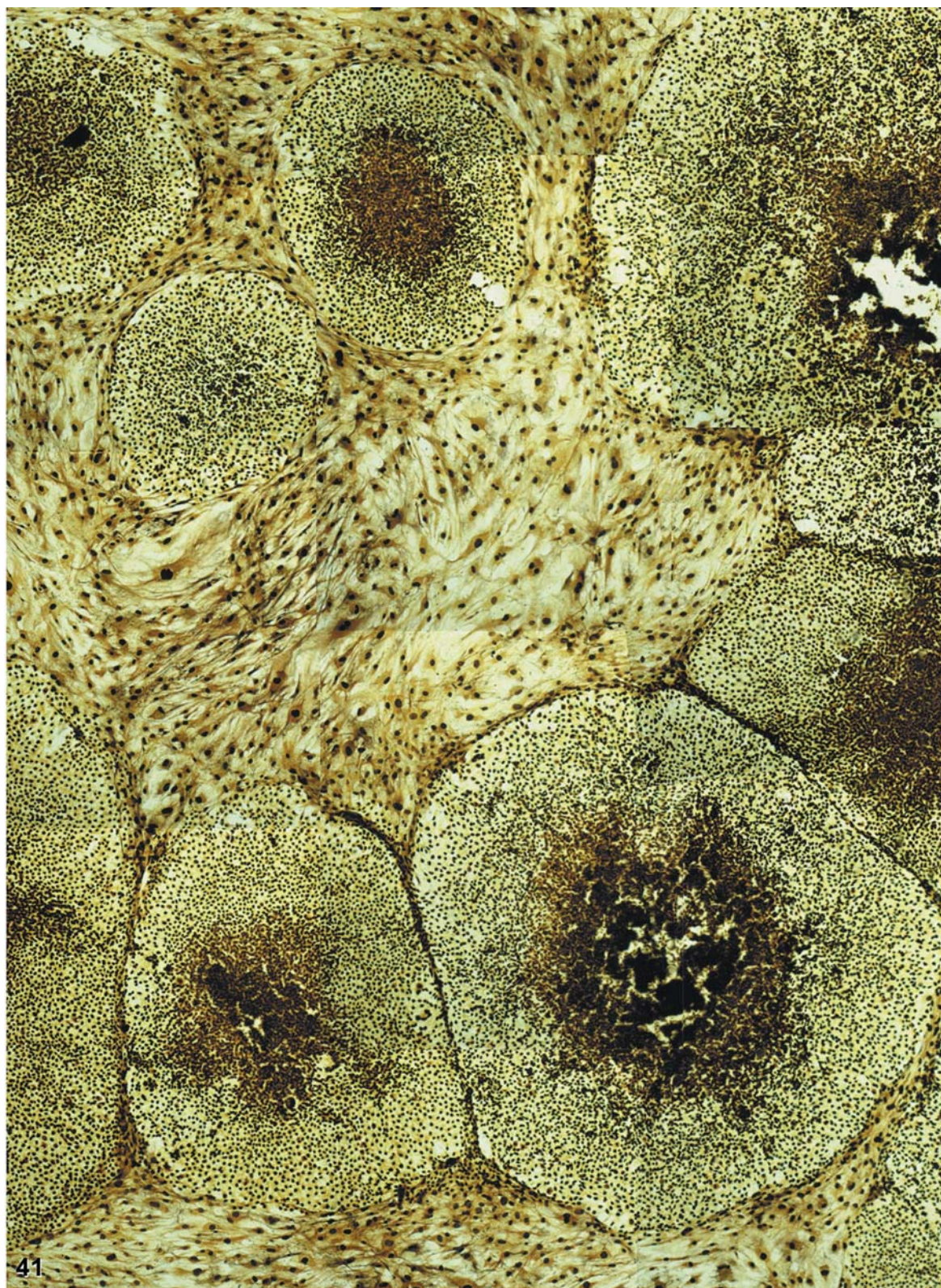
Figure 41. *Composite image of a KR-BC fibroblasts and HeLa cell co-culture. An inoculum of 10^5 KR-BC cells in 1 mL medium was cast over a monolayer of HeLa-cell colonies (with ~10-40 cells) and cultured for 10 days, with medium replacement every 2 days. A polygonal pattern of co-growth, dense growth of fibroblasts around HeLa cells and gaps along the boundaries of the HeLa colonies are clearly visible. Silver staining. ×50.*
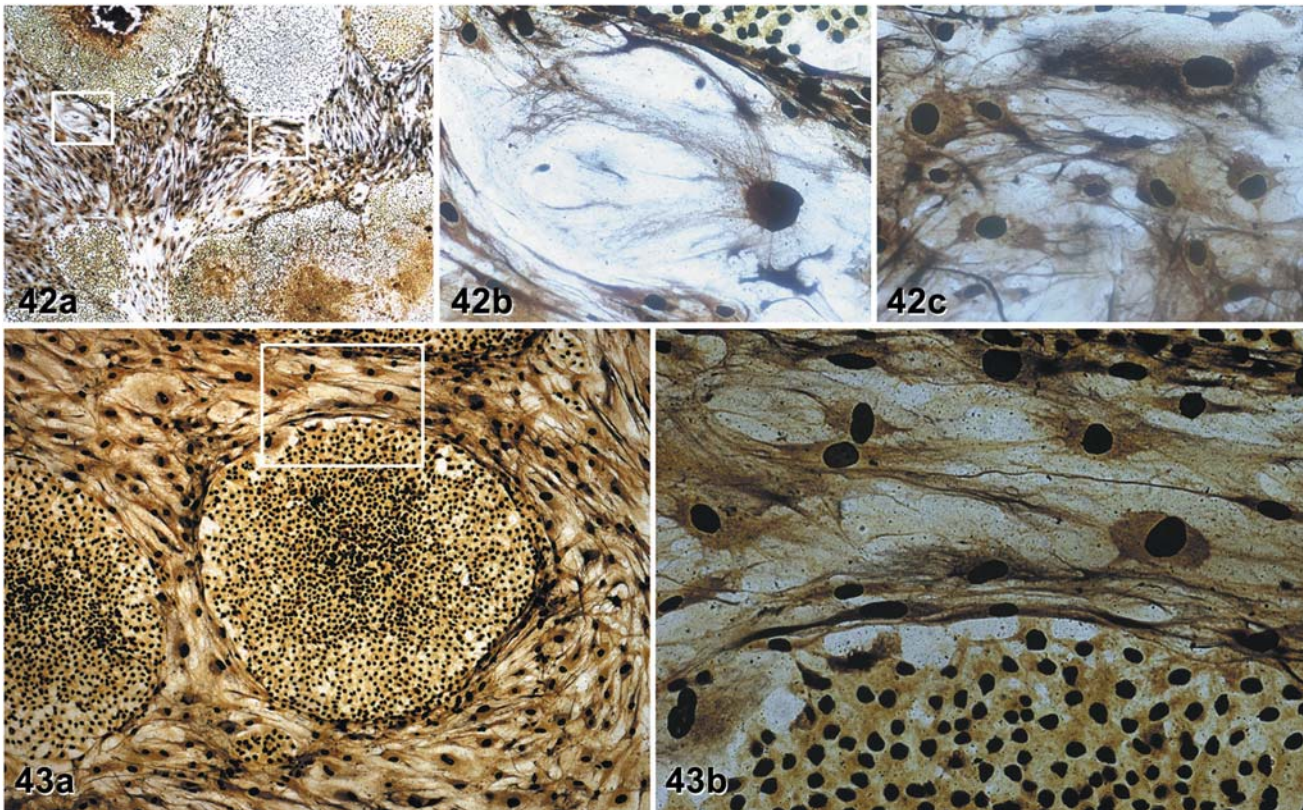
Figure 42. *a: Anarchic pattern of growth of KR-BC fibroblasts surrounding HeLa-cell colonies after 10 days of co-culture. b: High magnification (x200) of the left inset in part (a) (x20), showing a fibroblast with long argyrophilic projections. c: High magnification (x240) of the right inset in part (a), showing anarchic growth of fibroblasts with overlapping cells and intense argyrophilia. Silver staining.* Figure 43. *a: HeLa colonies surrounded by densely grown highly argyrophilic fibroblasts (×60), b: Detail of the inset indicated in part (a) showing the development of complex long cytoplasmic argyrophilic projections of fibroblasts and gaps along the boundaries of the HeLa-cell colony (×270). KR-BC fibroblasts and HeLa cells, 12 days of co-culture, Silver staining.*

7d. E-Cadherin. Neither HeLa cells nor fibroblasts expressed E-cadherin when cultured alone. Nevertheless, in co-culture for 11 or 14 days, E-cadherin appeared to be profoundly expressed at the peripheries of HeLa colonies surrounded by fibroblasts (Figure 44 a, b). Expression of E-cadherin was visible around parts of the HeLa cell nuclei, with some diffuse positivity in the cytoplasm, and rarely, in the nucleus (Figure 44c). In multinucleated HeLa cells, only a small part of the cytoplasm was E-cadherin-immunopositive, whereas the nuclei were negative.

