

Interactions between Human Fibroblasts and HeLa Cells *in vitro*

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Affinity toward each other was demonstrated in co-cultures between HeLa cells and fibroblasts originating from human tumor stromal or normal tissues. Both cell types in the mixed cultures (ratio 1:1, 1:2, 2:1) proliferated normally as shown by ^3H -thymidine labeling index estimation for up to 48 hr of co-culture. At ratios of fibroblasts: HeLa lower than 1:10, fibroblasts were eventually eliminated after serial passaging. It was shown that ^3H -nucleotides could be transferred between heterologous cells in either direction. Contact of cells was essential for this phenomenon. Transfer of the label from HeLa to fibroblasts required a longer interaction time and was evidently lower than the transfer from fibroblasts to HeLa. ^3H -thymidine incorporated into the DNA of either cell type could not be transferred from one cell to another. The model provides a means for studying neoplastic X normal (or tumour stromal) cell interactions *in vitro*.

Key-words: *Cell-to-cell interactions - Co-cultures - HeLa - Fibroblasts - Cell communication.*

INTRODUCTION

The study of cell-to-cell interactions *in vitro* may provide information on biological processes dependent on cellular recognition and cooperation; such as embryonic development and organization (40, 52), control of cell proliferation (1, 9, 20, 35, 65), wound healing (59), neoplastic cell behavior and metastasis (1, 2, 22, 39, 60, 64), carcinogenesis (27, 59), cell communication and transfer of specific signals between cells (6, 13, 16, 21, 23, 36, 38, 41, 43, 45).

Although tissue culture cannot simulate the complexity of the *in vivo* conditions, systems of mixed cultures seem effectively advantageous as a convenient experimental approach to the cell-to-cell interaction phenomena, since (a) they can be studied under controlled conditions, isolated from their environment in the organism, and, therefore, not influenced by homeostasis; and (b) combinations of any different cell types may be used to investigate functional differences due to external or genetically determined parameters.

The technique of autoradiography in monitoring nucleotide transfer from donor to recipient cells has been repeatedly used in several homologous or heterologous cell systems to study the aspects mentioned above (e.g. 12, 29, 36, 37, 41, 45, 51). In the present study we examine morphological features and the transfer of ^3H -nucleotides during interactions *in vitro*

between human fibroblastic and epithelial (HeLa) cells.

MATERIAL AND METHODS

Cells

Eight finite fibroblastic cell lines of those recently described (15, 17) were used (Table I). McCoy's 5a medium supplemented with 10% fetal bovine serum (Flow Labs., Irvine, Scotland), 10^5 IU/L penicillin, 10^5 $\mu\text{g/L}$ streptomycin and 2 mg/L amphotericin B was used throughout. All cells were free of mycoplasma and other contaminations, as shown by ^3H -thymidine labeling and autoradiography (57), and by microscopic examination.

Procedure of studying cell-to-cell interactions *in vitro*

Confluent fibroblast or HeLa cell cultures were detached of the plastic surface by means of a 0.1% trypsin (Difco, Detroit, MI, USA) 0.02% EDTA solution in Mg^{++} - Ca^{++} -free Hank's BSS, washed in medium once and mixed in suspension at appropriate densities and cell ratios for the various experiments. Cell mixtures were plated in 3 cm-diameter plastic tissue culture dishes (Flow) containing a glass coverslip and transferred in a humidified - 0.5% CO_2 , 36.8 °C incubator. Cells were examined daily and refed every third day or earlier if required. Coverslips were instantly washed in physiological saline pH 7.3, fixed immediately in methanol, air dried and stained with buffered Giemsa pH 6.8. Stained coverslips were mounted on slides and examined and photographed with a Reichert light microscope. HeLa and fibroblastic cells in mixed cultures were easily distinguished by their morphology and staining pattern.

