in vivo *4:* 109-114 (1990)

In Vitro and *in Vivo* Onco-suppressor Activity of Normal Cells on Cells Transformed with the H-*ras*1 Oncogene

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Abstract. *We have investigated mixed cultures of "normal" early passage Balb/c embryo cells and Balb/c 3T3 cells transformed by the human T24 H-*ras*1 oncogene. The presence of an excess of "Normal" cells could supress the phenotype of transformed cells* in vitro. *A similar type of suppression by normal cells could be shown* in vivo *on tumors induced by Balb/c 3T3 transformed cells. The suppressing effect of normal cells on T24 H-*ras*1 transformed cells could also be demonstrated by DNA synthesis inhibition experiments. It is suggested that normal cells could either carry or induce tumor inhibitory substances.*

Evidence from cell hybrid studies (1) and defects in human genes predisposing to cancer (2) have suggested the existence of onco-suppressor genes (3, 4). Moreover, related observations have suggested that proliferation of transformed cells can be inhibited by normal cells (5-9). The detailed characterisitics of this phenomenon and the molecular mechanisms are not known.

Early work by Stoker (5) showed that normal cells inhibited the growth of polyoma virus transformed cells. Parental normal rodent fibroblasts were also shown to have an inhibitory effect on their T24 H-*ras*1 oncogene transformed derivative cells (6). Moreover, malignant transformation of mouse primary keratinocytes by Harvey sarcoma virus is modulated by surrounding normal cells *in vivo* (10). The mechanism by which normal cells inhibit growth of morphologically transformed or malignant cells is not known, but at least in some cases it may not be caused by a diffusible factor (11). Requirement of cell-cell contact and functional channels for suppression (12) and the potential role of the human H-*ras*1 oncogene in the inhibition of gap junctional intercellular communication (13) have been suggested.

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Key Words: Onco-suppressor, H-*ras*1 oncogene.

In the present study we have investigated the effect of normal mouse cells on cells transformed by the human T24 H-*ras*1 gene *in vitro* and *in vivo*. We have found suppressor activity of normal cells both on the morphological transformation *in vitro* and the tumorigenic properties *in vivo* of T24 H-*ras* transformed Balb/c 3T3 cells.

Materials and Methods

Plasmids. Plasmid pAGT1 (12) was derived by inserting the 6.6 kb *Bam*H1 fragment containing the human T24 bladder carcinoma H-*ras*1 oncogene into the *Bam*H1 site of the plasmid pAG60 (Figure 1). Plasmid pAG60 (6.2 kb) contains the bacterial Tn5-encoded aminoglycoside phosphotransferase (*aph*) gene under the transcriptional control of the 5′ and 3′ signals of the *herpes simplex* virus thymidine kinase gene (HSV-1 *tk*).

Cells and transfection. All cells were maintained in Dulbecco's modified Mininum Eagles Medium (D-MEM) supplemented to 10% with Foetal Calf Serum (FCS) in a humidified incubator at 37°C in an atmosphere of 5% CO_2 in air. The Balb/c 3T3 cell line of mouse fibroblasts was transfected with plasmid pAGT1 using a modification (15) of the calcium phosphate technique (16) and colonies resistant to geneticin (200 μg/ml) were isolated. One cloned cell line, the BCAGT1-1 cell line, was further characterized and used in the experiments. The BCAGT1-1 cell line was derived after excising the tumor induced by BCAGT1-1 cells in a Balb/c mouse, plating the tumor cells in the presence of geneticin, picking out a geneticin resistant colony and propagating it further. The early passage Balb/c embryo cells were obtained from 12 day old mouse embryos.

Filter hybridizations. Southern blot hybridization analysis (17) was performed as follows. Total DNA was extracted from cultured Balb/c 3T3, BCAGT1-1 and BCAGT1-1T1 cells digested (20 μg each sample) with restriction endonucleases and analyzed on 1% agarose gel in TBE buffer (10mM Tris, 10mM Boric Acid, 1mM Na_2 EDTA, pH 8.0). The DNAs were transferred to nitrocellulose filter (Hybond$^{m}_{b}$ bond^{TM}, from Amersham) which was subsequently baked for 2 h at 80°C. The 6.6 kb *Bam*H1 fragment carrying the T24 H-*ras*1 oncogene was used as probe labelled by nick-translation with ^{22}P. Prehybridization, hybridization and exposure to X-ray film have been described elsewhere (18).

