Growth Inhibition of Oncogene-transformed Rat Fibroblasts by Cocultured Normal Cells: Relevance of Metabolic Cooperation Mediated by Gap Junctions[1]

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ABSTRACT

We have studied the proliferation of rat 208F cells (a derivative of Rat-1 cells) transformed by activated c-Ha-*ras*, v-*fgr*, v-*raf*, v-*fms*, or v-*src* oncogenes during cocultivation with an excess of early passage rat embryonic fibroblasts or immortal 208F cells. The total number and size of foci formed by oncogene-transformed 208F cells were strongly reduced by cocultured normal fibroblasts. The extent of growth suppression of transformed foci appears to be dependent on the type of transforming oncogene and on the type of normal fibroblasts rather than on the extent of gap-junctional communication between transformed and normal cells. Total inhibition of fluorescent dye transfer between normal and transformed cells by the 3β-O-hemisuccinate of 18α-glycyrrhetinic acid (18α-carbenoxolone), an inhibitor of gap-junctional communication in human fibroblasts, did not prevent growth inhibition of transformed cells in the cocultivation assay. Since adjacent cells remained electrically coupled in the presence of this inhibitor it is possible that the strongly reduced metabolic cooperation, as indicated by the lack of fluorescent dye transfer, is sufficient for mediating the growth-inhibitory effect of normal fibroblasts. 208F cell-conditioned medium, however, also caused strong growth inhibition of transformed derivatives, suggesting that the effect is in part mediated by release of stable growth inhibitor(s) from 208F cells.

INTRODUCTION

Intercellular communication mediated by gap junctions is assumed to be involved in modulation of cell growth, differentiation, tissue homeostasis, and synchronization of tissue reactions (1–3). Furthermore it has been suggested that normal cells in contact to transformed cells may exchange low molecular weight compounds through gap junctions, thereby modulating the neoplastic phenotype (4). The growth of various chemically and virally transformed cells is inhibited when they are in contact with normal cells of different types (5–10). This inhibition appears to be contingent on the presence of junctional communication between normal and transformed cells. Furthermore loss of the ability to communicate via gap junctions has been suggested to be decisive for progression to neoplasia by allowing initiated cells to escape from local growth control. Additional evidence for the role of intercellular communication in neoplastic transformation rests on observations that tumor promotors, such as 12-O-tetradecanoylphorbol-13-acetate, inhibit cell communication (for review see Ref. 11). From their studies of *in vitro* transformation of BALB/c 3T3 cells Enomoto and Yamasaki (12) concluded that disruption of gap-junctional intercellular communication plays an important role in the late phase (tumor promotion) of cell transformation. They found a good correlation between the ability of phorbol esters to inhibit gap-junctional communication and their ability to enhance transformation of BALB/c 3T3 cells. When transformation of BALB/c 3T3 cells is induced by chemicals or by transfection of

activated oncogenes, there is a selective lack of intercellular communication between transformed and surrounding nontransformed cells, although transformed as well as nontransformed cells can communicate among themselves (13, 14). On the other hand, when heterologous intercellular communication is established, a transjunctional flow of metabolites or signal molecules may result in a parasitic participation of invasive tumor cells in the metabolism of the normal host tissue. With an *in vitro* invasion assay, Bräuner and Hülser (15) found a coincidence of active tumor cell invasion with the ability of tumor cells to communicate with the host cells via gap junctions.

Recently a growth-inhibitory effect of primary fibroblasts was documented with v-*myc*-transformed quail muscle cells (9) or with v-Ha-*ras*-transformed BALB/c keratinocytes (10). In the latter cell system, however, cocultured established fibroblasts did not cause growth inhibition. In this paper we investigated whether gap-junctional communication is required for growth inhibition of oncogene-transformed rat fibroblasts caused by cocultured normal fibroblasts. In this context we wanted to find out whether immortalization of normal fibroblasts or the type of transforming oncogene influence the inhibitory effect. For this purpose we used early passage REF[3] or immortal, nontumorigenic rat 208F cells and studied their effect on 208F cells transformed by different types of oncogenes. By using REF cells we furthermore wished to test the hypothesis that cells initiated in transformation can be transformed only when intercellular communication is blocked. In this case normal and transformed 208F cells should exhibit no gap-junctional communication with REF cells. In contrast to 208F cells, REF cannot be transformed by transfection of only one transforming oncogene (16).