7e. DHFR. Although the boundaries of HeLa-cell colonies when alone did not stain intensely for DHFR (Figure 45), when such colonies were surrounded by fibroblasts, a very intense DHFR reaction was observed occupying a wide zone of about 5-12 HeLa cells from the point of contact with the fibroblasts towards the centre of the colonies (Figures 46, 47, 48).

The appearance of a very remarkable increase of strongly luminous mitotic cells within this zone indicated growth stimulation. HeLa cell nuclei stained dark blue, nucleoli darker blue and cytoplasm light blue. The cytoplasm and nuclei of the fibroblasts stained very faintly for DHFR (Figure 49), while some staining of nucleoli was seen at high magnification. Rarely, some cells of fibroblastic morphology around the HeLa-cell colonies showed intense cytoplasmic and nuclear DHFR staining, which may indicate that these cells were starting to enter the mitotic phase.

7f. Live staining of lipid. From the sixth day of co-culture, a progressive increase in the production and secretion of high numbers of lipid droplets by the encircling dense layer of fibroblasts was noticed (Figures 50, 51). These droplets accumulated only over the fibroblasts surrounding the HeLa-cell colonies and could be easily dislodged by agitation or moderate medium flow over them. When using methanol- or paraformaldehyde-fixed cell monolayers, these droplets dissolved and were not seen in fixed cells. In fixed HeLa cells, lipid staining was located in granules around the nuclei (Figure 52), while in fibroblasts it was located in cytoplasmic granules (Figure 53).
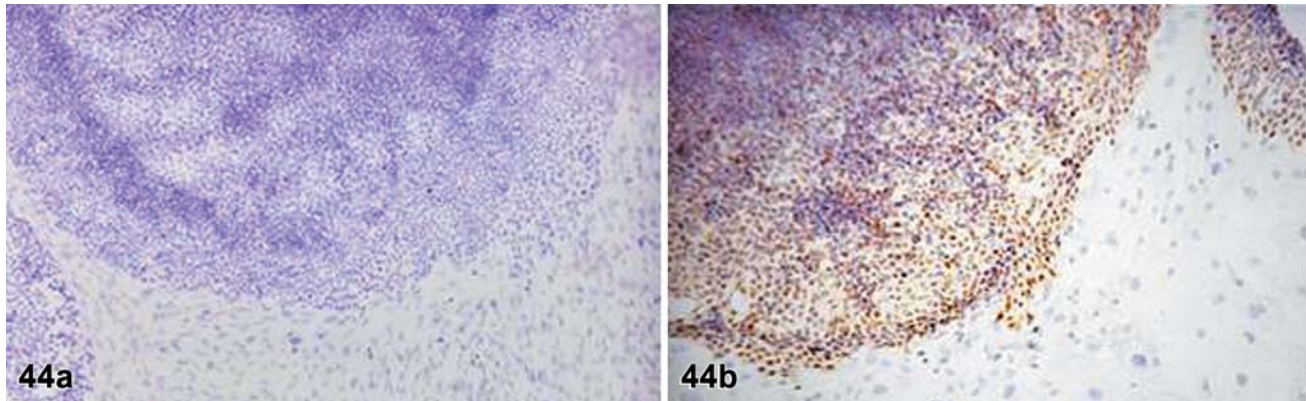
Figure 44. *HeLa colonies surrounded by KR-BC fibroblasts after co-culture for 11 days, stained for E-cadherin. a: negative control (x50). b: E-cadherin expression located at the HeLa colony periphery (x50).*

In co-culture, both cell types but even more so the fibroblasts, had higher numbers of lipid-stained cytoplasmic granules (Figure 54 a, b). In the dense layer of fibroblasts, lipid staining was diffuse (Figure 54 a, arrow) because of large lipid droplets that were loosely attached to the fibroblasts (Figure 51).

8. Apoptosis in HeLa-fibroblast co-cultures. Four distinctive phenomena were observed with increasing frequency after nine to ten days of co-culture close to the boundaries of HeLa-cell colonies surrounded by densely growing fibroblasts:

(a) detachment from the glass surface of groups of HeLa cells (Figures 43 a, b, 55, 56, 57, 58); (b) dilution of the HeLa cell density due to detachment after cell death and cytoplasmic shrinkage (Figures 32, 33, 41, 43, 57, 58); (c) areas of very dense growth of fibroblasts (close to HeLa-cell colony edges), with long, highly argyrophilic cytoplasmic projections and cell overlapping (Figures 42, 43, 55, 56); (d) regrowth of HeLa cells in areas of detachment but also within fibroblastic territories (Figures 57, 59, 60).

Small cytoplasmic fragments, mostly containing a darkly-stained small nucleus, were frequently observed from day 10 and were abundant after day 15 in areas of reduced HeLa cell density where, however, the fibroblasts did not show signs of distortion (Figures 61 a, b, 62 a, b, c; also Figure 56 upper part).

From the tenth day of co-culture, a continuously increasing occurrence of apoptotic features in HeLa cells was observed including nuclear fragmentation, cell shrinkage and long, thin projections (condensed basophilic cytoplasm), chromatin condensation, micronuclei, nuclear abnormalities, membrane-bound apoptotic bodies (small cells), and cellular membrane blebbing (Figures 62, 63, 64, 65). Cell fragments with similar features (fragmented nuclei, dark condensed cytoplasm and blebbing) were also observed during the first and second passages of fibroblasts isolated from human breast tumours (Figures 3 a, b, 4), most probably originating from apoptotic cancer cells.

Nuclear abnormalities were rarely observed in fibroblasts. These included nuclear fragmentation, nuclear gaps, nuclear membrane indentations and multinucleated cells (Figure 66 a, b, c, d, e).

Cytotoxic effects on HeLa cells were exhibited by all the finite fibroblastic cell lines used (Table I) and were not differentiated by fibroblast origin.

After 17 days, control HeLa-cell colonies covered the whole coverslip, piling up very extensively, and started to detach in batches, mainly at the colony centre, and did not have multinucleated cells, apoptotic features, regrowth or sparse growth. The pattern of HeLa cell detachment at the piled-up centre of HeLa colonies (*e.g.* Figure 26) was completely different from the detachment of HeLa cell groups from the HeLa-colony boundaries during their interaction with fibroblasts (Figure 41).

