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Original article

# Cytocidal effects of human fibroblasts on HeLa cells *in vitro*

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**Cocultivation of human fibroblasts and HeLa cells *in vitro* leads to the development of specific patterns of growth. These patterns depend on the cell density and cell-cell ratio in the initial mixed inoculum. Human fibroblasts can cause extensive nuclear fragmentation and cellular disintegration of HeLa cells *in vitro* after coculture for periods longer than 10 days, without subculturing, and with medium replacement every 2 days. This phenomenon is preceded by directional locomotion of the fibroblastic population parallel to the edges of and around HeLa colonies and by overgrowth of both cell types at the border sites. A dense border is thus developed around the HeLa colonies. In the absence of refeeding every 2 days, HeLa cells can overgrow, pass the dense border and form a new zone. Refeeding at this stage can again cause the formation of a second concentric dense border around the HeLa zone. This phenomenon may represent an *in vitro* metaphor of the invasive property of neoplastic cells. It also points out, however, the importance of feeding for the activation of fibroblasts against HeLa cells.**

**cell-to-cell interaction — human fibroblasts — HeLa cells — cytocidal effect**

## INTRODUCTION

The conservation of the physiological tissue state in a multicellular organism depends on the coordination of functions between cells of different types [26, 62]. Cell-cell interactions and communications play a significant role in this respect, since perturbation of cooperation may reflect a deviation from the physiological condition [19, 43, 67, 71, 72]. In a tumor there exist several different cell types which do not follow the homeostatic mechanisms of the organism, but which constitute a separate non-controlled cell community parasitizing on the physiological tissue. This variety of cells which undergo a non-controlled growth beyond normality may, apart from the numerous genetically heterogeneous malignant cell clones, also include mesenchymal fibroblasts. Malignant cells of a tumor come into contact with mesenchymal fibroblasts which compose a part of the tumor stroma. Fibroblasts derived from the tumor stroma were shown to exhibit various altered (abnormal?) characteristics including a defective contact inhibition in cell culture [12, 72], chromosomal abnormalities [14], prolonged *in vitro* growth [11], and some other properties which resemble those of primary mesenchymal tumors — the sarcoma — as suggested by van de Hooff [72]. Furthermore, many studies have suggested an immediate importance of the stromal fibroblasts, including those involved in tumor vascularization, for the process of tumorigenesis and tumor growth [10, 12, 27, 38, 57, 58, 67, 70, 72—75].

Since the *in vivo* situation of fibroblasts versus neoplastic cell interactions entails numerous complex parameters, including the interference of other cell types such as lymphocytes and macrophages, *in vitro* cocultivation of isolated fibroblasts with cancer cells has provided a convenient means for initial studies [*e.g.* 1, 3, 6, 13, 21, 40, 60, 63—65]. The present study deals with observations made in cocultures of a fibroblastic finite cell line (G-EP [11, 12]) with human malignant epithelial cells (HeLa).

## MATERIALS AND METHODS

One finite fibroblastic cell line (G-EP) of those recently described [11, 12] was used. The procedures for studying cell-cell interactions *in vitro* have also been described [13]. Low densities of cells at various fibroblast-HeLa ratios (1:20, 1:10, 1:5, 1:2, 1:1, 2:1, 5:1, 10:1 and 20:1, total number of cells $10^2$ to $5 \times 10^3$ cells per Petri dish) were mixed and plated in 32 mm diameter (8.03 $cm^2$ surface area) plastic petri dishes (Lux, Miles, USA) containing a 1-$cm^2$ glass coverslip. Low density was necessary in order to study cell-cell interactions for an extended period without reaching a confluent state too soon, thus avoiding the use of dissociating agents for passaging. The medium was replaced every 2nd day. Under these conditions, cells were cultured for up to 20 days. After 4 to 20 days the coverslips were fixed in methanol and stained with Giemsa or methyl violet.

