Inhibition and Stimulation of the Proliferative Activity of Tumor Cells with Different Degrees of Malignancy in Close Contact with Normal Embryonal Fibroblasts of Syrian Hamster

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Any transformed or tumor cell (TC) arising in carcinogenesis *in vivo* is surrounded by normal cells (NC) and in direct contact with them. These interactions in the early stages of carcinogenesis is likely to be responsible for tumor development and metastasis.

Investigations of the interactions between normal and tumor cells started more than 30 years ago, when Abercrombie and Ambrose described the contact inhibition phenomenon in a culture of normal cells [2,3]. A short time later, it was noticed that tumor cells lose such an ability or, at least, exhibit it to a lesser extent [2,4-6]. A more interesting finding was that normal cells are able to inhibit the growth of tumor cells [10,11,16]. Yet despite a large body of investigations concerning the interactions between TC and NC, the detailed picture remains unclear. There are some contradictory description of the phenomenon itself. Several authors have shown a "feeder effect" of NC, i.e., an enhancement of tumor cell growth [9,13,15,17,18], while others have demonstrated that NC are able to inhibit proliferation [4,12,13,16,18], to stimulate differentiation of TC [13], and even cause TC death [10,11,13]. These discrepancies seem to be due to the very different experimental models used. Moreover, well-known "immortalized strains" are often used as an NC model, although they can no longer be considered as normal in the full sense.

In view of all this, the aim of the present study was to develop an experimental model of the interactions of normal embryonal cells and TC and with the aid of this model to answer the following questions: 1) Is the effect of NC on TC stimulatory or inhibitory? 2) If both effects are present, what are the conditions for each of them? 3) Does the nature of NC-TC interactions depend on the malignancy of the tumor and, in particular, on its ability to metastasize?

For this purpose embryonal fibroblasts of Syrian hamster (HEF) in early generations *in vitro* were used as the model for NC, and spontaneously transformed *in vitro* HEF (STHEF) as well as STHEF variants with various degrees of malignancy selected *in vivo* were used as the model for TC [1,7]. Thus, the system designed consists of components derived from each other: HEF, STHEF, and *in vivo* selected low- and high-malignancy STHEF-derived strains.

MATERIALS AND METHODS

Embryonal fibroblasts of Syrian hamster were obtained by trypsinization of hamster embryos and grown in tissue culture *in vitro*. HEF of 3-6 passages

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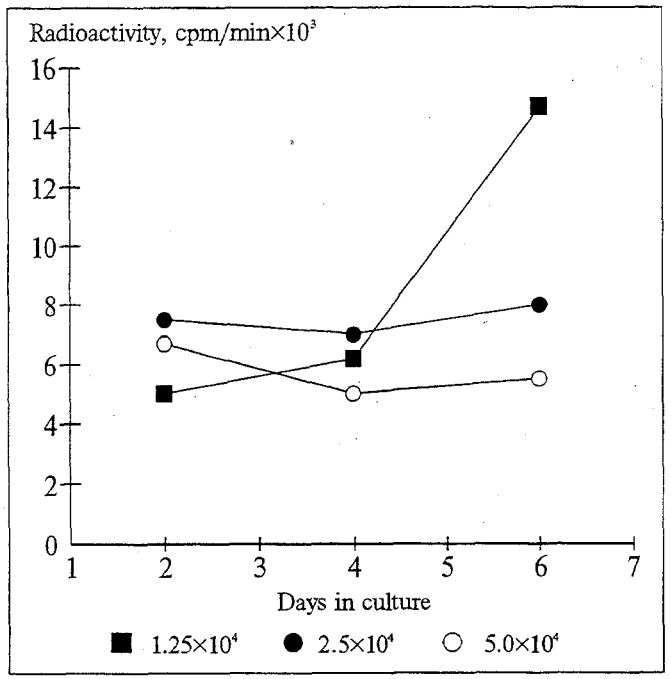

Fig. 1. Proliferative activity of HEF as a function of seeding density.

in vitro were used for the experiment. The following strains obtained in our laboratory were taken as TC or transformed cells: STHEF, STHEF-128, STHEF-162, STHEF-MLN-6, STHEF-MLN-8, and STHEF-75/18. The data on the malignancy of the cells studied are presented in Table 1.