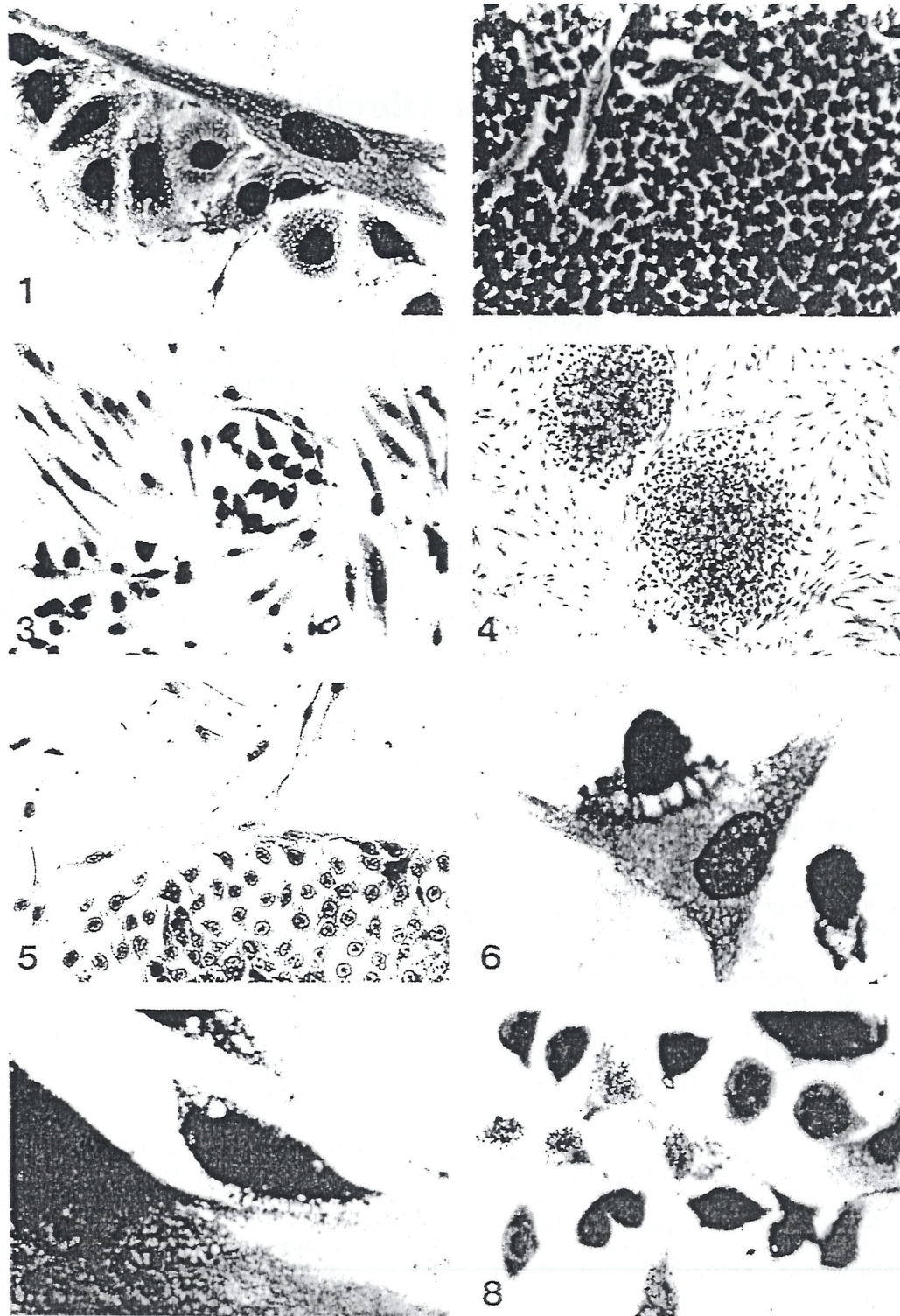


FIGURE 1. — Adherence of HeLa cells on the side edge of a fibroblast (G-EP) after 2 hr co-culture; $\times 900$, Giemsa.

FIGURE 2. — Absence of overlapping between fibroblasts (G-EP) and HeLa at a ratio 1:20 after co-culture for 48 hr; $\times 300$, Giemsa.

FIGURE 3. — Encircling of a small HeLa colony by actively moving fibroblasts (G-L), 48 hr after the addition of the fibroblasts over a dilute monolayer of HeLa cells; $\times 500$, Giemsa.

FIGURE 4. — Encircling of large HeLa colonies by actively moving fibroblasts (G-EP), 48 hr after the addition of the fibroblasts over a coverslip bearing HeLa colonies. Note that the density of fibroblasts around the HeLa colonies is considerably higher than in other districts; $\times 50$, Giemsa.

FIGURE 5. — Detail of a HeLa colony edge surrounded by human fibroblasts (MI-M). Details as in figure 4; $\times 500$ Giemsa.

TABLE I. — Human fibroblastic finite cell lines used in experiments of co-culture with HeLa cells.