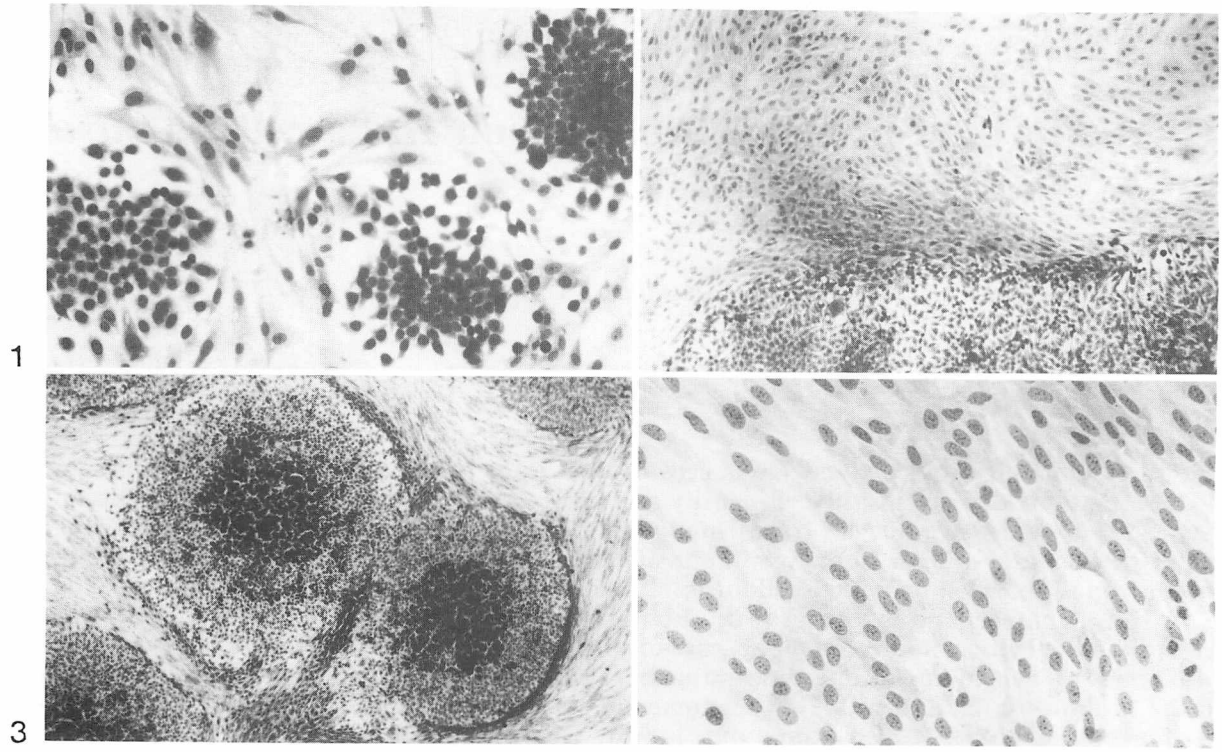


FIGURE 1. — HeLa cells and G-EP fibroblasts cocultured at a ratio 1:5 ($10^3$ cells per 8.03 $cm^2$) for 5 days. The faster growing HeLa cells have formed colonies surrounded by actively moving fibroblasts. × 200, Giemsa.

FIGURE 2. — HeLa cells and G-EP fibroblasts cocultured at a ratio 1:10 ($10^2$ cells per 8.03 $cm^2$) for 10 days. Overgrowth of both fibroblasts and HeLa cells is observed at the edges of HeLa colonies. × 40, Giemsa.

FIGURE 3. — HeLa and G-EP fibroblasts cocultured at a ratio 1:10 ($10^2$ cells per 8.03 $cm^2$) for 10 days. Overgrowth of both fibroblasts and HeLa cells at the edges of HeLa colonies gives rise to the formation of dense surrounding bow (DSB). × 25, Giemsa.

FIGURE 4. — A monolayer of G-EP cells ($10^3$ cells per 8.03 $cm^2$ cultured for 13 days) exhibiting contact inhibition of growth (absence of cell-cell overlapping) and parallel orientation of the cells. × 270, Giemsa.

Cell-free fibroblast-conditioned medium (FCM) was prepared by culturing G-EP cells to confluency, complete removal of fibroblasts by two successive centrifugations at 1500 rpm for 10 min, and pH readjustment with 7% $NaHCO_3$.

Cells were free of mycoplasma and other contaminations as shown by [$^3$H]-thymidine labeling, autoradiography and microscopy [66]. Autoradiographic procedures have been described previously [13].

Carmine-labeled HeLa cells were obtained by culturing the cells in complete medium containing 0.02% carmine (Fisher Scientific Co., Chicago, IL, USA) for 24 hr and washing twice in medium without serum. The procedure has been described by Epstein and Gilula [17].