9. Blockade of fibroblasts at G_2-M phase after 17 days of HeLa cell–fibroblast co-culture. After 17 days of co-culture of HeLa cells (colonies) with fibroblasts, extensive areas of the co-culture contained a high proportion of fibroblasts blocked in early to late (G_2-M) prophase (Figure 67 a, b) as shown by fading and disappearance of the nucleolus, disappearance of the nuclear membrane and by chromatin condensation into chromosomal regions (Figure 68 a-e). G_2-M nuclei were usually ovoid or exceptionally elongated (Figure 69 a-d), usually with a constriction (Figure 69 arrows). These observations show that the effects of HeLa cell–fibroblast interactions extend to alterations of the chromatin organization in fibroblasts.
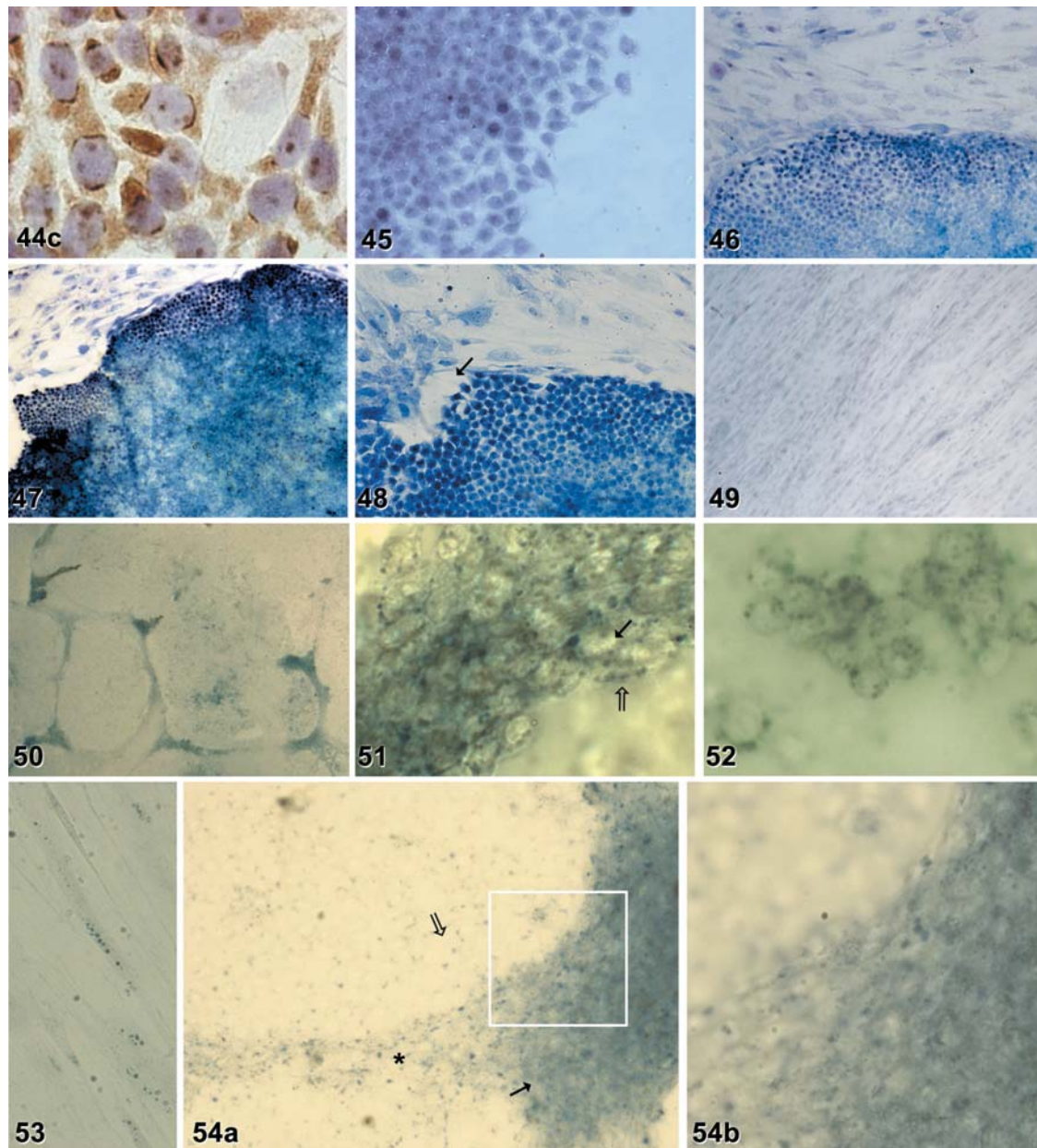
Figure 44c. *E-Cadherin expression in HeLa cells in co-culture with KR-BC cells for 11 days. Conditions were as described in the legend to Figure 23. ×500.* Figure 45. *Part of a control HeLa-cell colony stained for DHFR. Cells which have just divided are brightly stained (centre). ×100.* Figure 46. *HeLa-cell colonies surrounded by fibroblasts exhibit very intense DHFR staining perimetrically and in a wide zone of boundary HeLa cells. LA-BC fibroblasts and HeLa cells co-cultured for 9 days. Conditions were as described in the legend to Figure 23. ×60.* Figure 47. *b: Same as shown in legend to Figure 46. DHFR-staining with light 1-min Giemsa counterstaining. ×50.* Figure 48. *Gaps (arrow) along the boundaries of the HeLa-cell colonies in areas of anarchic fibroblast growth. LA-BC fibroblasts and HeLa cells cultured for 9 days. Conditions were as described in Figure 23. DHFR staining with light 1-min Giemsa counterstaining. ×80.* Figure 49. *LA-BC fibroblast monolayer stained for DHFR. ×30.* Figure 50. *Accumulation of lipid droplets over dense layers of fibroblasts surrounding HeLa-cell colonies. KR-BC fibroblasts and HeLa cells were co-cultured for 10 days. KR-BC cells (10^5 in 1 mL medium) were cast over a monolayer of sparse HeLa-cell colonies (6-10 cells/colony) on a 1×1 cm coverslip and co-cultured for 10 days with medium replacement every 2 days. Sudan Black staining. ×10.* Figure 51. *High magnification (from Figure 50) of the lipid droplets accumulated over the surrounding fibroblasts. By adjusting the focus, large homogeneously light-staining droplets were seen located over the fibroblasts (arrow). Fibroblasts carried small darkly-stained lipid cytoplasmic granules, which can also be seen here (double arrow). ×300.* Figure 52. *Lipid staining in HeLa cells was located in granules around the nuclei. Sudan Black staining. ×300.* Figure 53. *Lipid staining of cytoplasmic granules in KR-BC fibroblasts. Sudan Black staining, ×50. Similar lipid staining was obtained for all SF cell lines used.* Figure 54. *Lipid staining in KR-BC fibroblasts and HeLa cells co-cultured for 10 days. In the dense fibroblastic areas (arrow in part a), the large lipid droplets caused diffused staining (see part b), while in less dense areas of fibroblasts (asterisk) as well as in areas colonized by HeLa cells (double arrow), cytoplasmic lipid granules are obvious. Sudan Black staining. a: ×30; b: box from Figure 54a, ×80.*
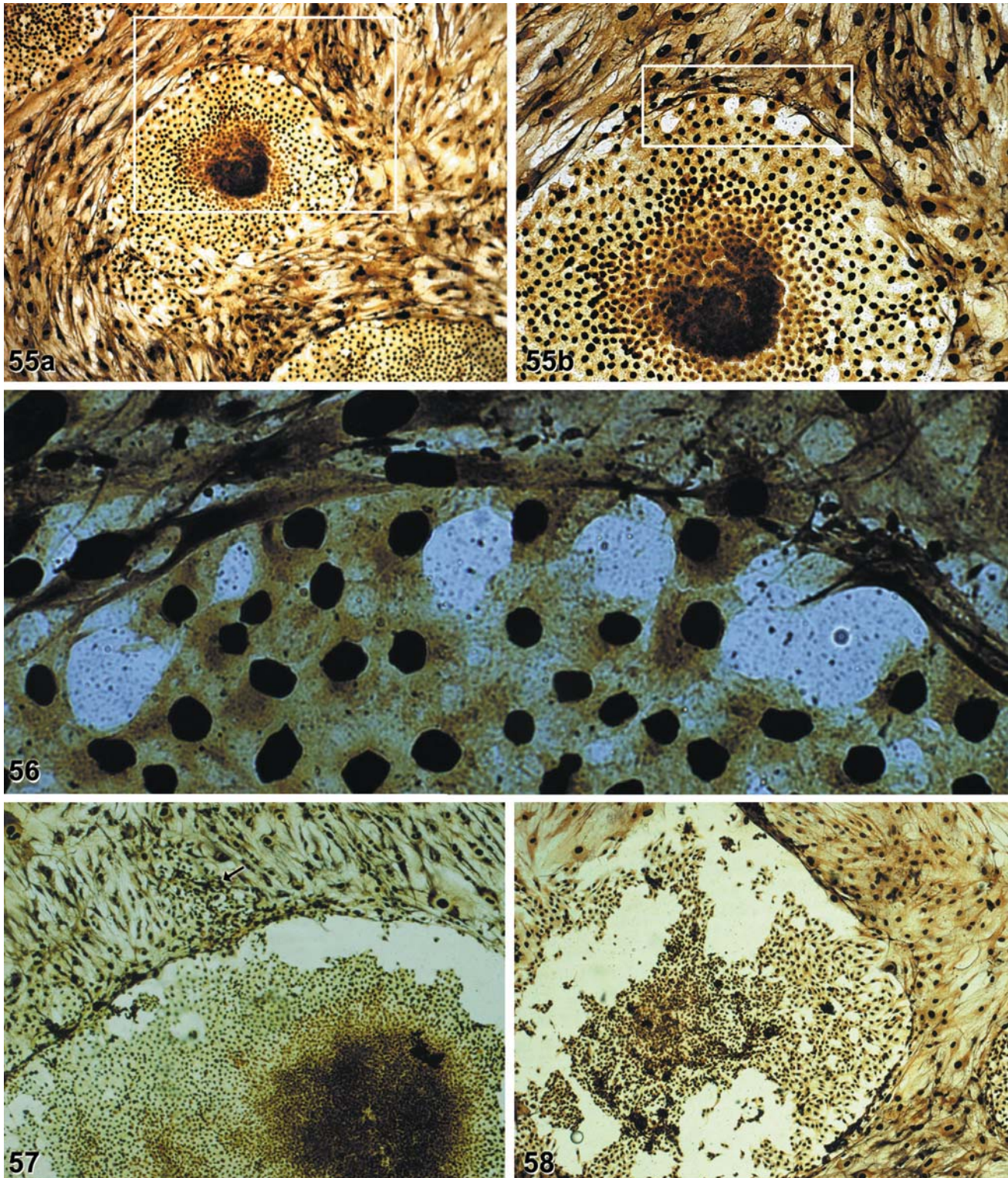
Figure 55. *Detachment from the glass surface of groups of HeLa cells from the peripheries of HeLa-cell colonies surrounded by densely growing highly argyrophilic fibroblasts. KR-BC and HeLa cells cultured for 10 days. Conditions were as described in the legend to Figure 50. Silver staining a: ×60; b: ×120. Figure 56. Higher magnification of the inset in Figure 54 b. ×600. Figure 57. As in Figure 55, after 12 days of co-culture. Larger empty areas of detached HeLa cells but also resurgence and growth of HeLa cells outside the dense surrounding fibroblast layer can be seen (arrow). Silver staining, ×30. Figure 58. As in Figure 55, after 12 days of co-culture showing extensive HeLa cell detachment. Silver staining, ×30.*
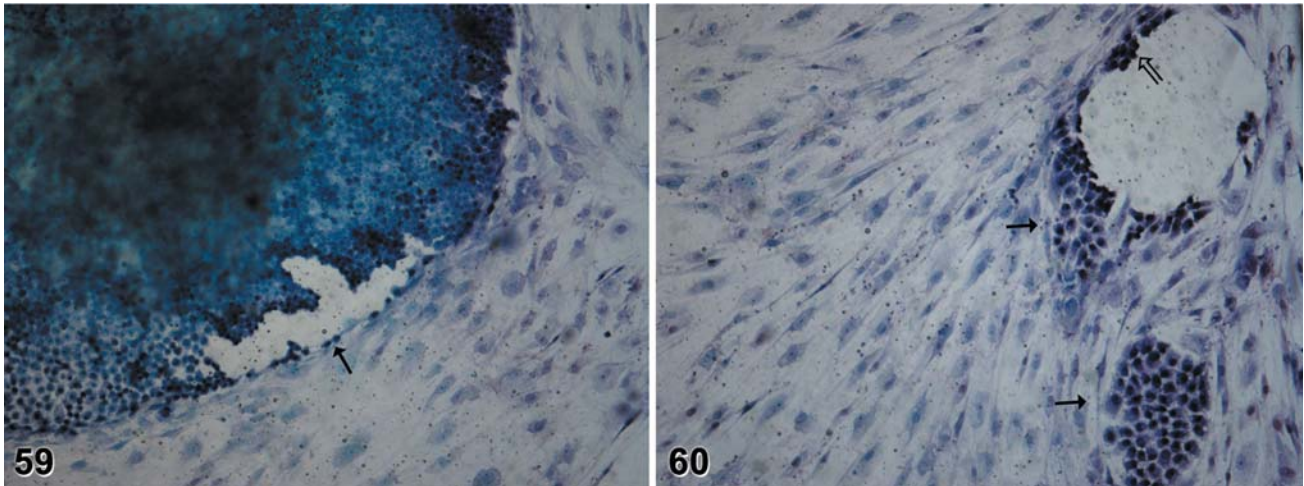
Figure 59. *As in Figure 55, after 10 days of co-culture, showing area of HeLa cell detachment, with some HeLa cells attached on the inner side of the surrounding stromal fibroblasts (arrow). DHFR staining with 1-min Giemsa counterstaining. ×50.* Figure 60. *As in Figure 55, after 12 days of co-culture, showing complete detachment of a small HeLa-cell colony, resurgence of two new HeLa colonies (single arrows) and regrowth of some HeLa cells on the side of the surrounding fibroblasts (double arrow). KR-BC fibroblasts and HeLa, DHFR staining with 1-min Giemsa counterstaining. ×140.*