Immunoblotting and immunohistochemistry. Proteins were extracted under denaturing conditions and subjected to polyacrylamide gel electrophoretic (SDS-PAGE) separation as previously described (17). Polypeptides were transferred from SDS-PAGE gels to nitrocellulose, for detection of *ras* p21 with MAb Y13-259 (20) and ^{125}I-protein A, as

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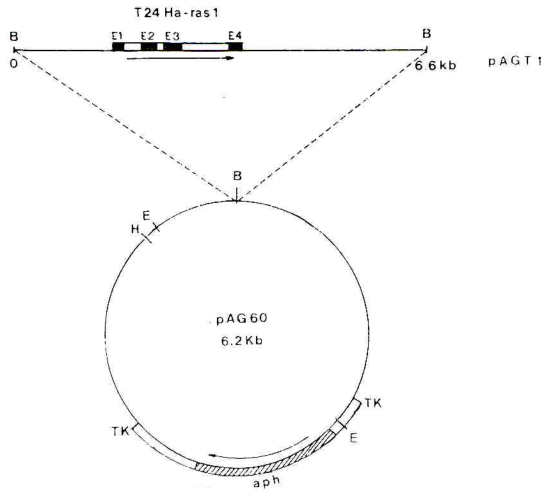

Figure 1. *Schematic representation of plasmid pAG60 and its derivative pAGT1 carrying the 6.6 kb* BamH*I fragment containing the human T24 H-*ras*1 oncogene in the* BamH*I site. TK = Thymidine Kinase,* aph = *aminoglycoside phosphotransferase, E =* EcoR*I, B =* BamH*I.*

Figure 3. *Autoradiographs showing Southern Blot hybridization analysis of recipient Balb/c 3T3 (BC3T3), transformed BCAGT1-1 and tumor derived BCAGT1-1T1 cells. The 6.6 Kb DNA fragment carring the H-*ras*1 gene labelled with ^{32}P by nick-translation was used as probe.*

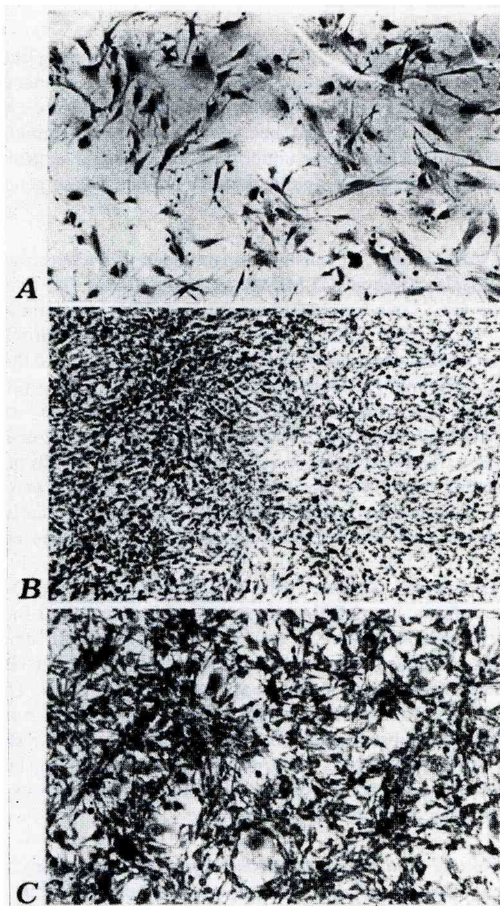

Figure 2. *Morphological appearance of mouse Balb/c embryo (a), Balb/c 3T3 (B) and BCAGT1-1 (C) cells. (X20).*

previously described (21). The rat RFHO6N1-1 cell line overexpressing the normal human H-*ras*1 gene was used as control (22).