## RESULTS

The main purpose of the initial experiments in this project was to examine the patterns of growth of both cell types (HeLa and fibroblasts) when cocultured for prolonged periods of time. Therefore, the ratio between the two cell types and the total cell number in the initial inoculum should ensure for both cell types: (a) unimpeded growth, and (b) ample surface for growth. At ratios of HeLa to fibroblasts 1:1 or 1:2 ($10^3$ cells per 32-mm diameter Petri dish or per 8.03 $cm^2$) G-EP fibroblasts were soon topo-inhibited and their growth ceased because of the coverage of the available surface by the faster growing HeLa cells. HeLa cells and fibroblasts, cocultured at a ratio of 1:10 or 1:5 ($10^2$-$10^3$ total cells per 8.03 $cm^2$), both grew efficiently (Fig. 1). When HeLa cells formed colonies most of them were closely surrounded by fibroblasts as has also been shown previously [13]. Similar behavior has been observed in fibroblasts when confronted with fibroadenoma epithelium [65]. The surrounding of HeLa cells by fibroblasts obviously depends on the number of fibroblasts present in the initial inoculum as well as on their growth rate.

The replication time of HeLa cells is 20-22 hr in this laboratory and that of G-EP fibroblasts 52-56 hr. After 10 days of coculture of G-EP and HeLa (cells ratio 10:1, $10^3$ cells; ideally 909 fibroblasts + 91 HeLa cells per Petri dish) the number of fibroblasts should speculatively be $909 \times 2^{4.61} = 22\,200$ cells and