10. Transfer of small molecules between HeLa cells and fibroblasts in co-culture. Transfer of ^{3}H-TdR from pre-labeled HeLa cells or fibroblasts to non-labeled fibroblasts or HeLa cells, respectively, was never observed in any cell ratio. Autoradiography of co-culture coverslips after 3 h to 20 days revealed that ^{3}H-labeled grains remained only on the nuclei of the pre-labeled cells (Figure 70 a, b). None of the fibroblastic lines used differed in their pattern of interaction.

Transfer of ^{3}H-UdR from fibroblasts to co-adhered HeLa cells, and through them also to HeLa cells that were not co-adhered to fibroblasts, occurred rapidly, at as early as 1 h or less of co-culture, until the density of ^{3}H-labeled grains in the HeLa cells reached almost the same as that in the fibroblasts (after 10 h) (Figure 71 a, b).

However, transfer of ^{3}H-UdR from HeLa cells to co-adhered fibroblasts occurred at a lower rate and the number of ^{3}H-labeled grains over the fibroblasts reached only 1/10 that over HeLa cells after 10 h (Figure 72).

Similar autoradiography results were obtained with G-EP, SE-L, PG-BC and TONS-F cells.

11. The HeLa–fibroblast co-culture battlefield after 17 days. After 17 and up to 20 days (Figures 73-75), the co-cultures presented the following features: (a) extensive areas of very dense growth of fibroblasts with a complex network of long highly argyrophilic projections and foci of overlapping and piling up cells. This feature started to appear from the ninth day of co-culture (Figures 43, 55, 56, 75); (b) areas with both fibroblasts and HeLa cells with a sparse pattern of growth including small colonies of HeLa cells, several abnormal or apoptotic HeLa cells, and rarely, fibroblasts with nuclear abnormalities (Figures 62, 66, 73, 74); (c) areas with a high percentage of fibroblasts blocked at the G_2-M phase of the cell cycle (Figure 67); (d) foci with unusually high numbers of multinucleated HeLa cells with apoptotic features (Figures 63, 64, 65); (e) areas with extensive disintegration of HeLa cells (Figures 62, 75); (f) areas of destroyed HeLa-cell colonies showing extensive detachment of HeLa cells, but also their regrowth in the same areas attached to surrounding fibroblasts (Figures 57, 58, 59, 60) or forming dense colonies (Figure 60).

These observations show that under biomechanical forces, communication and interactions, fibroblasts and HeLa cells influence each other's growth pattern and survival. These developments do not occur in clearly separated steps, since foci of all the above features co-existed after 17-20 days of co-culture, with some of them appearing as early as nine days after the start of co-culture.

The duration of time that the two cell types are in contact seems to topologically influence the emanation and sequence of specific events, which also depends on frequent replacement of medium.

Subculture of the co-cultures after the 17- to 20-day period produced either a mixture of the two cell types or a HeLa cell or fibroblast monolayer with contamination with the other cell type, or, rarely, only fibroblast cells. Some of the fibroblastic cell lines used (G-EP, AK, PL-BC) were able to completely destroy HeLa cells when the initial ratio of HeLa cells:fibroblasts was very low (<1:100); however, this phenomenon was not apparent for all the finite fibroblastic cell lines used. No association of this phenomenon with a specific origin of fibroblasts was
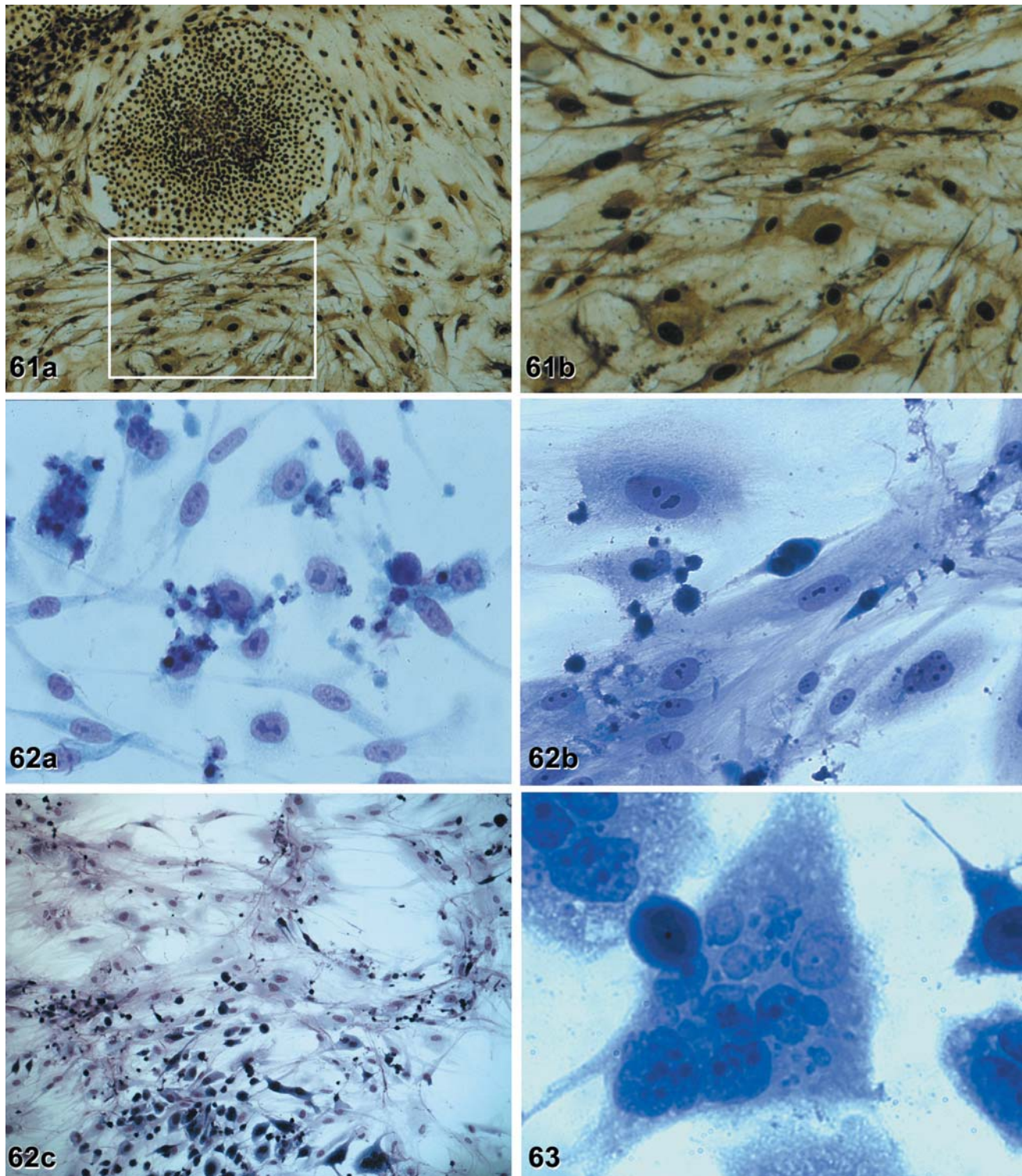
Figure 61. *As in Figure 55, after 10 days of co-culture showing detachment of HeLa cell groups (a), (b): magnification of the inset in Figure 61a showing a large area with many mini-cell fragments originating from HeLa fragmentation. These mini-cell fragments became more abundant after the 15th day of co-culture. KR-BC fibroblasts and HeLa, Silver staining, a: ×60; b: ×180. Figure 62. As in Figure 61 b, after 15 days of co-culture showing abundance of mini-cell fragments, intact fibroblasts and some reviving HeLa cells. Giemsa. a: G-EP fibroblasts and HeLa , ×320, b: ×320, c: KR-BC fibroblasts and HeLa, ×50. Figure 63. Apoptotic features of multinucleated HeLa cells after 15 days of co-culture with LA-BC cells. Conditions were as described in Figure 50. Giemsa, ×800.*
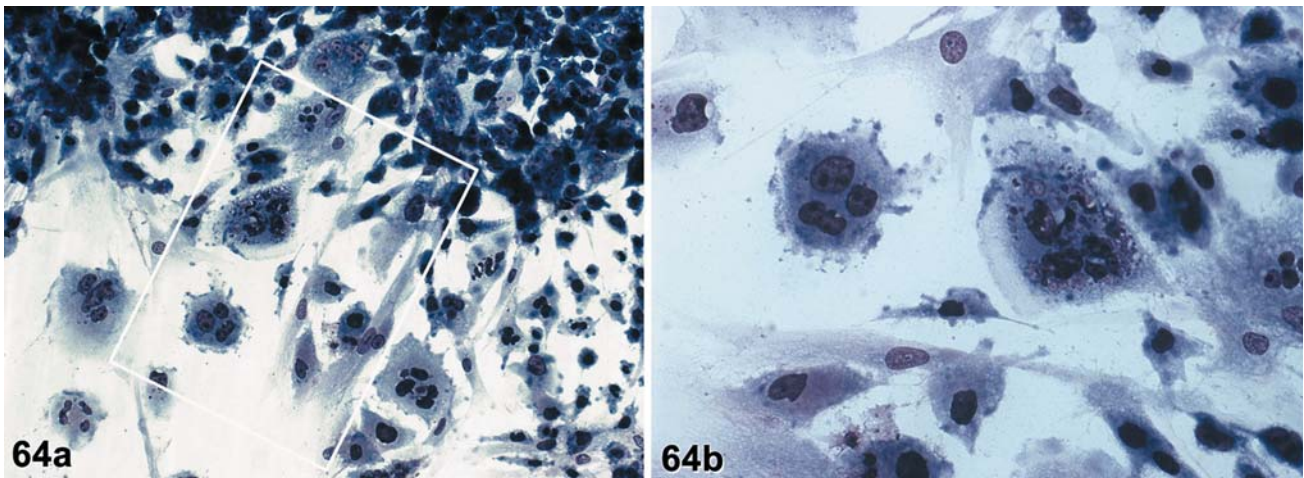
Figure 64. *Apoptotic features of HeLa cells after 15 days of co-culture with LA-BC fibroblasts. Nuclear fragmentation and intense cytoplasmic blebbing are apparent. Giemsa. a: ×120; b: higher magnification of the inset in a; ×252.*