Cell line designation	Site of specimen derivation	Case
AL-BC	Primary tumor	Infiltrating ductal carcinoma with lymph node metastases
CHA-EP	Normal breast epidermis	Infiltrating ductal carcinoma with lymph node metastases
CHA-L	Metastatic lymph node	Infiltrating ductal carcinoma with lymph node metastases
G-EP	Normal breast epidermis	Infiltrating ductal carcinoma with lymph node metastases
G-L	Metastatic lymph node	Infiltrating ductal carcinoma with lymph node metastases
MI-M	Primary lesion	Chronic mastitis
PG-M	Primary lesion	Chronic mastitis
SE-L	Metastatic lymph node	Infiltrating ductal carcinoma with lymph node metastases

Metabolic cooperation was examined by monitoring nucleotide transfer from donor to recipient cells (45). The donor cells were prelabeled with ^3H -uridine (0.1 $\mu\text{Ci}/\text{ml}$, sp.act. 21 Ci/mmol) or ^3H -thymidine (0.1 $\mu\text{Ci}/\text{ml}$, sp.act. 25 Ci/mmol, Amersham, UK) for 3-4 cell generations (3 days for HeLa; about 6 days for fibroblasts) and then thoroughly washed three times with "cold medium" and incubated for one further hour with "cold medium" (medium without the radioactive compound). This procedure resulted in homogeneously labeled cells and complete elimination of background grains in autoradiographs. In ^3H -thymidine-labeled cells radioactivity was entirely incorporated into the DNA in the nucleus; while in ^3H -uridine-labeled cells most of the radioactivity was incorporated into various RNA forms, allowing a pool of free nucleotides in the cytoplasm (5, 14). Labeled donor cells were mixed with non-labeled recipient cells, co-cultured as described above and coverslips were washed, fixed and processed for autoradiography as previously described (6). In order to find out whether the label in the recipient cells belonged to free ^3H -nucleotides or to ^3H -nucleotides incorporated into RNA, methanol-fixed coverslips were further treated with 20% trichloroacetic acid (3 changes, 1 hr each), dehydrated in methanol, autoradiographed and stained with Giemsa. The ^3H -thymidine labeling index was estimated, after autoradiography and staining, by calculating the percentage of cells with grains located over the nucleus.

RESULTS

Examination of coverslip cultures of premixed HeLa and fibroblasts (ratio 1:1), co-cultured for 3, 6 or 12 hr, showed a preferential adherence of HeLa cells around the attached fibroblasts rather than randomly on the available glass surface. This phenomenon seems to be independent of the fibroblast origination and could easily be confirmed either by adding HeLa cells in a dilute culture of pre-attached fibroblasts or by

utilizing a very diluted mixed cell suspension. In both cases, HeLa cells exhibited a highly remarkable affinity mainly for the side edges around the fibroblasts (Fig. 1), though aggregation in suspension may also occur between HeLa and fibroblasts. Overlapping of HeLa over fibroblasts, or vice versa, was never observed in mixed cultures incubated for 2 to 48 hr. The absence of overlapping was independent of the cell ratio or growth stage. Figure 2 shows a co-culture of HeLa X fibroblasts at a ratio of 20:1 for 48 hr where absence of heterologous overlapping is evident.

Preferential adherence (either aggregation during cell mixing or directional locomotion toward one another) between homologous cells (HeLa X HeLa or fibroblast X fibroblast) was not observed.

In order to examine whether or not fibroblasts could show a pattern of specific directional locomotion, especially in relation to their highly motive character when attached on a glass surface, fibroblasts in suspension were added over a culture of HeLa cells forming few and far between discrete colonies of 10-20 cells. Fibroblasts were attached on the glass surface randomly distributed in the space among HeLa colonies, and after complete spreading (approximately 3-4 hr) exhibited active motility. Coverslips of such co-cultures fixed and stained 10, 24, or 48 hr later showed a remarkable orientation of the moving fibroblasts by the borders of small or large HeLa colonies (Figs. 3, 4 and 5). Fibroblasts never invaded dense HeLa colonies. Figures 6 and 7 show numerous small processes connecting fibroblasts and HeLa cells. Active motility of fibroblasts attached on the glass surface has been repeatedly shown by time lapse cinematography and other studies (53, 54, 56, 62).