The cells were grown in Eagle's medium supplemented with lactalbumin hydrolysate (1:1), 10% calf serum, 0.3 mg/ml glutamine, and 0.08 mg/ml gentamicin. The same medium plus 5 nM HEPES buffer was used for the experiments.

The experiments were carried out in 96-well round-bottom plates (Linbro, Flow, U.K.). NC and TC were harvested with Versene, washed, diluted to the required concentration, and lethally γ-irradiated with a Stebel' apparatus (8 min, 10,000 rd). After the irradiation 5.0×10^4 HEF and 1.0×10^4 TC were mixed and placed in wells in 0.2 ml of the medium. TC in the same concentration without NC and γ-irradiated HEF without TC served as the controls. The cells were cocultured in an incubator at 37°C, 5% CO_2 with daily visual control. The medium was changed every 24 hours (1/2 of the well volume). Each experimental point was repeated four times.

The proliferative activity (PA) of TC was estimated by the incorporation into the cell nuclei of ^{3}H-thymidine (3-HTdR, 5 Ci/mmol, 0.25 μCi/well, diluted with 3.5 μg/ml unlabeled thymidine), which was added to the incubation medium for 20 hours. The cells were then washed, treated with Versene, and transferred to fiberglass filter by means of a cell harvester (LBK, Sweden). The incorporation of 3-HTdR (cpm/min) was estimated with a β-counter. The stimulatory or inhibitory effect of HEF on TC was calculated by the following formula:

$$\% = \frac{\text{cpm TC} - (\text{cpm TC/HEF} - \text{cpm HEF})}{\text{cpm TC}}$$

where cpm TC is the 3-HTdR incorporation into control TC; cpm TC/HEF is the incorporation of the label into TC in close contact with HEF, and cpm HEF is the incorporation of the label into the irradiated HEF.

RESULTS

First, the effect of the seeding concentration and, consequently, the density of cell contacts on PA was determined, as a reference point for further comparison with TC and transformed cells. Three seeding concentrations were chosen: 1.25×10^4 cells per well (no contact between cells at the moment of seeding); 2.5×10^4 cells per well (subconfluent state with individual contacts between cells); 5×10^4 cells per well (dense monolayer with closely packed spindle-shaped cells). 3-HTdR incorporation was estimated every 48 hours after 20-hour incubation during 6 days. The growth curves in Fig. 1 demonstrate that cells seeded in the first concentration exhibited a maximum PA between the 4th and 6th day of the experiment. The second seeding concentration resulted in a higher initial PA level, which increased gradually but did not reach that observed for the first concentration. When the cells were seeded in the third concentration, PA did not rise over the experimental period and even revealed a tendency to decrease or even come to a complete stop. Evidently, cells seeded with minimal and middle density proliferated actively, while in the case of the maximal seeding density, resulting in close contacts between cells, their proliferation was almost completely arrested.

To estimate PA of TC the latter were seeded (1.0×10^4per well) in close contact with a dense monolayer of HEF (5.0×10^4 cells per well). 3-HTdR

TABLE 1. Characteristics of STHEF Strains

Strain	TGA	EMA	SMA
STHEF (parent)	1.7–2.4	$\gg2.0\times10^6$	–
STHEF–128	the same	$>3.0\times10^6$	–
STHEF–162	the same	1.0×10^6	–
STHEF–75/18	0.7–1.2	10^4–10^5	++
STHEF–MLN–6	1.5–1.7	the same	+
STHEF–MLN–8	1.2–1.4	the same	+++

Note: TGA, tumorogenic activity, determined by quantitative transplantation test as log of 50% transplantation dose; EMA: experimental metastasizing activity; SMA: spontaneous metastasizing activity.

