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ARTICLE

Paracrine Effects of IL-4 Transfection on TS/A Adenocarcinoma Cells Mediate Reduced In Vivo Growth

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The *in vitro*/*in vivo* growth capacity and phenotype of TS/A and the IL4-transfected TS/A-IL4 cell lines were studied by cell cycle analysis, expression of ICAM-1/CD54, transferrin receptor/CD71 and E-cadherin and by histology of the primary tumors. TS/A-IL4, unlike the TS/A line, shows *in vitro* a marked increase in the fibroblastoid cell type and a decreased E-cadherin expression. Administration of conditioned medium containing IL4 obtained from the TS/A-IL4 cell line, stimulates CD54 expression in the TS/A cell line. TS/A-IL4 tumors grow more slowly *in vivo* and are ultimately rejected. These processes are accompanied by a marked increase in collagen and extracellular matrix proteins and increased recruitment and degranulation of mast cells. The paracrine effect of IL4, released by the transfected tumor cells, might be responsible for the reduced *in vivo* growth of the TS/A cell line in the presence of TS/A-IL4 cells. (Pathology Oncology Research Vol 5, No 2, 110–116, 1999)

Keywords: gene transfection, tumor growth, TS/A adenocarcinoma, IL-4

Introduction

The mouse mammary adenocarcinoma TS/A[1] was engineered to release IL4 by retroviral infection with the aim to reach at tumor site a critical concentration of this cytokine to counterbalance the immunosuppression generated by the incipient tumor growth.[2,3] In general, gene transfer often serves to increase the immunogenicity of poorly or non-immunogenic tumors and to produce an effective antitumor vaccine.[4] IL4 is a Th-2 cell derived cytokine that may mediate murine tumor rejection through the activation of host eosinophils.[5,6] Clinical trials of IL4 in cancer patients highlight systemic eosinophil degranulation based on the increase of major basic protein (MBP) in serum and urine.[7] Moreover IL4 performs a broad-spectrum of activities[8] that include B and T cell growth stimulation,[9] mast-cell turnover[1], induction of inflammatory macrophages,[11] regulation of collagen biosynthesis[10,12] and extracellular matrix proteins [13] and regulation of intercellular adhesion ICAM-1 molecule[14].

The knowledge of the biological aspects of *in vivo* growth of TS/A-IL4 line is mainly based on the results obtained by the group of G. Forni, concerning very early stages from tumor inoculation.[2] These data deal with a particular emphasis on the eosinophil component during the rejection of TS/A-IL4. Since inflammatory leucocytes need adhesion to ICAM-1 expressed on target cells via C11b to perform their cytotoxic activity, we have examined ICAM-1 expression on tumor cells of both lines, to examine the possible role of ICAM-1 in the process of tumor rejection.

Besides the *in vitro* study, the different characteristics of *in vivo* growth of TS/A and TS/A-IL4 lines have been analysed more in detail, with particular emphasis on advanced stages of tumor growth, proximal to last phases of rejection of TS/A-IL4 transfected tumor. This study has been carried out by histological analysis of primary tumor growth of both lines.

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Materials and Methods

Tumor transplantation

Single cell suspensions of the mammary adenocarcinoma TS/A line or the IL-4 transfected line (TS/A-IL4) were subcutaneously injected into Balb/c OlaHsd female mice (Harlan, Italy) at an amount of 10^5 viable cells/mouse.

Primary tumor

Primary tumors were measured with callipers and their weight were estimated by the following formula: $[(\mathbf{a}^2 x \mathbf{b} x R/6]$, where **a** and **b** are 2 perpendicular axes ($a<b$), and tumor density is assumed equal to 1.

Histology analysis

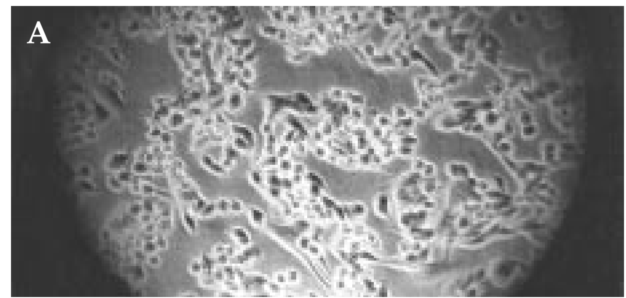
Slices of primary tumor were stained with Cajal-Gallego mounted in Canada Balsam, and were observed with a Orthoplan microscope (Leitz). "Blind" microscopy examinations were made separately by 2 independent workers who examined 5 microslides (each with 3 slices) per donor mouse.

