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THE USE OF *IN VITRO* ASSAYS FOR NEOPLASTIC TRANSFORMATION AND TUMOR PROMOTION

S. ROSA and M. BIGNAMI

Laboratorio di Tossicologia Comparata ed Ecotossicologia, Istituto Superiore di Sanità, Rome, Italy

Summary. - *We investigated growth control in mixed cultures of normal and oncogene-transformed mouse fibroblasts. NIH/3T3 transformed by* v-myc, *polyoma large T, polyoma middle T,* v-ras *and* v-src *showed comparable cloning efficiencies in agarized medium. However when cultivated with an excess of normal cells (Balb/3T3, C3H10T1/2 or primary rat and hamster embryo cells)* ras, src, *and middle T-transformed cells were able to form "foci" of transformation on the layer of density arrested normal cells, whereas* myc- *and polyoma large T-transformed cells lacked this ability. Addition of the phorbol ester tumor promoter, phorbol-12,13-didecanoate, rescued proliferation and focus-formation by these nuclear oncogenes-transformed cell lines. Evidence is presented and discussed supporting a main role of intercellular communication between normal and transformed cells in modulating suppression or expression of the transformed phenotype.*

Riassunto (Uso di saggi *in vitro* per lo studio della trasformazione neoplastica e della promozione tumorale). - *Il presente studio è focalizzato sui processi di controllo della crescita in colture cellulari miste di fibroblasti di topo normali e trasformati con oncogeni. Cellule NIH/3T3 trasformate da* v-myc, *large T di polioma, middle T di polioma,* v-ras *e* v-sarc *presentano efficienze di clonaggio confrontabili in terreno contenente agar. Tuttavia quando vengono coltivate in presenza di un eccesso di cellule normali (cellule Balb/3T3, C3H10T1/2 o colture primarie di embrioni di ratto e hamster), solo le cellule trasformate da* ras, sarc, *e middle T sono in grado di formare "foci" di trasformazione su un monostrato di cellule normali. Le cellule trasformate dagli oncogeni nucleari* myc *e large T di polioma recuperano la proliferazione e la capacità di formare "foci" dopo aggiunta del promotore tumorale estere del forbolo, forbolo-12,13-didecanoato. Sono presentati e discussi dati sperimentali che indicano un ruolo principale della comunicazione intercellulare tra cellule normali e trasformate nelle modulazione della soppressione o espressione del fenotipo trasformato.*

Introduction

The growth of normal cells in culture is a strictly regulated process and evidence suggests that cellular proliferation is regulated by environmental signals which are either the spatial restriction of cell-to-cell contacts or appropriate extracellular growth factors. Several conditions such as viral infections, exposure to chemical carcinogens or radiations, can dramatically change the growth properties of cells cultured *in vitro*. The loss or relaxation of growth control mechanisms and the consequent acquisition of new cellular characteristics by tumor cells define the process called "neoplastic transformation". The altered growth features acquired by neoplastic cells in culture are basically summarized into the following properties: 1) transformed cells proliferate at a higher saturation density than normal cells and produce discrete focal areas of growth on a dense monolayer of normal cells, the so-called "foci" of transformation; 2) transformed cells growth is anchorage independent, i.e. neoplastic cells form colonies when plated in agarized medium while normal cells do not; 3) transformed cells form tumors after injection into susceptible animals. These parameters of neoplastic transformation are utilized to define the characteristic properties of a transformed cell line.

Cells transformed by retroviruses represent an excellent biological system for studying the mechanisms of neoplastic transformation *in vitro*. Retroviruses carry in their genomes specific sequences, known as viral oncogenes (v-*onc*), that once integrated into the cellular genome are responsible for the transformed phenotype and confer to the cell population the ability to form tumors *in vivo*. In this paper we report data concerning the role of normal cells in modulating the expression of neoplastic transformation induced by viral oncogenes; in particular we determined whether the presence of an excess of normal cells could influence the proliferation of a small number of fibroblasts transformed by different oncogenes. Furthermore the ability of the tumor promoter phorbol-12,13-didecanoate (PDD) to enhance the transformation efficiency of various oncogenes was analyzed.

Results and discussion

By using either transfection or infection we isolated various NIH/3T3 clonal cell lines containing the oncogenes v-*myc* from MC29, v-*src* from Schmidt-Ruppin D and v-*ras* from raszip 6, respectively. Clonal cell lines of NIH/3T3 transformed by these viral oncogenes were characterized by the high level of expression of their respective oncogenes and by their anchorage independance as shown by their ability to clone in soft agarose [1]. When a small number (100 or 1000) of v-*onc* transformed cells were plated together with an 100-1000 fold excess of normal cells, only v-*src* and v-*ras* transformed cells but not v-*myc*-bearing cells were able to form distinct "foci" of morphological transformation onto the surrounding monolayer [2]. Several murine cell lines have been tested as normal partners in this co-culture assay: NIH/3T3, BALB/3T3, C3H10T1/2 and rat 1 cells, all displayed comparable inhibitory properties on v-*myc*- but not on v-*src* and v-*ras*-induced cell proliferation.