The ^3H -thymidine labeling index (0.1 $\mu\text{Ci}/\text{ml}$ for 2 hr of both cell types in mixed cultures, ratio 1:1,

FIGURE 6. — Cytoplasmic junctions extending from a HeLa cell toward a fibroblast (AL-BC), 2 hr after the addition of HeLa cells over a monolayer of fibroblasts; $\times 1,600$, Giemsa.

FIGURE 7. — Thread-like cytoplasmic junctions extending from a HeLa cell toward the edges of a fibroblast (SE-L), after co-culture for 7 hr; $\times 2,000$, Giemsa.

FIGURE 8. — Demonstration of absence of transfer of ^3H -thymidine from human fibroblasts (labeled AL-BC cells) to HeLa, after 72 hr of co-culture; $\times 900$, Giemsa.

1:2, 2:1; total cell density during plating: $8-10 \times 10^4$ cells/cm²), after co-culture for 10, 24 or 48 hr, did not differ from that in control cultures. This shows that inhibition in DNA synthesis did not occur in either cell type and, therefore, cell proliferation proceeded normally at this stage. Provided that cultures were refed regularly, examination of the mixed cultures daily for seven days, showed an increase in the number of both cell types.

In order to examine the effect of the HeLa cell density on the fibroblastic cell behavior in mixed cultures, (a) fibroblasts in suspension (10^5 cells/ml) were added over a confluent monolayer of HeLa cells, after medium replacement, or alternatively (b) a very dense HeLa cell suspension (10^6 cells/ml) was added over a very dilute monolayer of fibroblasts, so as to cover more than 80% of the available empty surface. In the first case it was observed that several fibroblasts