established. It should be noted, however, that all fibroblasts used were capable of inducing apoptosis and detachment of HeLa cells. Moreover, all of them produced lipid droplets when they were densely growing around HeLa-cell colonies.

Discussion

The interactions between malignant epithelial cells and the tumour microenvironment, including stromal fibroblasts, for solid tumours involve growth interdependence and abnormal growth control mechanisms, that advance tumourigenesis to invasion and metastasis (270, 271). It is generally held that carcinogenesis is the result of a series of initial genetic alterations in a target cell, which then causes modulations of the developing nearby tissue environment, thus facilitating the un-interruptive growth of a tumour (1, 3). Therefore, the existence of a tumour presupposes that abnormal cancer cells have surpassed the immune system's homeostatic mechanisms and have established a modified cancer-supportive microenvironment. Obviously, if the initial effort of a small colony of cancer cells fails to overcome the immune surveillance and to create an efficiently supportive microenvironment, the tumour will not develop (251). Therefore, there is a critical point at which a tumour will start developing, or succumb under the restraint of its microenvironment (6-8, 11, 46, 186, 251). The role of fibroblasts at this point is unclear since most relevant studies examined fibroblasts after the establishment of a fully-grown tumour. Such studies have shown that CAFs may be supportive or suppressive of tumour growth and progression to invasion and metastasis, as described in the Introduction.

However, the initial stages before the crucial point of overcoming the immune surveillance mentioned above have not been investigated. The present study constitutes a detailed description of the early interactions between proliferating fibroblastic cells of different origins with HeLa cells, based on a previously developed *in vitro* model (6, 9, 119, 186).

Stromal fibroblasts comprise a highly heterogeneic entity within the tumour microenvironment. Our previous studies showed that SFs isolated from human breast tumours may exhibit an intense tendency *in vitro* for overlapping growth, with loss of parallel cell growth and loss of contact inhibition (134, 142) (Figure 2). This property is also seen in normal skin fibroblasts, but only if they are co-cultured under certain conditions with HeLa cells (*e.g.* Figure 23). The present experiments showed that both normal skin- and tumour-derived fibroblasts change their characteristic properties stepwise during their cell–cell interactions with HeLa cells. An adaptable character for fibroblasts, compatible with the meaning of cell plasticity (92, 157, 272, 273), entailing reprogramming of their gene expression, protein synthesis and function, is thus also revealed in the present cell-interaction model.