Our studies were done with five 208F-derived cell lines transformed by the following oncogenes: (*a*) activated c-Ha-*ras*, coding for a membrane-bound defective GTPase (17); (*b*) v-*src* coding for a membrane-bound tyrosine kinase (18); (*c*) v-*fms* encoding the cell surface receptor for macrophage colony-stimulating factor 1 (19) which carries an intracellular tyrosine kinase activity; (*d*) v-*fgr* that encodes a protein-tyrosine kinase related to the *src* oncogene family (20); and finally (*e*) v-*raf* that codes for a serine-, threonine-specific protein kinase located in the cytoplasm (21).

MATERIALS AND METHODS

Plasmids and Chemicals. Plasmid pEJ carries the activated Ha-*ras*-1 gene of the human bladder carcinoma cell line EJ on a 6.6-kilobase *Bam*HI fragment (22), pSM-FeSV contains the complete Susan McDonough feline sarcoma provirus with the v-*fms* oncogene (23), pGR-FeSV harbors the complete Gadner-Rasheed feline sarcoma provirus with the v-*fgr* oncogene (24). Plasmid pv-raf contains a 4.2-kilobase fragment of 3611-murine sarcoma virus with the complete v-*raf* oncogene and the 5′ long terminal repeat region (25). Plasmid pY3 codes

[3] The abbreviations used are: REF, rat embryonic fibroblasts; ACO, 18α-carbenoxolone; FITC, fluorescein isothiocyanate.

for resistance to hygromycin B (26).

The sodium salt of 18α-glycyrrhetinic acid 3β-O-hemisuccinate (ACO) was a generous gift of Biorex Laboratories, Enfield, Great Britain. The compound has been shown to inhibit intercellular gap-junctional communication between human fibroblasts (27). Stock solutions were prepared in distilled water at a concentration of 4 mg/ml.

Cells and Culture Conditions. All cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (standard medium) at 10% CO_2 and 37°C. REF were prepared from 18-day-old embryos of BDIX rats and used at the 5th passage. Rat 208F cells are derived from Rat-1 cells defective for hypoxanthine phosphoribosyltransferase activity (28). Different oncogene-transformed cell lines were isolated after cotransfection of 208F cells with oncogene-coding plasmids as well as pY3 and after selection in medium containing 300 μg/ml hygromycin B (Boehringer Mannheim GmbH). After Southern blot analysis transformed cell lines harboring the lowest number of oncogene copies were chosen for further studies. The cell line F-RAS contains about 8 copies of the activated c-Ha-ras-1 gene, F-FGR cells harbor 4 copies of v-fgr gene, F-FMS cells contain one copy of v-fms, and F-RAF cells harbor one copy of v-raf. The cell line FSC-2, a xanthine-guanine phosphoribosyltransferase-positive derivative of 208F cells containing the v-src oncogene, was obtained from R. Schäfer, Zürich, Switzerland (29).

Analysis of Anchorage-independent Growth. Colony formation in semisolid agar medium was measured by seeding 10^2, 10^3, and 10^4 cells into standard medium in the presence of 0.15% (w/v) Difco Noble agar. Colonies 2–3 mm in diameter were counted after 3 weeks.

Cocultivation Assay. In cocultivation experiments 2×10^2 oncogene-transformed 208F cells were seeded in standard medium in 60-mm dishes. Eighteen h later fresh medium with 10^6 REF or 208F cells was added. During further routine cultivation the medium was renewed every 2 days to minimize depletion of growth factors. In some experiments the medium was replaced every day. After 2 weeks the number and mean area of transformed foci were determined after staining with Wright-eosin dye.

Labeling Cells with Latex Beads. The transformed cells could easily be distinguished from normal cells by their distinct morphology. In order to exclude all doubts, however, in some experiments the transformed cells were marked with FITC-conjugated dextran (M_r ~40,000; Sigma), which was endocytosed by the cells (7). Labeling was performed for 24 h in standard medium supplemented with 0.1% FITC-dextran. This cell labeling did not affect communication frequency or growth rate of the cells.