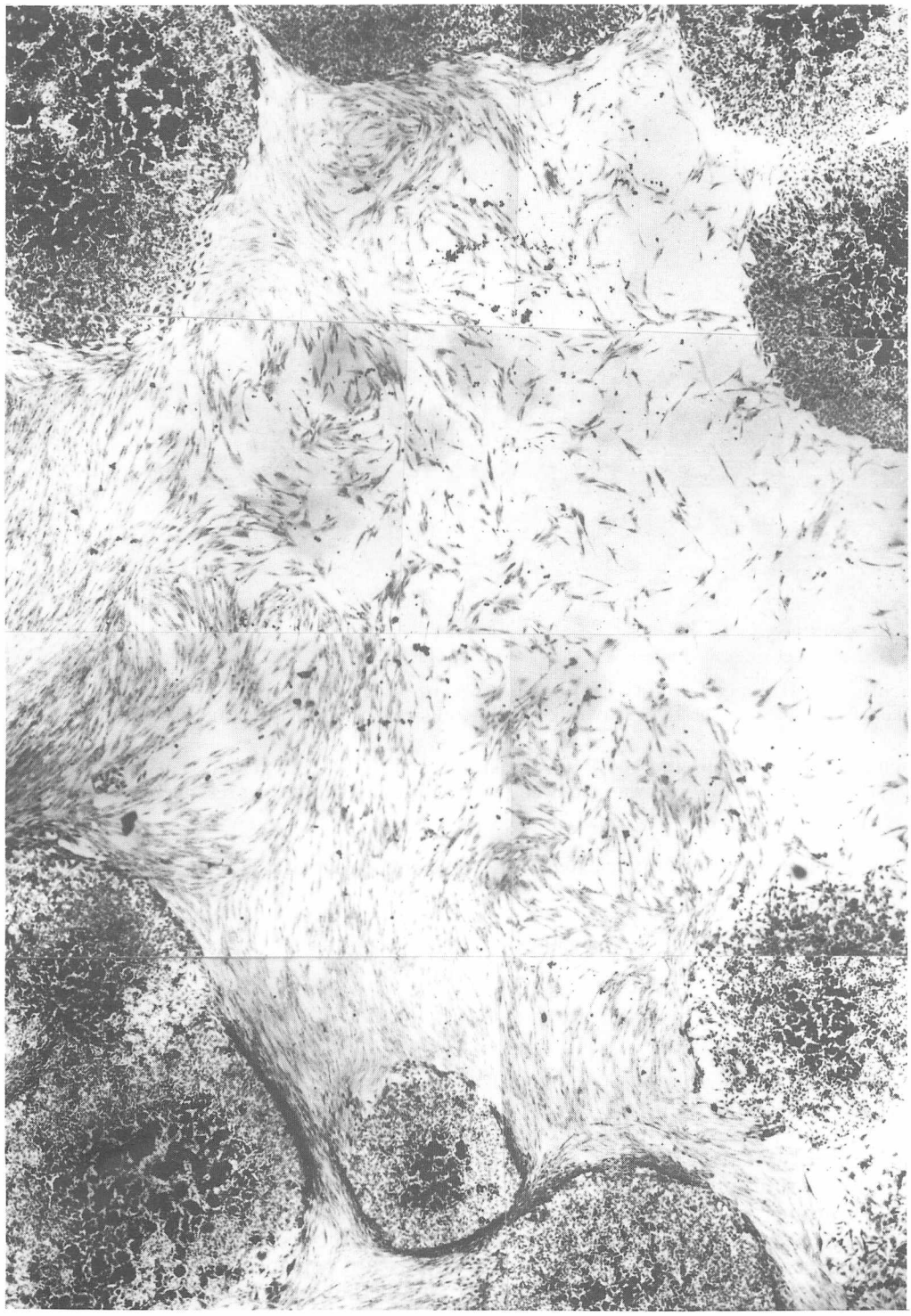

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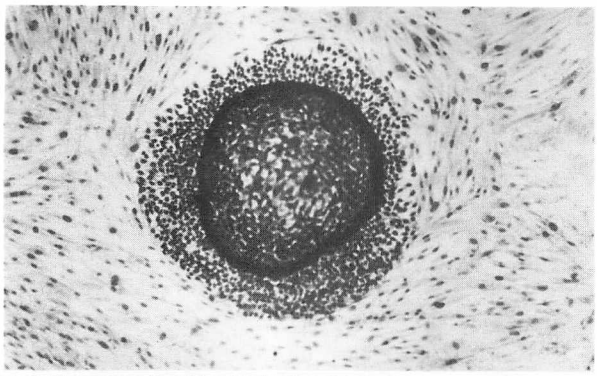
FIGURE 5. — Picture of a large coverslip area on which HeLa cells and G-EP fibroblasts were cocultured at a ratio 1:10 ($10^2$ cells per 8.03 $cm^2$) for 12 days. Note the presence of type A (lower part) and B (upper part of the figure) HeLa colonies distinguished according to the exhibited course of fibroblastic tropism as well as by the presence (A) or absence (B) of DSB. × 30, Giemsa.

the number of HeLa cells $91 \times 2^{12} = 372\,700$ cells, introducing the shorter replication times in the calculations, using the formula:

$$\text{Final number of cells} = \text{initial inoculum} \times 2^{\text{generations}}$$

Thus the expected G-EP: HeLa ratio after 10 days should be 1:16.7.

However, an examination of large fields of coverslip-cultures by counting the cells under large magnification showed a prominent growth of the fibroblast population raising the G-EP: HeLa ratio from 1:16.7 (expected) to approx. 1:4. This may indicate either an enhanced growth of fibroblasts, or an inhibition of HeLa cells, or both.



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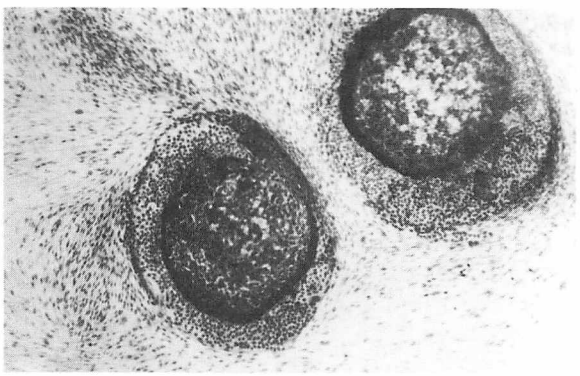

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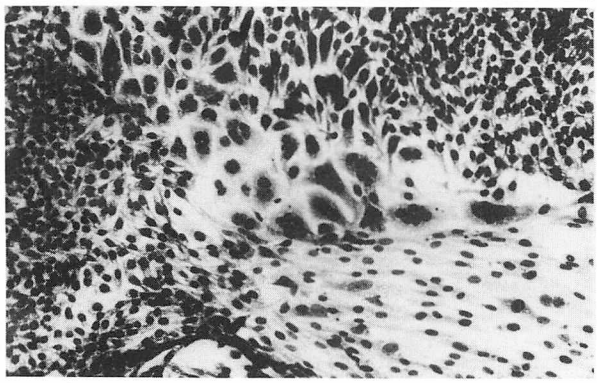