In view of the repeated observations that the presence of the activated *ras* oncogene is not sufficient to confer a transformed phenotype to primary rat or hamster embryo cell cultures [3, 4], it was interesting to clarify whether these types of primary cells were able to suppress the transformed phenotype of the "immortalized" and *ras*-containing NIH/3T3 cell line. The results, shown in Table 1, indicate that primary hamster and rat whole embryo cells similarly to the normal but "immortalized" cell lines described above, did not suppress the ability of v-*src* and v-*ras* NIH/3T3 cells to form "foci" of transformation on the surrounding monolayer. These results are consistent with the hypothesis that in primary normal cells at least two independent steps, the "immortalizing" *myc*-like event and the "initiating" *ras*-like event, are required for the full expression of the transformed phenotype.

The observation that morphological transformation was suppressed in the case of v-*myc*, a nuclear oncogene, but not in the case of v-*src* and v-*ras*, both cytoplasmic oncogenes, prompted us to investigate whether the suppressive ability of normal cells could be predicted on the basis of the nuclear or cytoplasmic localization of the oncogene proteins. To this purpose NIH/3T3 cells transformed by the nuclear oncogene large T of polyoma and by the cytoplasmic oncogene middle T of polyoma were constructed by infection with the viral vectors zipLT and zipMT, respectively. Different clones containing these two oncogenes showed comparable transformation characteristics as determined by their similar efficiencies in the formation of colonies in soft agarose (Table 2).

In a co-coltivation assay with C3H10T1/2, proliferation of large-T transformed NIH/3T3 cells was suppressed by normal cells (Fig. 1, a and b), while middle-T transformed NIH/3T3 (Fig. 2, a and b) were able to form dinstinct "foci" of transformation (Table 3). These data are in agreement with the hypothesis that transformation induced by nuclear oncogenes can be suppressed by normal cells while cytoplasmic oncogenes escape to this control.

Among the various possibilities to explain these observations we considered the hypothesis that cytoplasmic molecules concerned with growth regulation might be transferred directly between adjacent cells through cell-

Table 1. - *Focal outgrowth of* v-src *and* v-ras-*transformed NIH/3T3 onto hamster and rat primary cultures*

	5% serum			10% serum		
	C.E. on plastic (%)	Foci	Recovery (%)	C.E. on plastic (%)	Foci	Recovery (%)
Hamster primary cultures						
NIH/3T3 *ras*6	32	26	81	35	22	63
NIH/3T3 *ras*7	63	61	97	70	67	96
NIH/3T3 *src*1	63	60	96	52	51	98
NIH/3T3 *src*2	29	30	100	32	39	100
Rat primary cultures						
NIH/3T3 *ras*7	64	41	66	69	45	65
NIH/3T3 *src*1	62	49	79	52	42	80

Primary cell cultures: 3 x 10^5/60 mm dish; similar results were obtained with an inoculum of 6 x 10^5 tertiary cells
Oncogene-transformed NIH/3T3: 200 cells/60 mm dish

Table 2. - *Anchorage-independance of polyoma middle T- and large T-transformed NIH/3T3*

Cell line	C.E. in soft agar (%)
NIH/3T3	< 0.01
middle T	
NIH/3T3 S2	11
NIH/3T3 S4	13
large T	
NIH/3T3 SA	12
NIH/3T3 SB	19

10^2 - 10^3 - 10^4 cells were seeded in 0.33% agarose on a bottom layer of 0.5% agarose

Table 3. - *Focal outgrowth of polyoma middle T- and large T-transformed NIH/3T3 onto C3H10T1/2*

Cell line	C.E. on plastic (%)	C.E. on 10T1/2 (%)	Suppression (%)
middle T			
NIH/3T3 S2	64	66	0
NIH/3T3 S4	82	83	0
large T			
NIH/3T3 SA	65	0	100
NIH/3T3 SB	93	0	100

normal cells : 3 x 10^5/60 mm dish; transformed cells: 200/60 mm dish

cell channels (gap junctions) [5]. Therefore this phenomenon might depend on cell-cell contacts between normal and transformed cells i.e. on the ability to establish intercellular communication (IC). This hypothesis found support by a study on the level of IC between normal and transformed cells as measured by the Lucifer yellow transfer assay [6]. In fact, when normal cells were microinjected with the Lucifer yellow dye, spread of the fluorescent dye was observed from normal cells to v-*myc* transformed NIH/3T3 cells indicating that IC was present between these cell populations. On the contrary when normal cells cocultivated with v-*src* or v-*ras* transformed cells were microinjected, very little dye transfer was observed between these transformed cells and normal cells.