Communication Assays. Gap-junctional intercellular communication was measured by transfer of the fluorescent dye Lucifer yellow (Sigma) as described previously (30). Individual cells were injected iontophoretically with a hyperpolarizing current (Iontophoresis Programmer model 160; World Precision Instruments, Inc., New Haven, CT) using a 4% (w/v) solution of Lucifer yellow dissolved in 1 M LiCl. Homologous junctional communication was measured in almost confluent cultures 24 h after the cells were seeded. Communication frequency indicates the proportion of cells showing dye transfer to one or more neighboring cells of the first order. For each measurement of communication frequency 20 cells were injected. For measuring heterologous communication 5×10^3 FITC-dextran-labeled transformed cells were added after 6 h to about 3×10^5 normal cells in a 35-mm dish. Twenty-four h later 20 transformed cells were microinjected with Lucifer yellow and the communication frequency to surrounding normal cells was determined.

Ionic coupling between homologous or heterologous cells was qualitatively determined by using two microelectrodes filled with 3 M KCl. Electrodes were inserted into adjacent cells of an almost confluent monolayer. Current pulses of 10–20 nA with a frequency of 0.6 Hz were applied with one electrode and the resulting voltage deflections were registered with the second electrode in an adjacent cell.

Conditioned Medium. REF and 208F cells were grown to confluency in standard medium and washed twice with phosphate-buffered saline. Serum-free standard medium was added, harvested after 48 h of incubation, centrifuged for 10 min at $1500 \times g$ and stored at 4°C. During cultivation conditioned medium supplemented with 10% fetal calf serum was added to transformed 208F cells and renewed every 2 days.

RESULTS

Growth Properties of Normal and Transformed Cells. REF and 208F cells grow as monolayers of well spread cells that do not pile up even when they are maintained at confluency for several weeks. In contrast the cell lines F-RAS, F-RAF, F-FGR, F-FMS, and FSC-2 grow to high cell densities and exhibit the transformed phenotype of relatively small, spindle-shaped cells loosely attached to the surface of the culture dish. While REF and 208F cells do not proliferate in semisolid agar medium, the transformed 208F derivatives show cloning efficiencies between 52 and 79% (Table 1).

For cocultivation assays transformed cells were grown in the presence of a 5×10^3-fold excess of normal REF cells. Under these conditions the proliferation of transformed cells was strongly inhibited. The average colony size was drastically reduced (39 to 92% inhibition) as well as the number of colonies (25 to 79% inhibition) (cf. Table 2 and Fig. 1b as an example). In one experiment the growth medium of cocultured REF cells was changed every day. Under these conditions even higher percentages of growth inhibition were found: F-RAS, 74 (23)%; F-RAF, 91 (84)%; F-FMS, 100 (100)%; F-FGR, 100 (100)% inhibition of average colony size (colony number). Thus REF cells exert a strong growth inhibition on transformed 208F cells. This inhibition was even more pronounced when nontransformed 208F cells were used. Under these conditions the proliferation of oncogene-transformed 208F cells was totally inhibited by surrounding normal 208F cells. No foci of transformed cells were detected after 2 weeks of cocultivation (cf. Table 2 and Fig. 1c as example). All transformed cells seeded could be rescued from the excess of 208F cells after 3 days of cocultivation by trypsinization and reseeding the cells in hygromycin containing medium. At later times the monolayer of 208F cells could not be completely disrupted which prevented the outgrowth of transformed cells.

Gap-junctional Communication. Table 1 shows that the oncogene-transformed 208F-derived cell lines as well as the parental 208F cells exhibit close to 100% homologous gap-junctional communication. Probing heterologous communication revealed transfer of microinjected Lucifer yellow from transformed cells to REF cells (Table 2), although to a lower extent compared to homologous communication (Table 1). The transformed cells can be divided into two groups corresponding to the extent of heterologous communication with REF cells. F-RAS, F-RAF, and F-FMS cells exhibit heterologous communication frequencies of 70 to 75% whereas F-FGR and FSC-2 cells show heterologous communication frequencies of 20% (Table 2). There is no obvious correlation between the extent

Table 1 *Colony formation in semisolid agar medium and homologous gap-junctional communication of attached growing normal (REF and 208F) and oncogene-transformed cells*

Cells	Cloning efficiency (%)	Communication frequency (%)[a]
REF	0	90
208F	0	95
F-RAS	75	100
F-RAF	53	100
F-FMS	52	100
F-FGR	60	100
FSC-2	79	93

[a] Proportion of cells exhibiting transfer of microinjected Lucifer yellow to first order neighboring cells.