For immunostaining, paraffin tissue sections were deparaffinized and mounted on slides. Sections were washed with PBS and treated with the Y13-259 monoclonal antibody, goat anti-rat IgG, streptavidin peroxidase and DAB sequentially, as previosuly described (23, 24).

Growth inhibition in mixed cultures. To assay inhibition of DNA synthesis, transformed BCAGT1-1 cells were plated in 16 mm diameter wells of cells culture clusters (Costar) in D-MEM medium containing 10% FCS. Two hours later the medium was changed with or without the addition of the appropriate numbers of early passage Balb/c embryo cells. Cells were labelled for 4 h with 1 μCi/ml of ^{3}H-Thymidine (Amersham 18-23 Ci/mmol) at each time point. After labelling, cells were washed three times with ice-cold PBS, once with 5% ice-cold TCA and finally with 96% alcohol for 10 min on ice. Air dried cells were solubilized in 1 ml of 0.1 M NaOH for liquid scintillation counting.

Animals. Three to four week old Balb/c mice were used. Inoculation of mice with cells was performed as follows: cells were trypsinized and washed twice with PBS-saline, resuspended in D-MEM medium supplemented to 10% with FCS and counted using a hematocytometer. The appropriate numbers of cells were mixed and 0.2 ml inoculated subcutaneously into each Balb/c mouse.

Results

*Introduction of the human T24 H-*ras*1 gene into Balb/c 3T3 cells leads to moprhological transformation and tumorigenicity.* The human T24 H-*ras*1 oncogene was inserted into the expression vector pAG60 (Figure 1) which carries the ami-

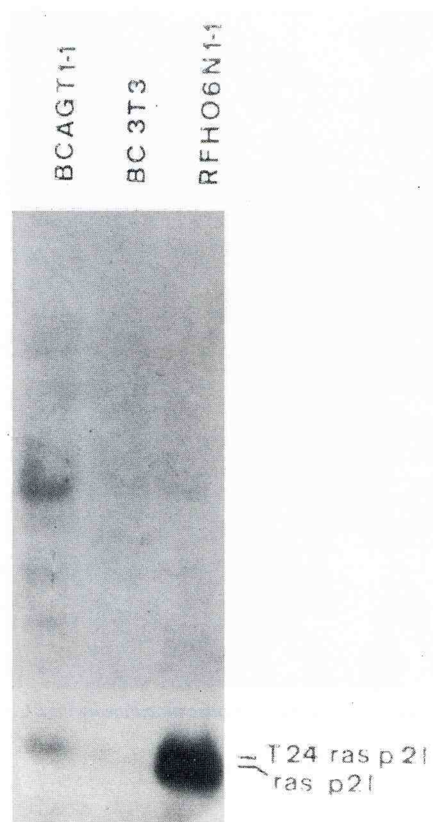

Figure 4. *Autoradiograph of immunoblot analysis of* ras *p21 proteins in BCAGT1-1 (Balb/c cells transfected with the mutant T24 H-*ras1 *gene), BC3T3 (Balb/c 3T3) and RFHO6N1-1 (208F rat embryo cells transfected with plasmid pHO6N1 carrying the normal H-*ras1 *gene) cells.*

noglycoside phosphotransferase (*aph*) gene as a selectable marker. The recombinant plasmid was introduced into the mouse Balb/c 3T3 fibroblast cell line by the calcium phosphate technique. A geneticin resistant clone BCAGT1-1 (Figure 2C), which was also morphologically transformed, was isolated and compared to normal Balb/c embryo cells (Figure 2A) and Balb/c 3T3 cells (Figure 2B). The presence of the exogenous T24 H-*ras*1 gene in the transfectant BCAGT1-1 and its derivative from the tumour BCAGT1-1 cells was tested by Southern blot hybridization analysis. As shown in Figure 3, both the BCAGT1-1 and BCAGT1-T1 cells carry the human T24 H-*ras*1 sequences, although the BCAGT-1T1 have these sequences amplified.