Differences between normal fibroblasts and CAFs regarding proliferation, contact inhibition of growth, gene expression and phenotypic profiles have been reported in many studies (9, 43, 53, 135, 168, 274, 275). Stromal fibroblasts ultimately acquire versatile properties that may be supportive, inhibitory or definitively destructive for cancer cells and their metastatic capacity (6-9, 46, 65, 80, 82, 92, 170, 186, 206, 213, 227, 251, 273, 276). Studies of *in vitro* interactions between isolated fibroblasts and cancer cells may therefore provide information on and explanations for their
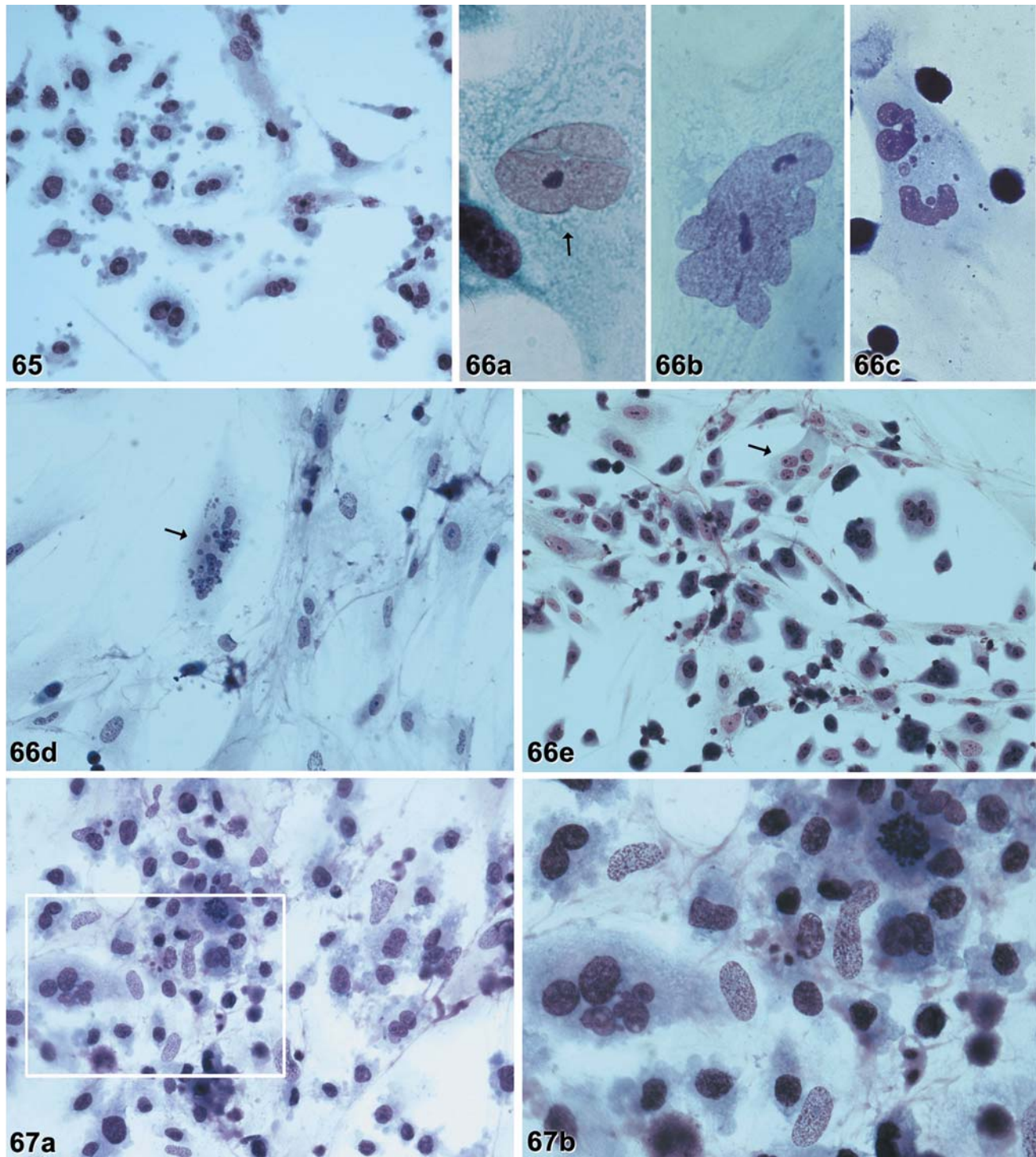
Figure 65. *Apoptotic HeLa cells after 18 days of co-culture with LA-BC fibroblasts. Cytoplasmic blebbing and bi-nucleate cells are visible. Conditions were as described in Figure 50. Giemsa, ×140. Figure 66. Nuclear abnormalities of various fibroblasts co-cultured with HeLa cells revealed by Giemsa staining. a: A nuclear constriction (arrow) frequently observed in all the stromal cell lines used. KA-BC fibroblasts and HeLa cells, ×1000. b: Abnormal nuclear shape of a KA-BC cell after 6 days of co-culture with HeLa cells, ×1000. c: Fragmented nucleus of a TONS-F cell observed during the attachment of a HeLa cell, ×500. d: Nuclear fragmentation of an LA-BC cell (arrow) observed after 15 days of co-culture with HeLa cells, ×100. e: Multinucleated TONS-F cell (arrow) after 15 days of co-culture with HeLa cells. ×120. Figure 67. a: Blockade of KR-BC fibroblasts in early-to-late prophase (G_2-M) after 18 days of co-culture with HeLa cells. Giemsa, ×120. b: Higher magnification of the inset in Figure 67a: top right: HeLa metaphase with chromosome non-disjunction; centre: two small HeLa cell fragments, left: multinucleated HeLa cell with abnormal nuclei; Four G_2-M fibroblast nuclei. ×240.*
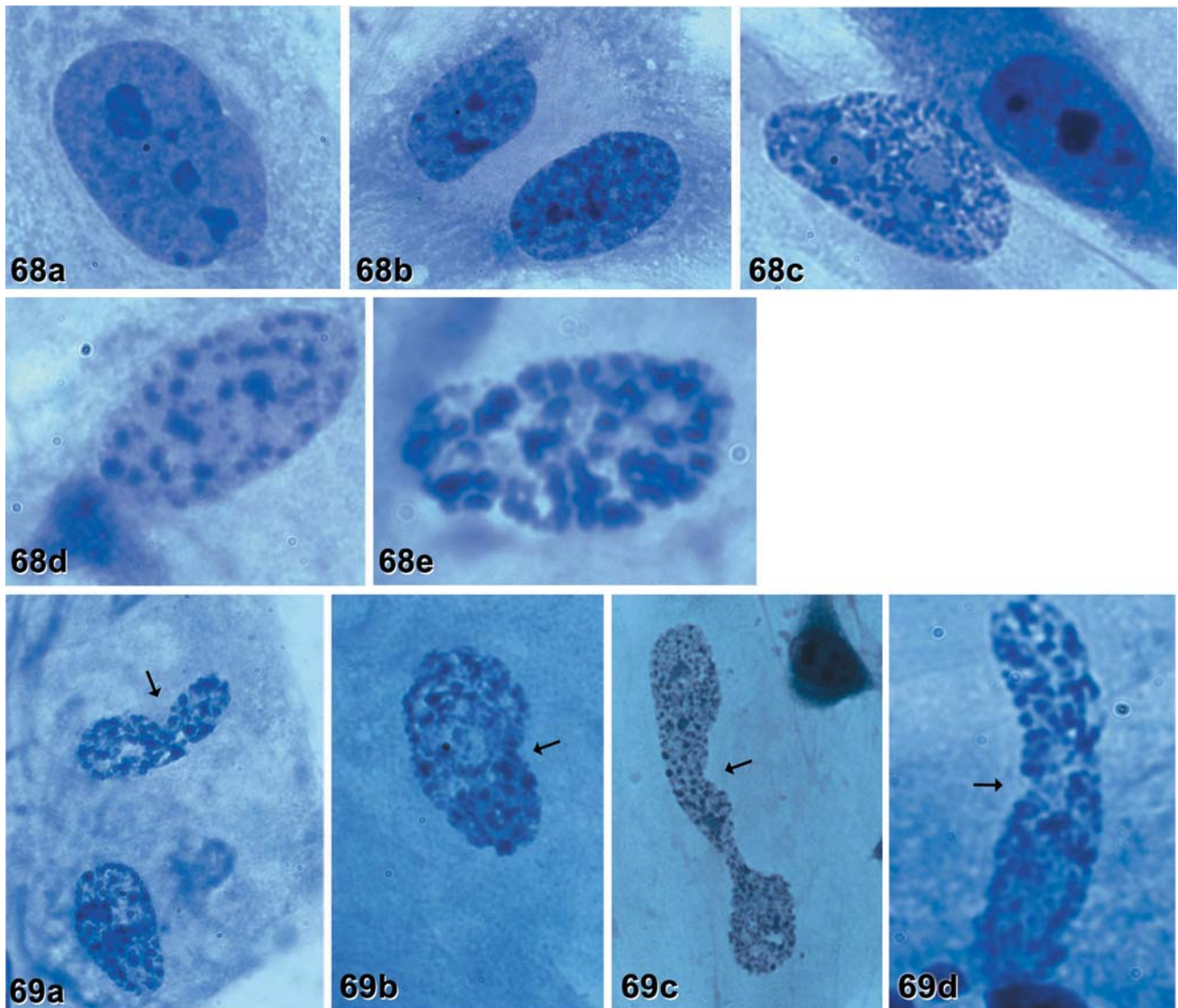
Figure 68. *Early-to-late (G_2-M) prophase nuclei of fibroblasts (KR-BC) after 18 days of co-culture with HeLa cells. Giemsa. a: Nucleus at early S-G_2 phase ×2000; b: early prophase nucleus with obvious nucleoli ×1000; c: early prophase nucleus with nucleoli starting to fade ×2000; d: disappearance of nucleoli ×2000; e: disappearance of nuclear membrane and clear appearance of nuclear chromosomal territories ×2000.* Figure 69. *G_2-M nuclei were ovoid (a, b) or exceptionally elongated, usually with a characteristic constriction (arrow) Giemsa, a, c: KR-BC fibroblasts and HeLa cells; b, d: LA-BC fibroblasts and HeLa cells. a: ×500, b: ×1000; c: ×500; d: ×1500.*

behaviour and function in the complex *in vivo* situation. The present results show that fibroblasts from various origins share common properties during the different stages of their interactions with cancer cells.

Confluent fibroblasts, which cease to divide, are supportive of cancer cell growth and are characterized as feeder layers, usually pre-irradiated (277). Fibroblasts also cease to divide if nutrient medium is not regularly renewed. Thus, the aim of studying the *in vitro* interactions between cancer cells and proliferating fibroblasts was justified and furthermore

supported by the fact that desmoplasia, as a characteristic feature of most tumours, actually consists of early-initiated fibroblast growth within the tumour microenvironment (9, 46, 51-55, 64, 79, 162, 278).