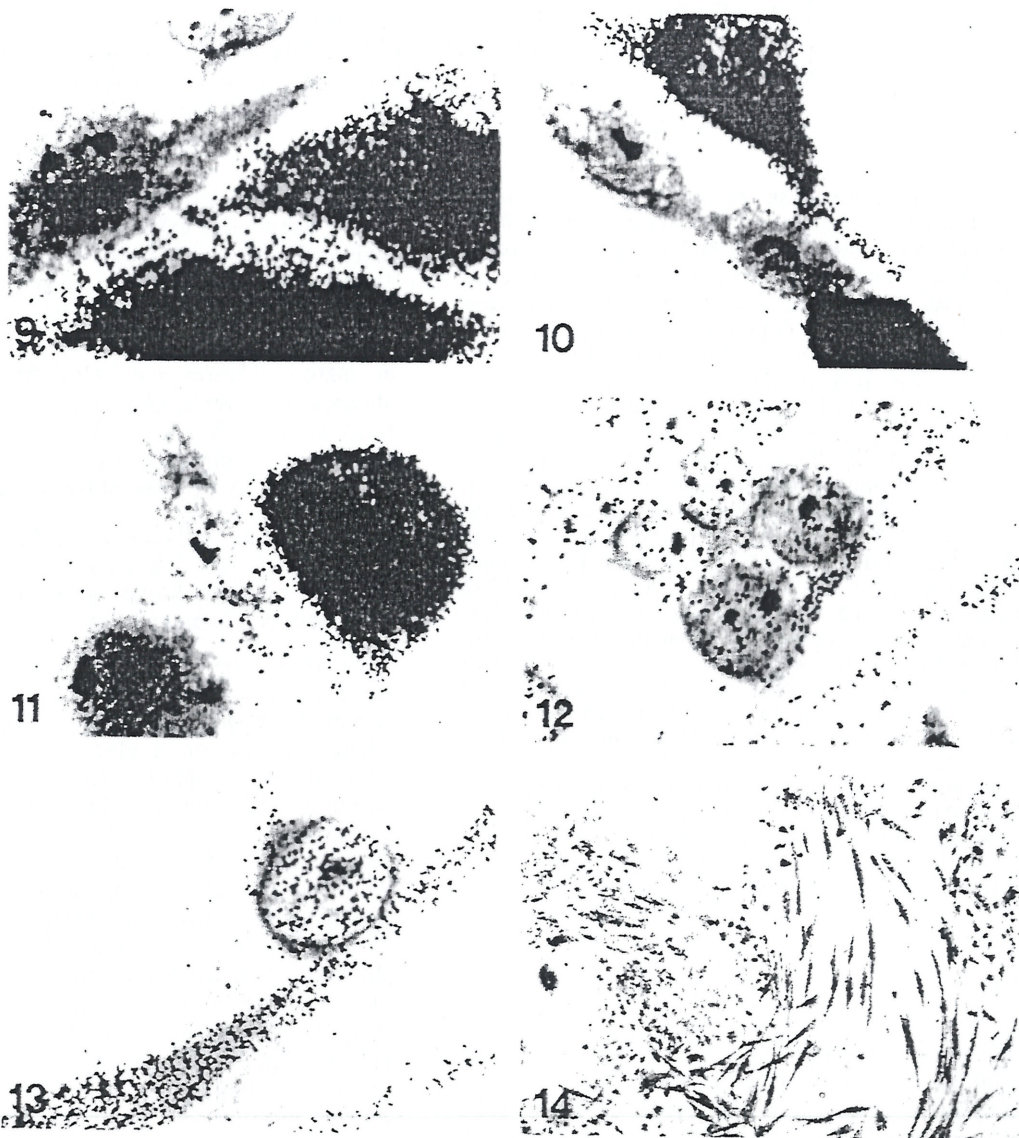


FIGURE 9. — Transfer of ³H-label carried by nucleotides from HeLa cells to fibroblasts (CHA-EP), after 3 hr co-culture; $\times 2,000$, Giemsa.

FIGURE 10. — As in figure 9, after 5 hr of co-culture; $\times 2,000$, Giemsa.

FIGURE 11. — As in figure 9, after 10 hr of co-culture; $\times 2,000$, Giemsa.

FIGURE 12. — Transfer of ³H-label carried by nucleotides from fibroblasts to HeLa cells after 2 hr of co-culture; $\times 2,000$, Giemsa.

FIGURE 13. — As in figure 12, after 5 hr of co-culture; $\times 2,000$, Giemsa.

FIGURE 14. — Transfer of ³H-label carried by nucleotides from HeLa cells to fibroblasts (CHA-EP), after 3 hr of co-culture. Labeled fibroblasts were added over a monolayer of HeLa cells forming small colonies. Note that HeLa cells adjacent to fibroblasts are heavily labeled; $\times 600$, Giemsa.

found a place squeezed among HeLa cells, but never on or underneath them. In both cases fibroblasts did not show any sign of proliferation in the presence of a confluent HeLa monolayer, though HeLa cells continued to grow in piles provided that the medium was frequently renewed. By serial passaging of such cultures, fibroblasts were eventually eliminated. Also, when the ratio of fibroblasts: HeLa (premixed before co-culture) was lower than 1:10 (1:15, 1:20, 1:30), for total cell densities higher than $8 \cdot 10^4$ cells/cm², rarely colonies of fibroblasts were found after 48 hr or after 7 days of co-culture; single fibroblasts were located wandering among and surrounded by HeLa cells (e.g. Fig. 2). Proliferation of fibroblasts ceased in such cultures, possibly due to contact topo-inhibition of growth (55), while HeLa cells flourished.

Transfer of ³H-label carried by thymidine incorporated into DNA was never observed in any cell combination, in either direction, between HeLa cells and fibroblasts co-cultured for 2, 24 or 72 hr (Fig. 8).

³H-nucleotides derived from ³H-uridine were found to be transferred in both directions between HeLa cells and fibroblasts through cell-to-cell contact and not through the extracellular environment. Transfer of labeled material from fibroblasts to HeLa was more pronounced than from HeLa to fibroblasts. Transfer of ³H-label carried by nucleotides from HeLa to fibroblasts is shown in figures 9, 10 and 11. Transfer of ³H-label from fibroblasts to HeLa is shown in figures 12, 13 and 14. In the cases of ³H-uridine-HeLa X fibroblast cell co-cultures for 2 hr, the number of grains located over the fibroblasts was as low as in the background, while after 10 hr, several grains were observed over the cytoplasm and the nucleus of fibroblasts (Figs. 10 and 11). In the cases of ³H-uridine-fibroblast X HeLa cell co-cultures for 2 or 5 hr, a high number of grains (over the background level) were located over the HeLa cells (Figs. 12 and 13). Co-culture for ≥ 10 hr increased both the number of grains over HeLa cells and the number of labeled HeLa cells. HeLa cells located near or adhering to fibroblasts were heavily labeled, while HeLa cells located in districts without fibroblasts were either unlabeled or lightly labeled (Fig. 14). Fibroblasts in their active wandering were obviously coming into temporary contact with many HeLa cells. Active passing of ³H-nucleotides between HeLa cells through junctions also occurs, since HeLa cells located in the central areas of small HeLa colonies surrounded by labeled fibroblasts were also found to be labeled, though fibroblasts do not penetrate HeLa colonies (Fig. 14). On the other hand, transfer of ³H-nucleotides between homologous cells has also been previously demonstrated (14, 45).