Expression of ras *p21 in cells and tumors.* Proteins extracted from BCAGT1-1 and control RFHO6N1-1 cells expressing the normal H-*ras*1 gene were analyzed by immunoblotting. Immunoblotting proteins were labelled with ^{125}I-protein A. As shown in Figure 4, BCAGT1-1 cells expressed the mutant *ras* p21 which migrates slower in the gel, whereas control RFHO6N1-1 cells express the normal p21 which migrates faster (20). The ability of monoclonal antibody Y13-259 to detect *ras* p21 by immunohistochemical analysis has been

Table I. *Plating efficiences of BCAGT1-1 cells in the presence and absence of geneticin and varying concentrations of early passage Balb/c embryo cells.*

No. of cells plated		No. of foci AV ± SD Geneticin	
BCAGT1-1	Early passage Balb/c embryo cells	−	+
50	0	42 ± 7.2	41 ± 5.5
50	10	43 ± 6.1	44 ± 9.9
50	10^2	43 ± 5.0	43 ± 6.3
50	10^3	40 ± 4.2	42 ± 8.1
50	10^4	35 ± 12	42 ± 7.6
50	10^5	22 ± 6.7	41 ± 10
50	10^6	16 ± 5.4	45 ± 4.4
50	5×10^6	0	43 ± 2.8
100	0	62 ± 8.4	77 ± 6.3
100	10	77 ± 9.0	75 ± 11
100	10^2	78 ± 4.2	85 ± 5.4
100	10^3	74 ± 4.3	84 ± 9.8
100	10^4	63 ± 7.1	87 ± 4.1
100	10^5	43 ± 7.9	92 ± 7.6
100	10^6	29 ± 6.8	91 ± 5.2
100	5×10^6	0	90 ± 3.4

previously described (20-22). BCAGT1-1 cells are tumorigenic in Balb/c mice. Sections of tumors were analysed by an immunohistochemical method for *ras* p21 expression (22). Representative immunohistochemical findings are shown in Figure 5. *Ras* p21 was detected in the cytoplasm as previously described (23, 24).

*Suppression of the T24 H-*ras1 *transformed phenotype by co-cultivation of normal and transformed cells.* To examine whether normal Balb/c embryo cells can inhibit the growth of the BCAGT1-1 cells, these cells were co-cultured. A constant number of BCAGT1-1 cells were mixed with a varying number of Balb/c embryo cells, and foci of morphologically transformed cells, typical of BCAGT1-1 cells, were observed ten days post plating. The results are shown in the Table I. As seen in the Table, for concentrations of 50 or 100 BCAGT1-1 cells/plate, the concentration of Balb/c embryo cells varied from 10 to 5x10^6. As expected, BCAGT1-1 cells obtained after transfection of Balb/c 3T3 cells with plasmid pAGT1 and with geneticin selection remain resistant to the antibiotic. At high densities of normal cells, 5x10^6 Balb/c embryo cells per 25 cm^2 flask, no foci of BCAGT1-1 cells could be detected in the absence of geneticin.

The ^{3}H-thymidine incorporation in the nuclei of the co-cultured cells was examined for different cell densities of normal cells, mixed with the transformed BCAGT1-1 cells. Comparison of the two rates of ^{3}H-thymidine incorporation between normal, transformed and co-cultured cells is illustrated in Figure 6. The ^{3}H-thymidine incorporation in the