TABLE 2. Effect of HEF on Proliferative Activity of TC of Different Malignancy (Results of Separate Experiments Are Are Expressed as % of Inhibition (−) or Stimulation (+) of Label Incorporation into TC versus control)

Cocultivation time, hours	Strain					
	STHEF	STHEF-128	STHEF-162	STHEF-MLN-6	STHEF-MLN-8	STHEF-75/18
24	-64, -79	-57, -56, -76	-60, -77, -89	-18, -31, -60, -54	-27, -16, -56, -70	-1, 28, -33, -31
48	-79, -86, -79	-75, -85, -52	-79, -89, -94, -70	-46, -54, -47, -40	-47, -35, -69, -67	-58, -56, -77, -75
72	-95, -94	-85, -82, -85	-92, -88, -84, -77	-77, -71, -63, -66	-40, -50, -50, -63	-68, -77, -74, -79
96	-82, -87, -82	-81, -70, -85	-82, -91, -91, -82	-68, -52, -66, -21	-16, +34, -23, +35 -26, -4	-19, -46, -40,
120	-76, -83, -83	-63, -81, -83	-90, -92, -96 -58	+30, +38, -21, -12	+70, +63, +86, -10, +75	+56, -17, -32,
144	-83	-83, -90, -81	-90, -87	+20, +5, +61, -13	+49, +53, +44 +28	+44, +24, +44,

incorporation was estimated every 24 hours during 6 days (Fig. 2). The results of individual experiments, expressed as an inhibition (-) or stimulation (+) of 3-HTdR incorporation into TC in close contact with NC (in % of control native TC), are shown in Table 2. Table 2 and Fig. 2 illustrate the difference in PA between TC strains used. PA of the parental strain STHEF and its low-malignancy variants STHEF-128 and STHEF-162 was considerably suppressed due to contacts with HEF over the whole period of observation (1-6 days). The reduction of PA for these strains ranged from 52 to 96%, with the lower values observed in the early period of coculturing (the first day) and the virtual arrest of proliferation at the later stages (2-6 days).