Table 2 *Inhibitory effect of REF or 208F cells on the growth of oncogene-transformed 208F cells*

Oncogene transformed cells	Transformed cells alone [av. colony size: c_2 (mm)]	Transformed cells cocultured with REF cells			Transformed cells cocultured with 208F cells	
		[Av. colony size: c_1 (mm)]	% of growth inhibition (R^a)	Communication frequency[b] (%)	% of growth inhibition (R^a)	Communication frequency[b] (%)
F-RAS	11.5 (170)[c]	7 (128)	39 (25)	75	100 (100)	95
F-RAF	6.7 (127)	1.9 (66)	72 (48)	75	100 (100)	100
F-FMS	13.5 (164)	1.7 (91)	87 (45)	70	100 (100)	65
F-FGR	4.1 (99)	0.8 (21)	81 (79)	20	100 (100)	20
FSC-2	3.7 (83)	0.3 (31)	92 (63)	20	100 (100)	40

[a] $R = 100 \frac{c_2 - c_1}{c_2}$ related to colony size or colony number.

[b] Proportion of transformed cells tested exhibiting transfer of microinjected Lucifer yellow to first order normal cells.
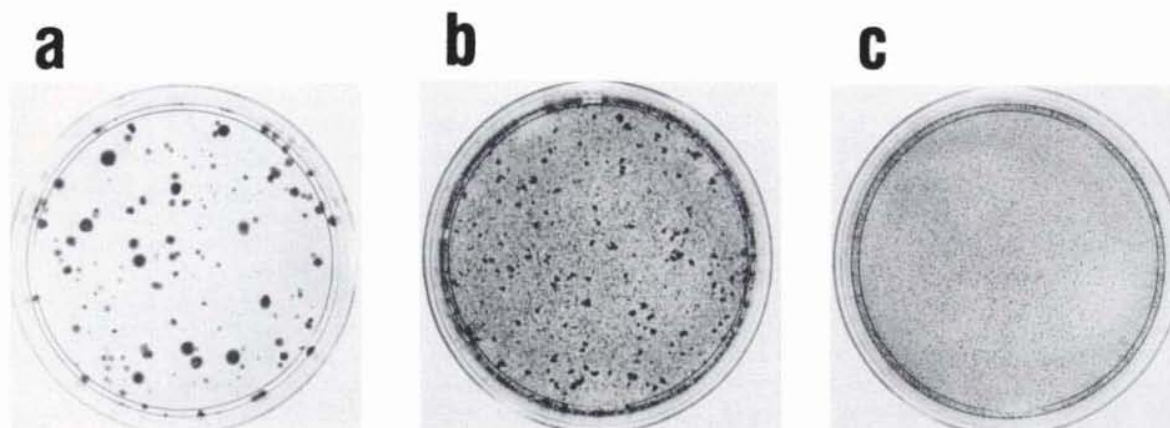
[c] Numbers in parentheses, colony number.

Fig. 1. Foci of F-RAF cells stained after 2 weeks of growth without cocultured cells (*a*). In the presence of cocultured REF (*b*), the average colony size is strongly reduced. No colonies were detected in the presence of cocultured 208F cells (*c*).

of growth inhibition caused by cocultured normal cells and frequencies of heterologous communication between transformed and normal cells.

Heterologous communication between oncogene-transformed 208F cells and their normal parental 208F cells was at least as high as shown for REF cells (Table 2). In the case of c-Ha-*ras*- and v-*raf*-transformed cells we found that the frequencies of heterologous communication to the parental cells were about 100%, *i.e.*, as high as detected for homologous communication (*cf.* Table 1). In order to make sure that communication frequencies did not change dramatically during cocultivation we measured heterologous and homologous gap junctional communication in cocultures of transformed foci and REF cells after 2 weeks of cocultivation. Such an experiment could not be carried out with 208F cells since no transformed foci could be detected after 2 weeks of cocultivation. Frequencies of heterologous communication between REF and transformed 208F cells were at least as high (40 to 100%) as measured 24 h after seeding the cells following the standard protocol. REF cells exhibited homologous communication at 90 to 100% after 2 weeks in cocultures. Transformed cells near the outer border of foci showed 100% communication whereas in the middle of transformed foci communication frequencies between 40% (F-FGR cells) and 100% (F-FMS cells) were measured. Thus frequencies of gap junctional communication did not change very much between cocultured cells during 2 weeks of cocultivation. The measurements of dye transfer 24 h after onset of cocultivation are representative for the intercellular communication during the following 2 weeks. Thus far our results show that growth inhibition of transformed cells by surrounding normal cells and heterologous gap-junctional communication

correlate in this cell system. In order to analyze whether or not there exists a causal relationship between these parameters the same cocultivation experiments as above were performed in the presence of an inhibitor of gap-junctional communication.