DISCUSSION

Though it is obscure which *in vivo* parameters of the homeostatic process can be reproduced and maintained *in vitro* when cells of one, two or more different types are set in co-culture, the single fact that these cells

will be isolated and studied under controlled conditions is an advantage for examining the effects of several biophysical and biochemical factors. One of the most extensively studied aspects of cell-to-cell interactions *in vitro* has been cell locomotion. Adequate evidence has suggested that in epithelial X mesenchymal interactions *in vitro*, cell locomotion is affected (1, 3, 9, 53, 56, 62, 65). Neoplastic epithelial X fibroblastic cell systems *in vitro* have been used by most workers in this field for the study of malignant invasion and metastasis (34, 49, 54).

The results presented suggest that human fibroblasts and HeLa cells in mixed cultures interact, and that small molecules such as nucleotides pass from cell to cell in either direction but with a different quantitative rate influenced by many factors (pool sizes, rates of RNA synthesis, etc.). The fact that nucleotides pass extensively from fibroblasts to HeLa cells might be explained on the basis of a "preferable feeding dependence" of HeLa apposed to fibroblasts instead of synthesizing nucleotides *de novo*. This phenomenon is not identical to metabolic cooperation between deficient and non-deficient cells (45, 58). It may be considered as a property of HeLa to utilize fibroblasts as suppliers of small molecules in support of their cellular metabolic economy. The remarkable preferential adherence of HeLa on the edges of human fibroblasts may also be attributed to this property. On the other hand, it has been a common experience to utilize fibroblast cultures as feeder layers for the facilitation of growth of several cell types *in vitro* (e.g. 4, 25, 30). Another parallel explanation may be based on a genetically determined property of the membranes of the two cell types, leading to a strong affinity when the two cell types coexist and are allowed to interact under *in vitro* conditions. The elucidation of the drastic modifications in the cell surface organization of cancer cells (23, 42) may explain this phenomenon.

The passing of ³H-nucleotides from HeLa to fibroblasts is at present unexplained. This phenomenon requires a longer time of co-culture to occur than the passing of nucleotides from fibroblasts to HeLa and, moreover, the label over the recipient fibroblasts never reaches a high density level. It is also interesting that nucleotides transferred from HeLa cells are used by the recipient fibroblasts for the synthesis of RNA. This may be indicative of a functional role played by the fibroblast during the interaction, or after a given interval. The present observations also suggest that, except the prerequisite of cell-to-cell contact, the phase in the cycle of each interacting cell may be a determinant of the result, since in longer interaction intervals the cell phase combinations of interacting cells are increased.

Abolishment of contact inhibition of growth in culture (1, 55) has been considered as an indication of declination from the normal cell growth regulation (1, 19, 63) and/or in many cases as a sign of malignant transformation (1, 46, 47).

In a tumor both malignant epithelial cells and stromal fibroblasts surpass the host's regulatory homeostatic mechanisms and create a new cell system with new properties, presumably governed by some peculiar and

still obscure growth control processes. This general definition of malignancy included stromal fibroblastic cells as part of the outgrown cell population, although these cells are not considered as neoplastic. Nevertheless, the close contact and continuous interaction between epithelial and fibroblastic elements in a tumor predispose for an interest in studying further the role of the stroma in the control processes within the tumor. In this direction of thought, it should be necessary to mention that several authors have pointed out many aspects of the important role fibroblasts may play in a tumor (10, 11, 16, 18, 24, 26, 28, 50, 61).

We believe that stromal fibroblasts derived from a tumor differ from normal fibroblasts in both their growth patterns and longevity *in vitro* (15, 17). The increasing evidence that fibroblasts derived from the skin, other body sites, or the tumors of cancer patients exhibit *in vitro* different properties in comparison to fibroblasts from normal individuals (7, 8, 31, 32, 33, 44, 48), supports this aspects. The results presented did not discriminate between fibroblasts originating from breast tumor tissues and fibroblasts from normal tissues of breast cancer patients, regarding the patterns of their interaction with HeLa cells. The utilization of fibroblasts from healthy individuals, as well as other types of cancer cells, in similar experiments seems to be justifiable.

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