From the initial stages of their *in vitro* interactions, the intense affinity between cancer cells and fibroblasts clearly creates a topologically heterogeneous environment on the glass culture surface, giving rise to the question whether fibroblasts in contact with cancer cells acquire properties different from those of fibroblasts growing or wandering far
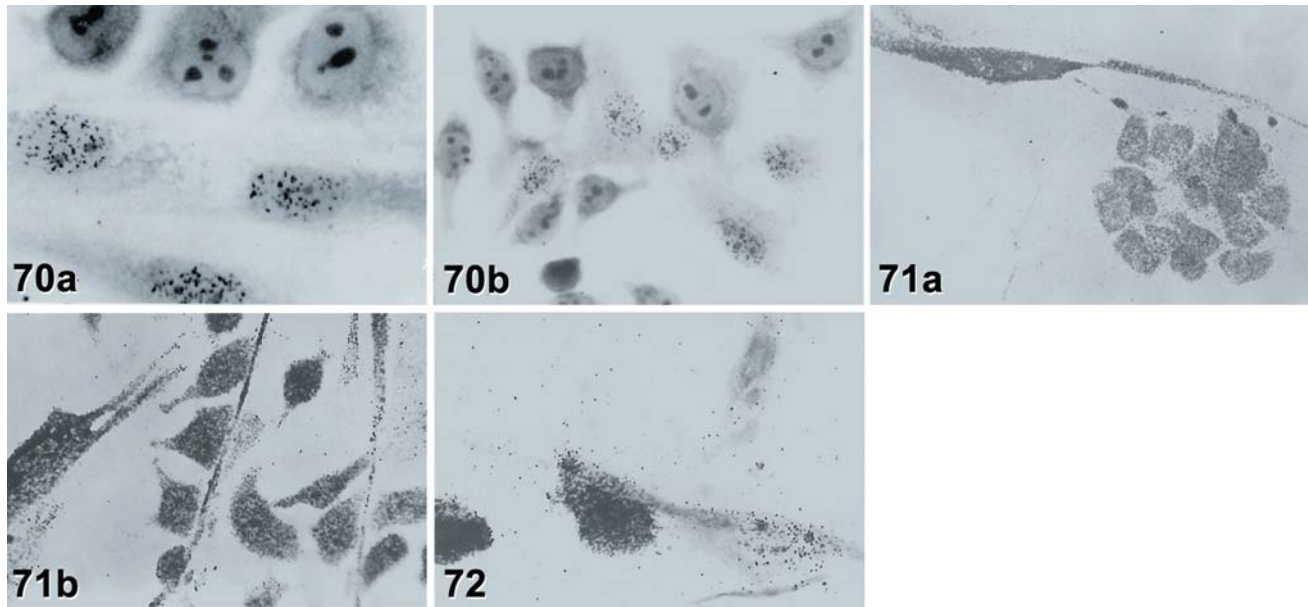
Figure 70. *Autoradiography of a co-culture of HeLa cells with ³H-TdR-pre-labeled G-EP fibroblasts. ³H-TdR-G-EP (10³ cells in 1 mL medium) were cast over a monolayer of small (6-40 cells) HeLa-cell colonies and cultured for 15 h (a). There is no transfer of grains from the fibroblasts to HeLa cells. Identical results were also obtained if 10³ HeLa cells were mixed with ³H-TdR-labeled fibroblasts before plating and cultured for 15 h (b) or 15 days. Same results were obtained with AK, PG-M, SE-L, TONS-F and PL-BC cells. a: x500, b: ×240. Figure 71. Autoradiography of a co-culture of HeLa cells with ³H-UdR-pre-labeled G-EP fibroblasts. ³H-UdR-G-EP (10³ cells in 1 mL medium) were cast over a monolayer of small (6-40 cells) HeLa-cell colonies and cultured for 10 h (a). Extensive transfer of grains from a heavily labeled fibroblast to all the cells of the HeLa-cell colony can be seen. ×200. The same phenomenon was observed when HeLa cells were cast over a monolayer of ³H-UdR-pre-labeled G-EP fibroblasts and cultured for 10 days (b: ×200). Figure 72. Autoradiography of a co-culture of ³H-UdR-pre-labeled HeLa cells with SE-L fibroblasts. HeLa cells (10³ cells in 1 mL medium) were cast over a non-confluent monolayer of SE-L fibroblasts and cultured for 10 h. A HeLa cell has attached to the side of a fibroblast and grains have already been transferred to the fibroblast, with a higher accumulation in the nucleoli. ×200.*

away. There is evidence that stromal cells adjacent to tumours, stroma close to tumours, and stroma more than 15 mm from tumours display differentially-expressed genes (279) and histological heterogeneity (71, 72). Additionally, various other types of heterogeneity have been described (9, 43, 65, 82, 133, 135). Indeed, in our *in vitro* experiments, both cell types first contacted side by side and then showed intense proliferation and various changes in their protein expression profile, dependent on their topology. This phenomenon, involving active fibroblast locomotion, proliferation, cell adhesion and contact guidance, is reminiscent of biomechanical morphogenetic processes during embryogenesis and organ formation (146, 147, 280). Furthermore, it shows that the fibroblasts acquire functions which are specific to their position. As can be seen from Figures 11, 12, 13, 23, 24, 42, 61 and 64, in the course of their interactions with HeLa cells, fibroblasts first circumscribe HeLa-cell colonies, then actively proliferate and finally cause the appearance of apoptotic foci, specifically at the boundaries of HeLa-cell colonies. Thus, the present results indicate the development of a restraining role of the fibroblasts in cancer progression. The affinity of the fibroblasts for HeLa cells may be interpreted as an indigenous property of all kinds of mesenchymal cells to provide a supportive background for epithelial cells. On the other hand, the intense proliferation of the HeLa cells located at the outer edge of the colonies may indicate a feeding relationship (119) or competition for nutrients (281). Indeed, in our model, it was shown that the growth of HeLa cells at the periphery of colonies is enhanced if the colonies are surrounded by proliferating fibroblasts. This was shown by the expression of Ki-67 proliferation antigen and of DHFR, an enzyme associated with the *de novo* synthesis of amino acids and nucleic acid bases in the S phase of the cell cycle (282, 283). However, this friendly cooperation is reversed only a few generations later, as evidenced by extensive disintegration characteristics at the edges of the HeLa-cell colonies (Figures 48, 55, 56, 57, 58, 61). This phenomenon may indicate the capacity of certain fibroblasts to recognize the malignant cells and to cause their destruction by apoptosis or mitotic catastrophe (284) (Figures 43, 48, 55, 61, 62, 63, 64). Despite, however, this aggressive effect exerted by the fibroblasts, the HeLa cells managed to grow again and started forming new colonies (Figures 57, 60, 62, 75).
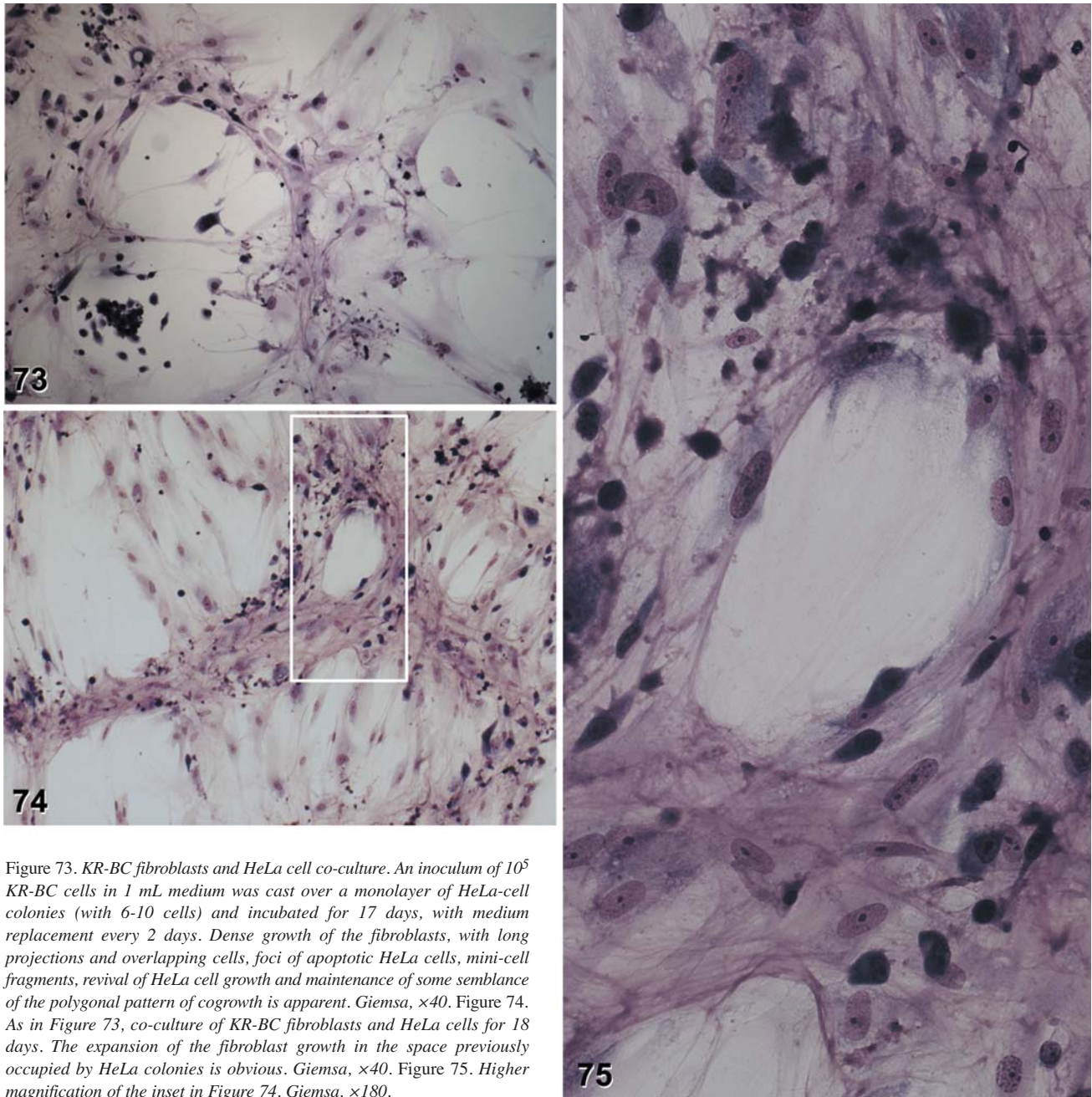
Figure 73. *KR-BC fibroblasts and HeLa cell co-culture. An inoculum of 10⁵ KR-BC cells in 1 mL medium was cast over a monolayer of HeLa-cell colonies (with 6-10 cells) and incubated for 17 days, with medium replacement every 2 days. Dense growth of the fibroblasts, with long projections and overlapping cells, foci of apoptotic HeLa cells, mini-cell fragments, revival of HeLa cell growth and maintenance of some semblance of the polygonal pattern of cogrowth is apparent. Giemsa, ×40.* Figure 74. *As in Figure 73, co-culture of KR-BC fibroblasts and HeLa cells for 18 days. The expansion of the fibroblast growth in the space previously occupied by HeLa colonies is obvious. Giemsa, ×40.* Figure 75. *Higher magnification of the inset in Figure 74. Giemsa, ×180.*