Effects of 18α-Carbenoxolone on Gap-junctional Communication. ACO is a water-soluble derivative of 18α-glycyrrhetinic acid that has been shown to inhibit gap-junctional communication between human fibroblasts in a reversible manner (27). The mechanism of action of this compound is not clear. It appears unlikely that glycyrrhetinic acid and its derivatives cause inhibition of junctional communication by activation of protein kinase C or by increasing the cytosolic Ca^{2+} content (31). It has been proposed that glycyrrhetinic acid derivatives intercalate into the plasma membrane and bind to the gap junction connexon, thereby inducing a conformational change which results in closure of the channel (27).

In order to use ACO in cocultivation experiments over 2 weeks we tested the stability and inhibitory effect of this compound on cell proliferation. We found that ACO did not lose its inhibitory activity on fluorescent dye transfer in 208F cells over 3 days in culture medium at 37°C. Above a minimal concentration of 20 μM ACO in the medium the junctional communication of 208F cells was inhibited in a concentration-dependent manner as measured by transfer of Lucifer yellow (data not shown). Inhibition was complete at a concentration of 50 μM ACO. The inhibitory effect became detectable after 2 h and reached its maximum after 4 h. At a concentration of 57 μM ACO total inhibition of dye transfer in all cells tested (REF, 208F cells, and all transformed 208F cells) was observed (*e.g.*, see Fig. 2). Likewise heterologous gap-junctional communication between REF, 208F cells, and transformed 208F cells was
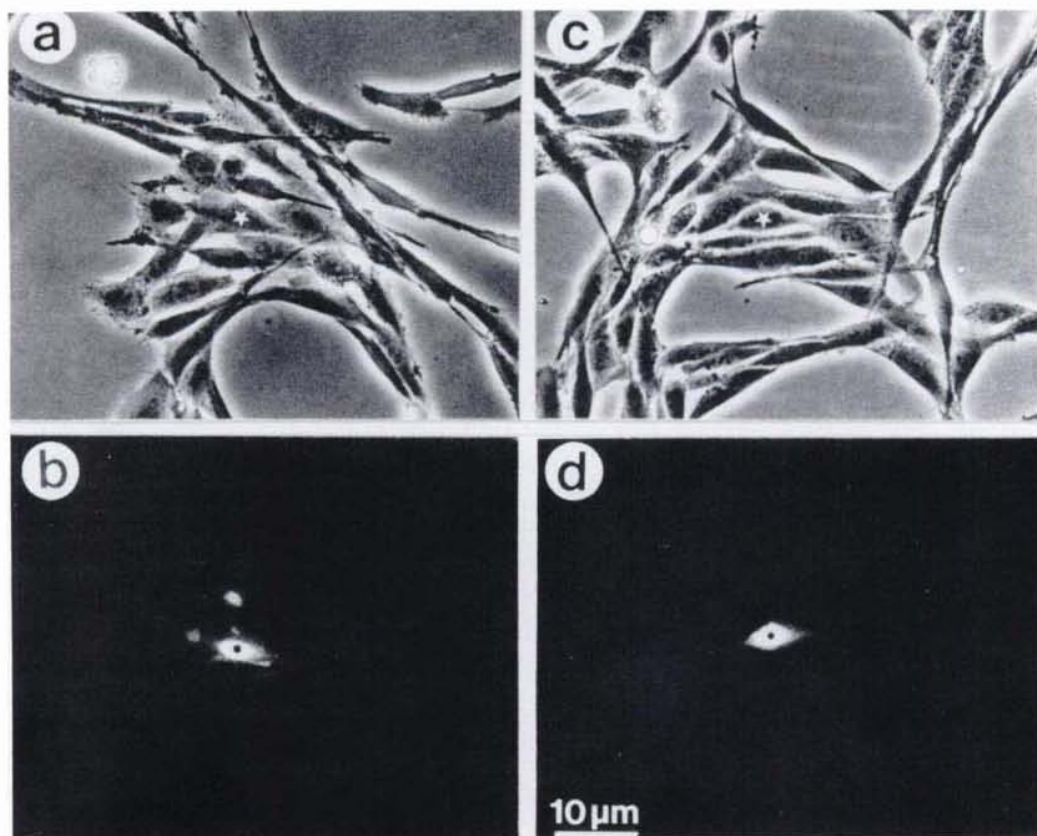
Fig. 2. Transfer of Lucifer yellow in F-RAF cells before (a, b) and after incubation in the presence of ACO (57 μM) for 4 h (c, d); a and c, phase-contrast micrographs; b and d, fluorescence micrographs of the same area 2 min after injection of Lucifer yellow, •, microinjected cell.