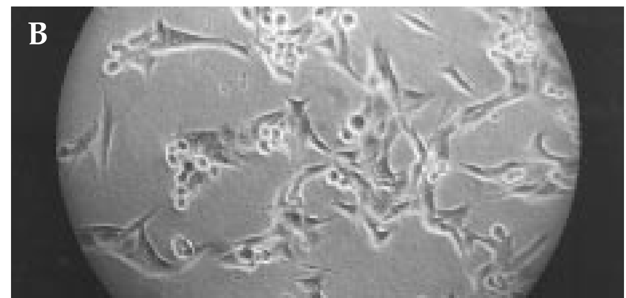
Cell culture

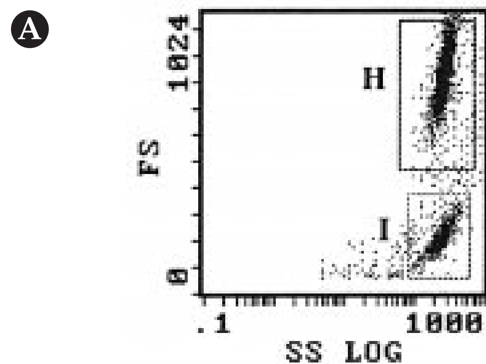
Cell lines (TS/A and TS/A-IL4) were kindly supplied by the group of G. Forni (CNR Centro di Immunogenetica ed Oncologia Sperimentale, Torino, Italy). Vials of the original lines were maintained in liquid N_2; from them cells were obtained, *in vitro* maintained and used for the experiments reported in the present work. Both the cell lines, for the passages and the experiments, were maintained in complete medium (CM) consisting in RPMI-1640 Medium (SIGMA Chemical CO.) supplemented with 10% FBS (HyClone Europe, Holland), 2 mM L-glutamine (HyClone Europe, Holland), 50 µg/ml Gentamicin Sulfate solution (Irvine Scientific, Santa Ana, CA) and for TS/A IL 4 CM added with 2 mg/ml Geneticin solution (SIGMA Chemical CO.). Cells from confluent monolayers, were removed from flasks by 0.25% Trypsin solution (SIGMA Chemical CO.), washed twice and the pellet diluted with CM or PBS. Cells viability was determined by the trypan blue dye exclusion test.

Conditioned medium

IL4-containing medium was prepared by collecting supernatants obtained from TS/A-IL4 cells *in vitro* maintained 96 h in culture medium without geneticin. An aliquot of the supernatant, before use, was analysed for IL4 content by an ELISA kit. IL4-containing medium was then diluted with fresh tissue culture medium to a concen-







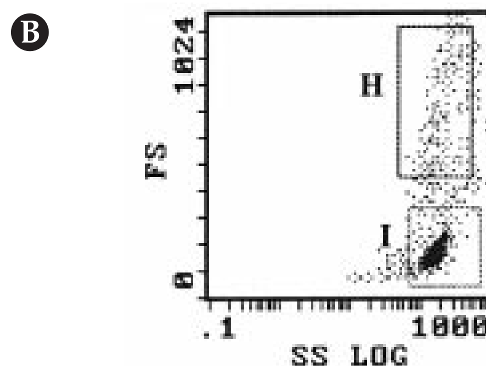


Figure 1. *Morphology analysis of TS/A cells in vitro.* ***Top.*** *TS/A (A) and TS/A-IL4 (B) cells were seeded on a plastic surface in CM; 48 h later, flasks were analysed by inverted microscope and the relative pictures are shown.* ***Lower.*** *TS/A (A) and TS/A-IL4 (B) cells, harvested from flasks after 72 h incubation, appropriately diluted in PBS are analysed by flow cytometry. Morphology is reported as dot density of combined signals FS and logSS to evidence differences in size. Region I = small-size cells, Region H = large-size cells.*

tration corresponding to that produced by the IL4 engineered cells in 72 h incubation, and added to cells sown on plastic plates.