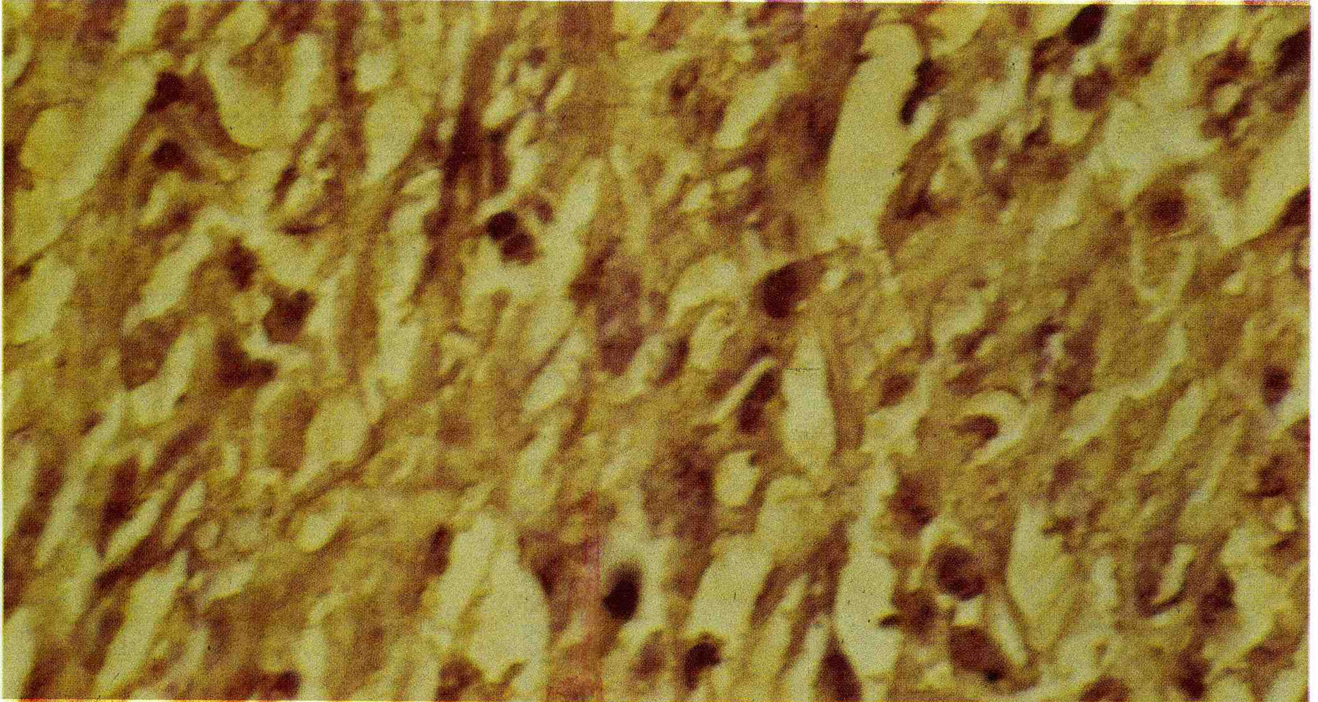

Figure 5. *Immunohistochemical detection of p21* ras *oncogene product on a fibrosarcoma developed on a Balb/c mouse inoculated with BCAGT1-1 cells. (x63).*

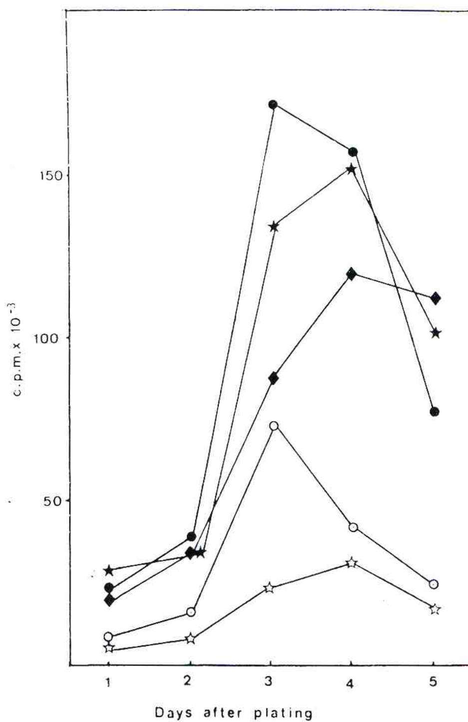

Figure 6. *DNA synthesis inhibition of BCAGT1-1 cells after co-cultivation with early passage Balb/c embryo cells. BCAGT1-1 cells were cultivated either alone (◆–◆) or with high density Balb/c embryo cells, (●–●, 4×10^4 or ★–★, 2×10^4). Balb/c alone (○–○, 4×10^4 or ☆–☆, 2×10^4) were used as controls. Cells were labelled with ^{3}H-TdR, as described in Materials and Methods.*

normal cells remained low and decreased by day 4. The rate of the proliferation of the transformed BCAGT1-1 cells increased very rapidly, remaining very high during the experiment. In the co-cultured cells the ^{3}H-thymidine incorporation showed intermediate levels when compared to normal Balb/c embryo and transfected BCAGT1-1 cells, suggesting a suppression effect of normal on transformed cells.