blocked (Fig. 3). The same results were obtained after 2 weeks of cocultivating transformed 208F cells and REF. Clonal growth of transformed cells, as measured by average size and number of colonies after 2 weeks of cultivation, was reduced by maximally 10% in the presence of 57 μM ACO.

Cocultivation experiments of oncogene-transformed cells and normal cells were performed in the presence of 57 μM ACO. Medium containing the inhibitor was renewed every 2 days to avoid a decrease in concentration of biologically active ACO. As shown in Table 3 the presence of the ACO did not significantly alter the inhibitory effect of normal cells (REF or 208F cells) on the clonal growth of onocogene-transformed 208F cells. These results appear to be at variance with the hypothesis that gap-junctional transfer of metabolites is required for the inhibitory effect of normal cells on transformed cells. Inhibition of gap-junctional communication by 18α-glycyrrhetinic acid was detected in cultured human fibroblasts by an assay of metabolic cooperation assay using the transfer of argininosuccinate between cells as a measure of junctional communication (31). Since Lucifer yellow (M_r 457) has a molecular weight similar to that of argininosuccinate (M_r 425) we wanted to determine whether the gap junctional transfer of ions is also inhibited by ACO. Thus electrical coupling between cells was measured in the presence and absence of ACO.

Table 4 shows that in the presence of 57 μM ACO, a concentration that totally inhibits Lucifer yellow transfer between cultured rat fibroblasts, ionic coupling could still be detected in all cells assayed. The extent of ionic coupling was diminished in the presence of ACO, however (not shown in Table 4), and the input resistance of the cell increased within seconds after contact of the inhibitor with the cells.

Effect of Conditioned Medium on Focal Growth of Oncogene-transformed 208F Cells. Could the growth inhibition of transformed rat cells by normal rat fibroblasts be due to other types of intercellular communication not mediated by gap junctions? In Table 5 we compared the effects of culture media conditioned by REF or 208F cells on the proliferation of oncogene-transformed cells.

Cultivation of transformed cells in REF-conditioned medium resulted in a slight increase of cloning efficiencies. The additional appearance of mainly small clones is responsible for the observed slight decrease of average colony size of transformed cells. In contrast, 208F conditioned medium caused strong growth inhibition (Table 5). Conditioned medium was harvested after cultivation of normal cells in serum free medium for 48 h in order to arrest proliferation of the cells and to minimize consumption of medium components during this time. Cultivation of transformed cells in conditioned medium was carried out after addition of fresh serum, in analogy to control cultures growing in standard medium. Conditioned medium harvested after 48 h from normal cells growing in serum containing standard medium showed an inhibitory effect that was at least as high as observed with serum-free conditioned medium. The results suggest that the observed inhibitory effects of cocultured 208F cells on oncogene-transformed cells are at least in part mediated by 208F-conditioned medium. Clonal growth of transformed 208F cells was not inhibited in medium supplemented with purified plasma membranes of REF or 208F cells (100 μg of protein/ml of medium; data not shown).