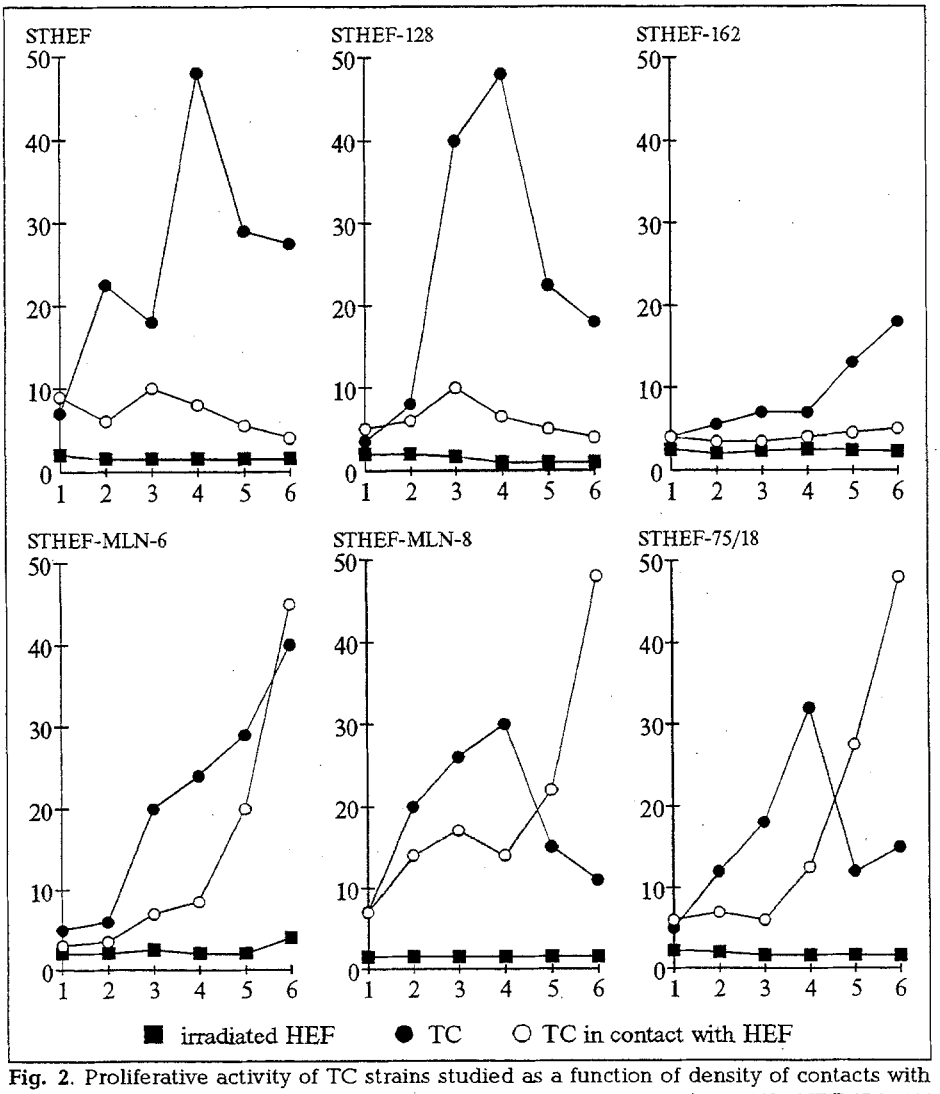

Fig. 2. Proliferative activity of TC strains studied as a function of density of contacts with normal cells. Abscissa: days in culture; ordinate: radioactivity (cpm/min×10³). HEF (5.0×10⁴ per well) seeded 24 hours before the experiment were irradiated and TC (1.0×10⁴ per well) seeded. The data are presented as the average of 4 replicate wells.

The proliferative activity of STHEF-MLN-6 cells was somewhat different. During the first day of coculturing with irradiated HEF, the inhibition of PA ranged from 18 to 60%, but on average was lower than that for the low-malignancy variants. The inhibition gradually increased and reached the maximal value on the third day (63-77%). We then observed (4th day) a reduction of the inhibitory effect of HEF, while a stimulatory effect (by +5 to +40%) was observed in some experiments starting from the 5th day.

In the case of STHEF-MLN-8 cells the PA dynamics was similar to that for STHEF-MLN-6 cells, except that the maximum of inhibition was detected on the 1ts-2nd day and accounted for 16-67%. A stimulatory effect of HEF on PA was observed on the 4th day in 2 out of 4 experiments, and on the 5th day STHEF-MLN-8 proliferation was enhanced in all experiments.

The proliferative activity of STHEF-75/18 was suppressed during the first 4 days, with maximum inhibition on the 3rd day (68-79%). The inhibitory effect decreased gradually on the 4th day (4-46%) and switched to stimulation (up to 75%) starting from the 5th day.

Thus, PA of low- and high-malignancy strains of TC in contact with a dense monolayer of NC is seen to be different. For example, PA of STHEF and its low-malignancy variants due to contact with HEF was considerably suppressed over the whole experimental period, and in some cases this suppression increased gradually. The more malignant strain STHEF-MLN-6 turned out to be less susceptible to the inhibitory action of HEF; this suppression declined during the experimental period and the PA of the strain in the late stages was even higher compared to intact cells. The high-malignancy strains STHEF-MLN-8 and STHEF-75/18 exhibited a low susceptibility to the inhibitory action of HEF from the start point of the experiment and gradually overcame it. Moreover, the PA was substantially increased due to the contact with HEF for 4-6 days.

We contend that the system used makes it possible to investigate the interactions between normal (irradiated) fibroblasts and cells of the same origin transformed *in vitro* as well as their high- and low-malignancy variants selected *in vivo*.

The data obtained show that, unlike the TC studied, normal cells (nonirradiated control) in a dense monolayer exhibit no PA. When cocultured with normal HEF, the PA of transformed STHEF was suppressed during the whole cultivation period to the point of complete arrest. Thus, cell interactions in the system, all components of which are derived from each other, demonstrate the existence of a proliferative control which NC exert over themselves (in a dense monolayer) and, to a greater or lesser extent, over TC. We found the inhibition of TC proliferation immediately after their contact with HEF cells. Evidently, normal cells in close contact with each other are able to transmit a PA inhibition signal to either normal or tumor cells. An essential difference of high-malignancy tumor cells is their reduced sensitivity to such an influence. Moreover, the strains with metastasizing phenotype due to selection *in vivo* (STHEF-MLN-8 and STHEF-75/18) gained the ability to escape the proliferative control from the normal cell environment, and, probably, to use its resources. The stimulation of tumor cell proliferation by normal fibroblasts is of great interest, especially in view of the lack of such an influence on low-malignancy strains of tumor cells.

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