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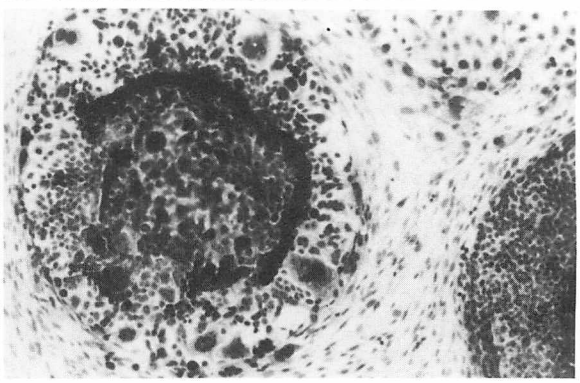

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FIGURE 6. — Coculture of HeLa cells and G-EP fibroblasts (ratio 1:20, $10^3$ cells per 8.03 $cm^2$) for 14 days. Medium was not renewed for the last 6 days. Note the overgrowth of HeLa cells extended around the pre-formed DSB. × 50, Giemsa.

FIGURE 7. — Coculture of HeLa cells and G-EP fibroblasts (ratio 1:20, $10^3$ cells per 8.03 $cm^2$) for 18 days. Medium was not renewed from the 8th to the 14th day but was renewed on the 15th day. Note the presence of two concentric DSBs. × 25, Giemsa.

FIGURE 8. — A focus of a group of multinucleated HeLa cells at the edge of a HeLa colony surrounded by fibroblasts. × 120, Giemsa.

FIGURE 9. — A colony of HeLa cells containing many multinucleated HeLa cells and exhibiting a DSB. Similar culture to that described in Fig. 6. × 70, Giemsa.

Two distinct types of HeLa colonies (A and B) were observed with regard to the exhibited course of fibroblastic tropism and to the morphological characteristics of cell growth at the borders between HeLa colonies and surrounding fibroblasts.

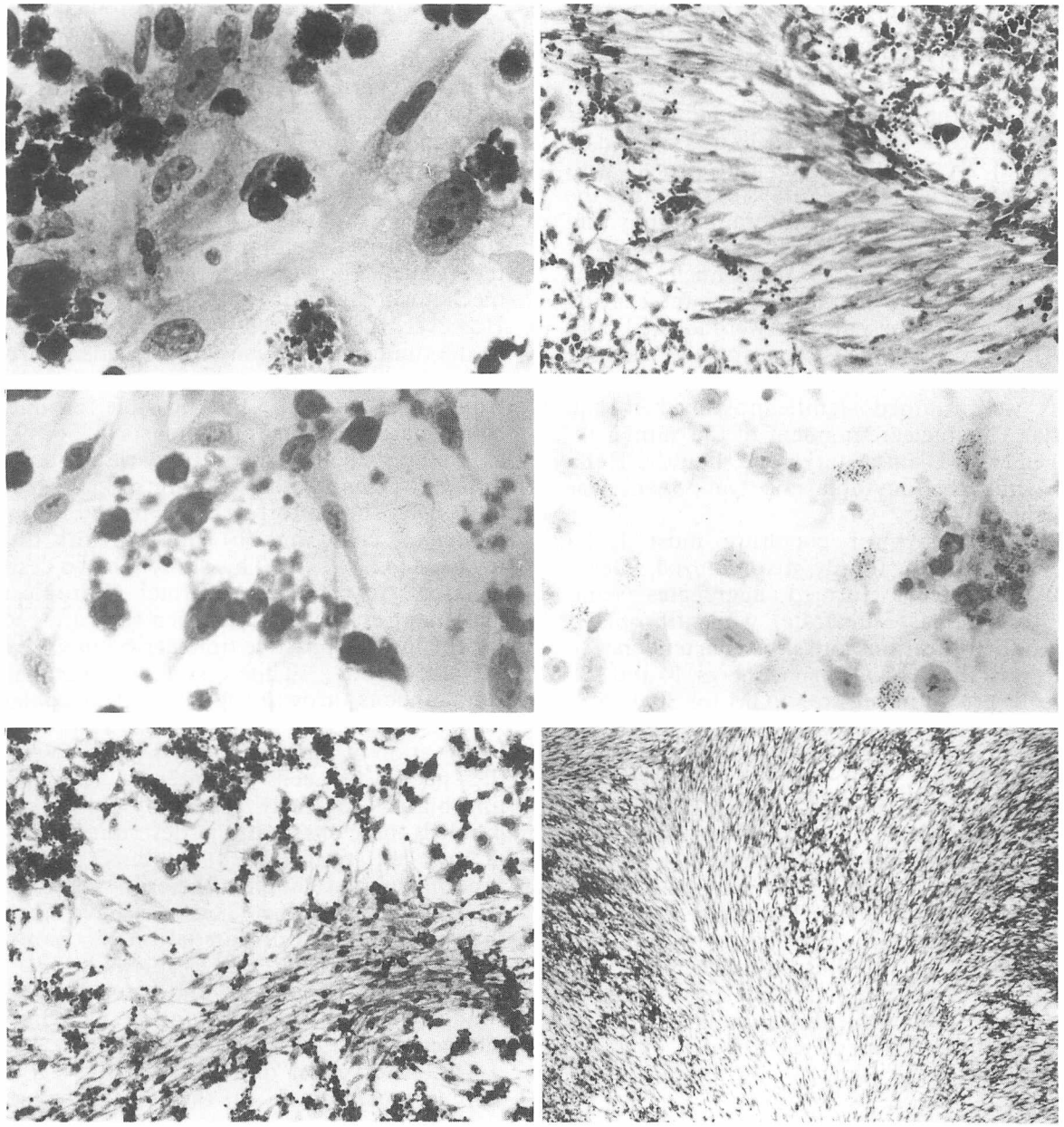
Type A HeLa colonies exhibited considerably increased growth and assembly of fibroblasts near and around them. A very dense surrounding bow (DSB) (Figs. 2 and 3) eventually developed in all these colonies provided that the medium was replaced every 2 days. Figs. 2 and 3 show stages of the development of DSB. This structure consisted of (a) densely packed and each other overlapping HeLa cells, and (b) fibroblasts on the outer side of the colony with profound overlapping. G-EP cells cultured under similar conditions without HeLa cells never showed cell overlapping (Fig. 4).