DISCUSSION

The data presented in this paper confirm that the proliferation of oncogene-transformed rat 208F fibroblasts can be inhib-
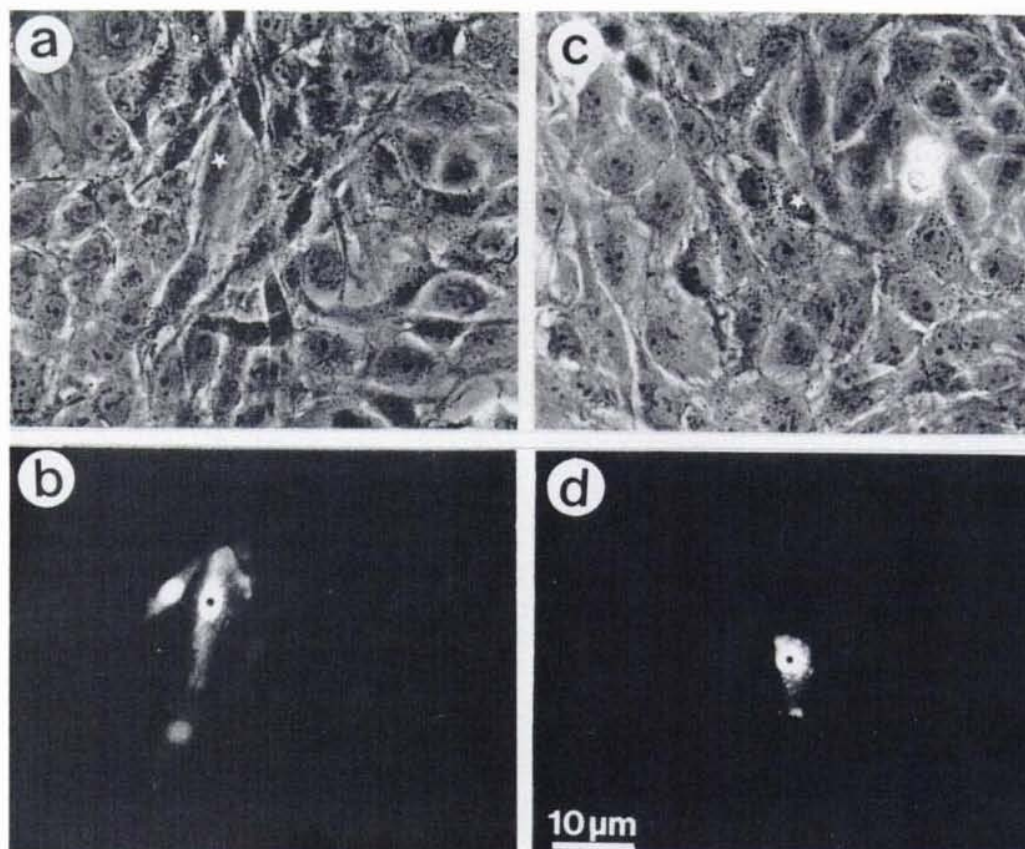
Fig. 3. Transfer of Lucifer yellow between F-RAF and 208F cells before (*a*, *b*) and after incubation in the presence of ACO (57 μM) for 4 h (*c*, *d*); *a* and *c*, phase-contrast micrographs; *b* and *d*, fluorescence micrographs of the same area 2 min after injection of Lucifer yellow. •, microinjected cell.

Table 3 *Inhibitory effect of cocultured REF and 208F cells on the growth of oncogene-transformed 208F cells in the presence of ACO (57 μM)*

Oncogene-transformed 208F cells	% of growth inhibition $(R)^a$ in			
	Cocultures with REF cells		Cocultures with 208F cells	
	No ACO	+ACO	No ACO	+ACO
F-RAS	39	44	100	88
F-RAF	72	87	100	100
F-FMS	87	67	100	100
F-FGR	81	69	100	100
FSC-2	92	100	100	100

^{a} *R*, colony size (*cf.* Table 2).

ited by confluent normal cells. Cocultured immortal 208F cells cause an even stronger inhibition of clonal growth than REF cells. Compared to homologous communication we have observed a decrease of heterologous gap-junctional communication between transformed 208F cells and REF or 208F cells as measured by Lucifer yellow transfer. The gap-junctional communication between c-Ha-*ras*- or v-*raf*-transformed 208F cells and cocultured 208F cells was found to be very similar compared to homologous communication among the transformed cells. This is in contrast to previous results that there is a complete block of communication between v-Ha-*ras* (8)-, activated c-Ha-*ras* (14)- or v-*src* (8)-transformed mouse 3T3 cells and cocultured nontransformed 3T3 cells. For none of the transformed rat fibroblasts studied in this paper a total loss of heterologous communication to cocultured normal cells was found. The extent of decreased heterologous communication seems to depend on the transforming oncogene of the transformed partner. Heterologous communication in this system of

transformed and normal cells does not correlate with the extent of growth inhibition caused by cocultured normal cells. Thus we conclude that the inhibitory effects of normal cells on transformed cells (5–10) may not be mediated in all cell systems only by gap-junctional communication.