*Suppression of tumour formation of the T-24 H-*ras1 *transfected cells by co-inoculation with normal cells.* Inoculation of BCAGT1-1 cells into syngeneic Balb/c mice gave rise to malignant tumors, which grew rapidly and eventually killed the animals. This rapid development of malignancy makes it unlikely that genomic alterations other than acquisition of the T24 H-*ras*1 gene are required in order to trigger this malignant phenotype. As demonstrated by immunohistochemical analysis, these expressed *ras* p21 at high levels (Figure 5).

In order to examine whether the observed inhibition of the transformed phenotype of BCAGT1-1 cells by normal cells was also seen *in vivo,* we undertook a series of grafting experiments using BCAGT1-1 cells alone or in combination with the Balb/c mouse embryo fibroblasts. As mentioned above, in the absence of Balb/c embryo fibroblasts BCAGT1-1 cells yield highly invasive tumors. However, in mice engrafted with BCAGT1-1 together with Balb/c embryo cells, malignant growth of BCAGT1-1 is inhibited (Table II).

Inhibition of malignant growth by Balb/c embryo fibroblasts, initially observed macroscopically, was confirmed histologically.

Table II. *Onco-suppressor effect of early passage Balb/c embryo cells on the tumorigenic BCAGT1-1 cells carrying the human T24 H-*ras1 *oncogene.*

Number of cells in the inoculum		Days of tumor Appearance (AV ± SD)	
BCAGT1-1	Early passage Balb/c embryo cells		
10^3	–	21, 21, 21, 29, 29, 34	(26 ± 6)
10^3	3×10^5	34, 34, 44	(37 ± 6)
10^3	5×10^5	36, 44, 46	(42 ± 4)
10^3	10^6	33, 40, 40, 44	(39 ± 5)
10^4	–	18, 22, 22, 22, 22, 27	(22 ± 3)
10^4	3×10^5	21, 23, 23, 23, 27, 27	(24 ± 3)
10^4	5×10^5	22, 22, 22, 27, 27, 28	(25 ± 3)
10^4	10^6	20, 29, 32, 43, 43, 44	(35 ± 10)
10^5	–	13, 15, 15, 18, 20, 22	(17 ± 4)
10^5	3×10^5	15, 15, 16, 16, 16, 23	(17 ± 3)
10^5	5×10^5	15, 15, 15, 17, 19, 21	(17 ± 3)
10^5	10^6	15, 15, 22, 24, 24, 25	(21 ± 5)
10^6	–	8, 12, 14, 14, 14, 18	(13 ± 3)
10^6	3×10^5	12, 12, 14, 17, 18	(13 ± 3)
10^6	5×10^5	12, 12, 15, 15, 19, 23	(16 ± 4)
10^6	10^6	12, 14, 15, 15, 17, 28	(17 ± 6)

Discussion

The inhibition of growth of transformed cells due to the presence of surrounding normal cells has been examined by many investigators (5-9, 25-27). In many cases normal cells can act as an onco-suppressor modulating the cancer phenotype and the growth of transformed cells when they are co-cultured in mixture and the appropriate cell density is reached. The mechanisms of this type of suppression are not known. Experimental evidence has suggested that cell-cell contacts via plasma membrane glycoproteins carrying terminal galactose residues are important for the evaluation of the proliferation of cultured human fibroblasts and presumarly of the accelerated synthesis of collagen type III (30).

Another mechanism suggested by Loewenstein (31) is mediated by gap junctional transfer of intracellular growth-regulatory molecules. Evidence to support this mechanisms has been reported by several groups (13, 27, 32, 33).

The nature of the suppression caused by normal cells on tumorigenic cells operating *in vitro* and *in vivo* is still unclear. However, it is unlikely that this inhibition is due to non-specific competition between cells simply for space or nutrients. It seems more likely that this inhibition depends on intracellular exchange of specific inhibitory factors. We feel that efforts to identify these factors and the genes coding for them is an important area of research.

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Received February 15, 1990
Accepted February 23, 1990