Type B HeLa colonies (Fig. 5) did not readily attract wandering fibroblasts or, alternatively, fibroblasts located near such colonies neither showed a disposition to encircle them, nor developed an enhanced growth.

If medium was not replaced for 6 or more days in a coculture of HeLa cells and fibroblasts intensely presenting the phenomenon of DSB, a profound growth of HeLa cells outside the DSB was observed while surrounding fibroblasts were persuaded away (Fig. 6). If the medium was replaced in this culture, a second DSB started to form in several colonies since a dense gathering of fibroblasts around the HeLa colonies once more developed (Fig. 7). If medium was not replaced at this stage, HeLa cells overgrew until they exhausted the medium completely, while fibroblasts lagged behind. This can be explained by the high feeding demands of fibroblasts as compared to HeLa cells, or by the ability of the HeLa cells to feed on fibroblasts but not the opposite.

In districts between HeLa cells and fibroblasts foci with groups of multinucleated HeLa cells were occasionally observed (Figs. 8 and 9). Multinucleated HeLa cells are also usually observed in HeLa cultures, though never in groups.

Examination after 12 or more days of many HeLa colonies raised among fibroblasts revealed foci of destroyed HeLa cells (Fig. 10). These foci presented the following characteristics: (a) HeLa cells were loosely located on the glass surface and exhibited a tendency to form longer than usually cytoplasmic



10 11 12 13 14 15

FIGURE 10. — A focus of destroyed HeLa cells among fibroblasts with a normal appearance. Nuclear and cytoplasmic fragmentation is obvious. HeLa and G-EP cells (ratio 1:10, $10^2$ cells per 8.03 $cm^2$) were cocultured for 12 days, with medium renewals every 2nd day. × 700, Giemsa.

FIGURE 11. — Coculture of HeLa cells and G-EP fibroblasts (ratio 1:10, $10^2$ cells per 8.03 $cm^2$) for 18 days, with medium renewals every 2nd day. Multiple foci of 'mini cells' are shown. × 120, Giemsa.

FIGURE 12. — Magnification of a focus of 'mini cells' in a culture similar to that described in Fig. 11. × 450, Giemsa.

FIGURE 13. — Autoradiograph of a coculture of HeLa cells with $^3$H-TDR-labeled G-EP fibroblasts. Conditions were as described in Fig. 11. The $^3$H-grains are exclusively located over the nucleus of the G-EP fibroblasts. × 450, diluted Giemsa.