The contact between fibroblasts and HeLa cells, in the present study, was shown to be necessary for activating the process of protein expression changes in both cell types. Obviously, the overproduction of a specific protein (*e.g.* NSE, DHFR, E-cadherin) should require the overexpression not only of the specific gene but also of the series of genes involved in the provision of all necessary elements for the synthesis of that specific protein. Thus, such 'activated' cells should be expected to exhibit increased metabolic and transcriptional activity (285) and intense protein synthesis, being evident by their intense cytoplasmic basophilia and argyrophilia (Figures 42, 43, 55, 56). Cytoplasmic silver staining in these interaction experiments reflects an increased transcriptional activity for proliferative and functional needs of the cells (286, 287). Silver staining was sensitive in detecting material that could not be demonstrated by Giemsa, *e.g.* the long strongly argyrophilic fibroblastic projections. Nevertheless, basophilic and argyrophilic cytoplasm, as shown by Giemsa and silver

staining, respectively, means that the cell is actively producing proteins. It should be mentioned that numerous proteins associated with many fibroblastic functions are argyrophilic (such as fibronectin type III, collagen, and ribosomal proteins). Additionally, protein synthesis in the fibroblasts may be assisted by frequent replacement of the medium and through direct cell-cell communicative exchanges of large and small molecules with HeLa cells (80, 119, 288-290). Moreover, this process may assist in the synthesis of specific proteins. The fast transportation of free ^{3}H-UdR from fibroblasts to HeLa cells shows active metabolic cooperation between the two cell types for RNA synthesis. The contribution of the frequent replacement of the medium to the process of alteration of protein expression in the fibroblasts is evident since interruption of nutrient supply results in fibroblasts changing to a non-proliferating state where they are used just as a feeder layer by HeLa cells (Figures 5, 6, 7). Consequently, these fibroblasts, *i.e.* CAFs, that may or may not express α-SMA (291), can only serve as fodders for the cancer cells. It is interesting to note that suppression of α-SMA in stromal cells resulted in suppression of breast cancer metastasis *in vivo* (292). The feeding role of CAFs may also be supported by their properties, to express mainly genes of structural proteins (25, 29, 68, 86, 87, 129, 293), to be located in areas of cancer-cell proliferation such as the invasion front (53, 82) and to preselect and prepare a tissue to accept metastatic cancer cells (169, 270, 276, 294).

All these observations are compatible with the supportive nature of CAFs, however, they do not exclude or diminish the notion of stromal fibroblasts potentially becoming aggressive against cancer cells, opposing or destroying them. There are indeed numerous examples of such a spectrum of effects of fibroblasts against cancer cells, both *in vitro* and *in vivo*, as described in the Introduction. On the other hand, the tumour microenvironment is a region of ambiguous, positive and negative responses to various factors *e.g*. IL-6 (295).

The expression of E-cadherin in the periphery of HeLa cell colonies surrounded by fibroblasts for 11 days or longer in this study (Figure 44) suggests a direct regulation of E-cadherin by the fibroblast interaction. This may indicate alterations of epithelial barrier permeability and cellular-junction properties, as previously shown by the modulation of the expression of E-cadherin in cancer cells by fibroblasts in several co-culture systems (296-300). E-Cadherin is not expressed in several types of tumours but its expression may be associated with suppression of tumour growth (301). The involvement of E-Cadherin in tumourigenesis, metastasis and tumour suppression is a complex phenomenon, varying among different cell types and tissues (302-304). Remarkably, remodeling of stromal-specific proteins and E-cadherin expression in the epithelioid cells of benign prostate hyperplasia has been shown to facilitate the transfer of epithelial-secreted proteins to the stroma (305).

The production and release of lipid droplets by the dense fibroblast layers surrounding the HeLa-cell colonies is a novel finding. Although the function of lipids in cancer remains unclear, they evidently play a significant role in cancer progression (306, 307). Adipogenesis is related to fibrosis, and the interconversion between stromal fibroblasts and adipocytes is involved in carcinogenesis (308, 309). Furthermore, adipocytes contribute to breast cancer invasion (310). From our experiments, it may be suggested that, during their close interaction with HeLa-cells, fibroblasts produce lipids in their cytoplasm that are finally released and accumulate on their upper cell surface (Figures 50, 51, 54).

The apoptotic features observed at the border of HeLa-cell colonies after ~10 days in co-culture with fibroblasts clearly constitute an effect of the bidirectional communication and interactions between the two cell types in contact since conditioned medium did not induce these cytotoxic effects (6, 119, 186). The presence of foci with many multinucleated HeLa cells possibly indicates that in these particular areas, close to the edge of HeLa colonies, as well as to the dense fibroblast bow, specific factors affect the process of mitosis leading to incomplete telophase without cytoplasmic division. This view is supported by the high frequency of nuclear abnormalities observed in HeLa cells in the present study, and also by the nuclear atypia in cancer cells surrounded by CAFs (311).

NSE consists of a large group of hydrolytic enzymes with variable staining intensity in different cells and tissues (312). They may present peculiarities in staining due to their sensitivity to pH, osmolarity and fixation method (313, 314). NSE is localized in the cytoplasm, with diffuse staining, or in lysosomes, with spherical droplet staining (313, 315). Since carbonic anhydrase (CA), an enzyme ubiquitously-expressed in almost all cells (265, 316, 317), is abundantly stained with the NSE reaction used, it was necessary to use acetazolamide, a specific inhibitor of CA, so that true NSE expression could be revealed (263, 264, 314). NSE was used as a general marker to show that most probably a very wide range of proteins are produced by the encircling fibroblasts during their interactions with HeLa cells, as also shown by the intense silver staining. It should be noted that CA and specific esterases have been suggested to play a role in cancer progression (316-318).

The blockade of the fibroblasts at the G_2-M phase of the cell cycle, after only 17 days in co-culture with HeLa cells, shows a time-dependent effect on the nuclear membrane, nucleolus and chromatin organization of the fibroblasts. The importance of the observation of such nuclear features has been stressed for cancer cells (319-321), but never before for fibroblasts following their interaction with cancer cells *in vitro* or *in vivo*. The separation of the chromosomes in nuclear regions relates to gene expression and gene expression changes in transcriptional activity (319, 322). The possible

involvement of TGF-β signaling in this phenomenon (323), leading to alterations within the tumour microenvironment, should be further investigated.

The above discussion points to an indigenous common property of fibroblasts: namely a remarkable adaptability through the selective expression of certain proteins, thus facilitating specific functions under certain environmental conditions or under the influence of specific signaling. This is well-supported by many studies (45, 102, 105, 117, 324-329). Therefore, it could be postulated that the tumour fibrovascular stroma consists of many heterogeneous subpopulations of fibroblasts at different stages of proliferation and with a wide spectrum of alterations in protein expression (329, 330), whatever their designation is considered to be (CAFs, TAFs, myofibroblasts, mesenchymal stromal cells, mesenchymal stem cells, stromal fibroblasts, etc.). The phenomenon of the epithelial–mesenchymal transition and the involvement of other cell types were not included in this discussion.

Considering their strong affinity for cancer cells (119, 331, 332), as well as their various functions within the tumour microenvironment, mesenchymal cells have recently emerged as potential therapeutic targets or means against cancer (77, 104, 113, 153, 222, 333-342).

Enriching our knowledge on the *in vitro* and *in vivo* interactions of cancer cells with other tumour micro-environment cellular elements, and using new methodologies, such as delicate 3D culture (147, 249, 343-346), will be particularly useful for a more accurate simulation of the *in vivo* situation, allowing the mechanisms of specific interactions to be elucidated.

Conclusion

The protein-expression patterns of both fibroblasts and HeLa cells in *in vitro* co-culture depend on the topology, cell-to-cell contact, cell-to-cell ratio, duration of their interaction and avoidance of disruption of proliferating capacity of the fibroblasts due to contact inhibition or nutrient deprivation. Interactions of the two cell types gradually lead to changes in their growth and survival patterns. Fibroblasts develop a remarkable pattern of overgrowth around HeLa-cell colonies, express NSE, show intense silver staining and development of a complex network of long highly basophilic-argyrophilic projections, and produce lipids. At this stage, the signs of overgrowth of HeLa cells at the boundaries of HeLa-cell colonies give way to signs of apoptosis with gradually increasing detachment of HeLa cell groups.

It is certain that the co-culture of fibroblasts and HeLa cells *in vitro* produce a series of interactions, starting with acquaintance and mutual affinity, continuing with communication and effects on each other's protein expression and ending with a fight for survival. The important prerequisite for these observations is the enabling of fibroblast proliferation.

The present results justify an in-depth investigation of the conditions under which the flexible and industrious nature of fibroblasts can be manipulated in such a way that these cells can be utilized against cancer cells *in vivo*.

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