The mechanisms by which oncoproteins in transformed cells are able to cause a suppressive effect on junctional communication to normal cells are not known. Since gene products of all onocogenes tested appear to be part of the signal-transducing

Table 4 *Ionic coupling in homologous and heterologous cultures*
ACO (57 μM) was added 4–5.5 h before measurements.

Cell lines	No ACO	+ACO
208F/208F	12/12	$5/5^a$
F-RAF/F-RAF	6/6	5/5
F-FGR/F-FGR	13/13	6/6
208F/F-RAF	^{b}	6/6
208 F/F-FGR	6/6	5/6

^{a} Number of ionically coupled cells/number of measurements.
^{b} Not determined because of 100% dye coupling.

Table 5 *Influence of REF- and 208F-conditioned medium on the growth of oncogene-transformed 208F cells*

Oncogene-transformed cells	% of growth inhibition $(R)^a$ caused by	
	REF-conditioned medium	208F-conditioned medium
F-RAS	19 (0)	100 (100)
F-RAF	21 (0)	66 (83)
F-FMS	10 (0)	67 (88)
F-FGR	8 (0)	79 (78)
FSC-2	0 (0)	38 (52)

^{a} *R*, colony size (colony number) (*cf.* Table 2).

pathway associated with phospholipid turnover (32–35), transformation-induced elevated levels of 1,2-diacylglycerol or inositol-1,4,5-triphosphate or both might mediate the decrease of gap-junctional communication reported here (36, 37). Furthermore it has been described for normal human epidermal keratinocytes that epidermal growth factor and transforming growth factor β inhibit gap-junctional communication (38). Transformation-induced alterations of growth factor production or secretion could be responsible for the decreased communication (39). These interpretations require that heterologous communication should be more sensitive to these signals than homologous communication.

Alternatively the decrease of heterologous communication between transformed and normal cells could be caused by transformation-associated cell surface alterations that prevent cell-cell recognition between normal and transformed cells (40). Such changes include variations of the extracellular matrix (41), cytoskeleton (42), surface proteoglycans (43), or membrane fluidity (44). These changes could preferentially affect heterologous communication. Our observation that heterologous communication between normal and transformed rat fibroblasts appears to be dependent on the type of transforming oncogene supports the latter possibility.

In our experiments complete inhibition of metabolic cooperation between normal and transformed rat fibroblasts by the glycyrrhetinic acid derivative ACO did not extend similarly to electrical coupling between these cells. Thus we cannot rule out that even the strongly inhibited metabolic cooperation or ion transfer between cells might still be sufficient to mediate the growth inhibition inflicted upon transformed fibroblasts by surrounding normal cells. It is unlikely, however, that regulatory compounds with molecular weights around 400 or larger pass through gap junctions in this process, since transfer of Lucifer yellow is blocked completely by ACO. ACO may decrease the opening time of gap junction channels to such a large extent that transfer of Lucifer yellow is no longer detectable although electrical coupling can still be demonstrated.

In addition to gap-junctional communication other forms of interaction among cells may contribute to the observed growth inhibition, for example humoral factors and cell-cell interactions. We have observed that conditioned medium of 208F cells causes decreased clonal growth of transformed 208F cells. Thus stable, secreted growth inhibitor(s) may be involved. In contrast conditioned medium of REF shows no effect. For human fibroblasts (45), mouse embryo fibroblasts (46), and 3T3 fibroblasts (47) secreted growth inhibitors have been described. Since transformed 208F cells are inhibited by glutaraldehyde-fixed 208F and REF cells to the same extent (about 80%, data not shown) we suppose that growth inhibition by 208F-conditioned medium may enhance an inhibitory effect caused by components of fibroblast membranes. It has been reported (48) that the growth of human diploid fibroblasts is inhibited by a membrane-bound glycoprotein isolated from contact-inhibited cells. Furthermore membrane-anchored precursors of transforming growth factor α are able to react with epidermal growth factor receptors on adjacent cells thereby transmitting a growth-regulatory signal (49). This could also be the case with negative growth factors. Until now purified REF and 208F membranes did not inhibit growth of transformed 208F cells. We suppose that the potentially effective membrane component has not remained intact during the preparation procedure and the co-cultivation assay.

The cell system presented here appears to be very powerful for dissecting the mechanism(s) by which adjacent normal cells can suppress proliferation of transformed cells.

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