FIGURE 14. — Disorganization of HeLa colonies and destruction of HeLa cells in a coculture of HeLa vs. G-EP fibroblasts after 20 days. Fragments of HeLa cells form aggregates semi-attached to the glass surface. × 300, Giemsa.

FIGURE 15. — Disorganization of HeLa colonies in a coculture of HeLa cells and G-EP fibroblasts after 20 days. HeLa cells are wrinkled and detached from their territories, while the fibroblasts layer morphology shows normal confluence. × 60, Giemsa.

projections; (b) fibroblasts were found interspersed among HeLa cells; (c) many HeLa cells showed extensive nuclear and cytoplasmic fragmentation (Fig. 10), and after 12—14 days of coculture, fragments of disintegrated HeLa cells took the form of 'mini cells' (Figs. 11 and 12).

The origin of the nuclear-cytoplasmic fragments and of the 'mini cells' from HeLa cells was secured by coculturing [$^3$H] TdR-labeled fibroblasts with unlabeled HeLa cells. After 12 days of coculture the coverslips were processed for autoradiography and the label was observed exclusively over the nuclei of the fibroblasts and never over HeLa nuclei or their fragments (Fig. 13). When using carmine-bearing HeLa cells, the carmine particles were located both over the HeLa cells and the HeLa fragments after the 12-day-coculture period. HeLa cells as well as their fragments were stained significantly darker than fibroblasts. The nuclear fragment of the 'mini cells' was stained with Giemsa dark blue, like the HeLa nucleus, while their cytoplasmic component was stained light pink.

After 15—20 days of coculture most HeLa colonies were almost totally disorganized. Dead, wrinkled HeLa cells formed aggregates semi-attached to the glass substrate, while fibroblasts maintained their positions and showed a tendency to expand toward the evacuated space (Figs. 14 and 15).

HeLa cells grown in cell-free FCM for 30 days (5 passages undergone) showed no sign of cell disintegration or alteration in morphological pattern.

The experiments presented in this report were conducted using G-EP fibroblasts derived from the normal breast epidermis of a female patient with infiltrating ductal carcinoma with metastases in the lymph nodes [11, 12]. G-EP cells exhibited a normal growth *in vitro* forming parallel arrays without cell overlapping (Fig. 4) [12]. Future experiments will deal with other human finite fibroblastic cell lines to find out whether they also exhibit similar properties to G-EP when cocultured with HeLa or other neoplastic cells.

## DISCUSSION

In most neoplastic processes the organism is unable to confront the disturbance of cell growth in the particular site of a tumor through the defensive mechanisms which are effective in other sites of the organism. The influence of normal cells on the growth of neoplastic cells *in vitro* is of immediate interest since tumors grow among normal tissues *in vivo*.

There is adequate evidence of the variation in the abilities of tumor cells to adhere to and interact with other cells *in vitro* [1, 50, 59, 76], as well as of the differences in the distribution and survival of metastatic cells implanted in different organs [22, 31, 52, 54]. The interacting endothelial cells which possess unique antigenic differences that reflect their tissue origin [51, 69] are, to a considerable degree, responsible for these variations.

The process of tumor cell invasion and metastasis has been extensively studied from many aspects [29, 41, 45, 46, 52, 53]; however, there is no strong or even slight experimental evidence concerning the existence of a defense mechanism other than the host macrophage and the NK-cell activity [29] against tumor cell proliferation and invasion in the microenvironment around the viable active tumor cell in the organism. It has been suggested that stromal cell development within a tumor may represent a process of the organim's defensive mechanism against tumor cells [61, 70, 74, 75]. However, the observed increased collagen synthesis in the tumor stroma may be a means to protect the tumor from the immune system of the host [8]. Apart from these aspects, the possible production of collagenase by stromal fibroblasts [4, 36, 49, 72, 77], as well as their growth in variable amounts in different tumors [5, 8, 72], are processes which (a) have been shown to be stimulated by the epithelial malignant cells, and (b) interfere with tumor cell invasion [42, 52, 72]. This study aims to describe the behavior of human normal fibroblasts and malignant epithelial cells when they are cocultured for the longest possible time period *in vitro* without subcultivation. This system parallels the simultaneous growth of stromal fibroblasts and neoplastic cells in a tumor *in vivo*. The main observation of the present work is the destruction of the neoplastic cells apparently caused by the fibroblasts. An early report by Eagle *et al.* [16] parallels these observations, describing that a human diploid cell strain (WI38) inhibited the growth of superinoculated human heteroploid cancer cell (HeLa). Several other examples have since shown that a malignant cell population can be prevented from proliferating by surrounding normal mesenchymal fibroblasts under *in vivo* or *in vitro* conditions by processes obviously involving cell-cell interactions [7, 9, 15, 24, 33, 34, 44, 48, 76].