Several recent papers [7, 8] have suggested that tumor promoters can enhance "focus" formation in particular experimental settings thus prompting an analysis of the effects of tumor promoters in our system. When cocultures of C3H10T1/2 and v-*myc* transformed cells were treated with tumor promoters (12-O-tetradecanoylphorbol-13-acetate (TPA), PDD, mezerein, etc), an almost complete recovery of focus formation by v-*myc*-bearing cells was observed [1, 2]. Similarly, large T-induced cell transformation was also rescued at a high percentage in the presence of the tumor promoter PDD (Fig. 1 c and Table 4), while no effect was observed in the case of middle T-induced cell transformation (Fig. 2 c). The mechanisms by which PDD exerts this effect is not entirely clear as tumor promoters can elicit a wide range of effects on cultured cells. Howewer considering that PDD can inhibit various form of IC we suggested that PDD rescued the growth of v-*onc* transformed cells by inhibiting IC with normal cells. Junctional communication capacity of normal as well

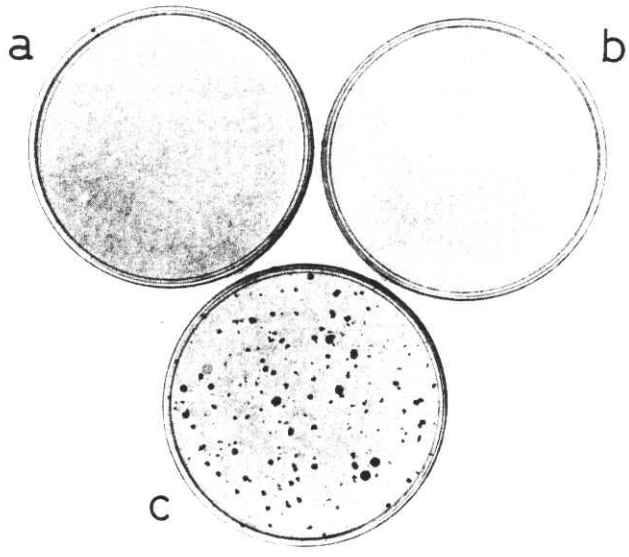

Fig. 1. - Focal outgrowth of polyoma large T-transformed NIH/3T3 onto C3H10T1/2; a) C3H10T1/2 alone, b) mixed cultures of C3H10T1/2 and large T-transformed NIH/3T3, c) PDD-treated mixed cultures of C3H10T1/2 and large T-transformed NIH/3T3.

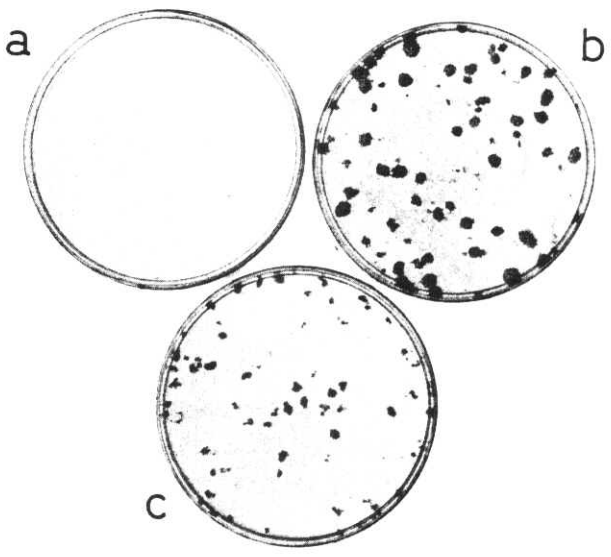

Fig. 2. - Focal outgrowth of polyoma middle T-transformed NIH/3T3 onto C3H10T1/2; a) C3H10T1/2 alone, b) mixed cultures of C3H10T1/2 and middle T-transformed NIH/3T3, c) PDD-treated mixed cultures of C3H10T1/2 and middle T-transformed NIH/3T3.

Table 4. - *Recovery of "focus-formation" by the tumor promoter PDD*

Cell line	C.E. on plastic (%)	C.E. on C3H10T1/2 (%)	Recovery (%)
large T			
NIH/3T3 SA	42	28	67
NIH/3T3 SB	75	63	84

normal cells: 3 x 10^5/60 mm dish; transformed cells: 200/60 mm dish

oncogene transformed cells was measured in the presence of tumor promoters. As previously reported [9], PDD blocked IC both in cellular populations of normal and transformed cells, although with different efficiencies in different transformed clones [3]. These results are therefore in agreement with a critical role of IC in the modulation of cellular growth.

We finally asked whether the relationship between IC and focus formation *in vitro* might also apply to tumorigenicity *in vivo*. We tested the ability of various cell lines to form tumors when injected subcutaneosly into nude mice. All *src* and *ras* transformed clones were tumorigenic 1-2 weeks after injection, while animals injected with NIH/3T3 were tumor free up to 25 weeks after injection. v-*myc* containing clones formed no tumors or slowly growing tumors that appeared after 3 weeks [6]. In conclusion the *in vitro* ability of *ras* and *src*-transformed cells to form foci onto normal cells was accompanied by a highly tumorigenic potential *in vivo*. The reduced or absent tumorigenicity of *myc*-transformed cells suggests that growth restriction may occur also *in vivo* at the site of injection. The close similarity of *in vivo* and *in vitro* growth behaviour is consistent with the possibilty that IC with adjacent normal cells is also occurring *in vivo*.

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