The observation of HeLa cells with a different colony morphology in the present experiments provides evidence for the heterogeneity of the HeLa cell population *in vitro*. From the locomotive behavior of wandering fibroblasts, proliferating among these colonies, it is inferred that the heterogeneity of HeLa morphology reflects an altered fibroblast response. Thus, tumor cell heterogeneity [32, 47] seems also to abide in the presented *in vitro* system.

Cocultivation of HeLa cells with fibroblasts can be divided in two phases: The first phase is characterized by cell-cell contacts, parallel growth of HeLa colonies surrounded by fibroblasts, overgrowth of both fibroblasts and HeLa cells at the sites of contact. The second phase is characterized by the adverse effects of fibroblasts on HeLa cells. HeLa colonies show a high degree of morphological disorganization and cell disintegration at the sites of this influence. The appearance of this phenomenon not earlier than the 10-12th day of cocultivation may mean that interactions involve the gradually increasing production of a factor that eventually

influences fundamental processes of HeLa cell growth and cell division, and finally results in the destruction of HeLa cells. Contact between the cells is a prerequisite for the occurrence of the phenomenon. As multinucleation and polyploidization may be implicated in the defense against loss of genetic material under adverse conditions of cell growth [20, 30, 39], the presence of multinucleated HeLa cells in HeLa versus fibroblast cocultures may also denote the struggle for survival of HeLa cells.

The accelerated growth of both fibroblasts and HeLa cells at the borders of HeLa colonies during the first phase of interaction leads to the assumption that growth promotion factors are produced which are effective on both cell types. Obviously, simple renewals of the medium cannot be considered as the reason for this stimulation of growth at this particular site, since if such an explanation were correct, a homogeneous growth of cells (at least for the fibroblasts) would be expected. Growth stimulation of endothelial cells by simultaneous culture with sarcoma 180 cells in diffusion chambers was reported earlier by Atherton [2]. Furthermore, it has been suggested that neoplastic cells may induce proliferation of fibroblasts not only inside the tumor, but also in a wide area around the tumor [5]. On the other hand, the bias of the tumor cell toward a modification of the physiological environment in favor of its further proliferation has been implicated in the processes of tumor angiogenesis [28, 56] and metastasis [23, 41, 52, 54]. It is not known how the suggested loss of selectivity of junctional communication in cultured breast cancer cells [18] may interfere with these processes. Stoker *et al.* [65] in an elegant experiment of interactions between fibroblasts and epithelial cells *in vitro* suggested that loss of territorial integrity and resistance to fibroblast invasion may be a feature of the malignant change. If so, invasion of the tumor by the stroma may be as important as is the reverse. Such studies indicate that altered fibroblast locomotion may determine the functional (acquired or genetic) necessity in a tissue environment predisposed to tumorigenesis.

As the time of interaction proceeded in the experiments presented, after about 10-12 days of coculture the first signs of HeLa cell destruction appeared provided that both cell types had not reached an over-confluent growth stage. Since parallel cultures of HeLa cells at logarithmic, confluent or over-confluent culture stages have never shown similar destruction patterns, it might be inferred that fibroblasts were the source of factors influencing HeLa integrity and survival.

In conclusion, the cell type, cell density, cell/cell ratio, and time of cocultivation of two different mixed cell population — an epithelial and a fibroblastic one — are parameters which influence the behavior (growth, morphological characteristics, locomotion, survival, and induction of cell-cell interaction-related phenomena) of both cell types. It is suggested that under certain *in vitro* conditions human fibroblasts can produce factors which are destructive for human neoplastic cells.

In view of the present results and findings on the effect of human fibroblast interferon against malignant cells [25, 35, 37], as well as of the increasing interest in the origin, role, and properties of stromal fibroblasts in tumors [4, 12, 57, 61, 70, 72] further studies of stromal-neoplastic cell interactions are justifiable.

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