Quantitative determination of IL4 in culture medium

Aliquots of supernatants from cell culture, at 72 h of incubation, were collected and stored at –20°C until use. The quantitative determination of IL4 released in medium was performed as indicated in the protocol of an ELISA kit (BioSource International Cytoscreen Mouse Interleukin-4, CA, USA). Experiments were performed in triplicate and data expressed in pg/ml were extrapolated from standard curves run with each assay.

Antibody staining and flow cytometry

Cells growing adherent to plastics, were washed with PBS and subsequently harvested after a 15 min incubation at 37°C, using 0.2% EDTA in HBSS buffered with 0.1 M Hepes.[15] Cells were washed twice with PBS, filtered by 30 µm nylon mesh and adjusted to $1x10^7$ cells/ml. Cells viability, as determined by the trypan blue dye exclusion test, was above 90%.

Antibody staining of single cell suspensions was performed using the anti-ICAM-1 fluorescein isothiocyanate-conjugated monoclonal antibody clone 3E2 (4 $\mu g/10^6$ cells CD54-FITC Pharmingen, San Diego, CA), the anti-CD71 fluorescein isothiocyanate-conjugated monoclonal antibody (1 $\mu g/10^6$ cells CD71-FITC, SIGMA Chemical CO.) and anti E-cadherin (5 $\mu g/10^6$ cells DECMA-1, SIGMA Chemical CO.). The controls were performed with cells (autofluorescence) and cells stained with an isotype control (IgG2a-FITC, SIGMA Chemical CO.). After 30 min staining at 4°C, unbound MoAb was discarded by washing the cells twice with PBS (0.5% BSA-0.1% NaN_3). The resulting pellet was diluted with PBS (0.5% BSA-0.1% NaN_3), formalin added and analysed within 5 days. Flow cytometry was performed with an XL-Coulter Electronics, acquiring 10,000 events per sample (as indicated in legends), and analysing them by the software supplied. Electronic gates were set to exclude 98% of the cells in the isotype control.

PI staining and cell cycle

Aliquots of $1x10^6$ cells in 500 µl PBS were fixed with 70% EtOH, and maintained at 4°C until flow cytometry examination (within 5 days). For the analysis, fixed cells were washed, maintained for 2 h in PBS and the pellet stained overnight with 1 µg/ml PI, 40 µg/ml RNase in PBS. Each sample was analysed by XL-Coulter Electronics (Miami), stored in listmode and elaborated by the software Multicyle XL-Coulter Electronics.

Statistical analysis

Data were submitted to computer-assisted statistical analysis using the t-test for grouped data, Tukey-Kramer Multiple comparison test and Student-Newmann-Keuls analysis of variance (Instat).

Animal studies

Animal studies were carried out according to the guidelines currently in force in Italy and according to the DHHS Guide for the Care and Use of Laboratory Animals.[16]

Table 1. **Flow cytometric characterisation of TS/A cells**

	CD54		*E-Cadherin*		*CD71*		*Cell Cycle*		
	% positive	*mean channel*	*% positive*	*mean channel*	*% positive*	*mean channel*	*% G_0/G_1*	*% S*	*% G_2-M*
TS/A									
region I	71.1±2.58	2.9±0.1	42.0±3.3	7.7±0.8	81.3±1.0	1.9±0.02	78.1±0.9	21.9±0.9	0±0
region H	7.4±3.9	3.7±0.8	17.4±1.2	21.1±2.8	91.5±0.2	4.2±0.07[a]	22.7±1.6	26.3±2.0^	50.9±3.5^
TS/A-IL4									
region I	74.5±5.8	2.4±0.2	25.7±2.7*	2.6±0.6	87.6±1.0	1.5±0.01	74.9±2.4	25.1±2.	0±0
region H	10.5±1.2	4.4±0.7	6.4±2.7	22.9±8.6	89.4±0.6	3.3±0.06[a]	11.5±4.1	54.5±0.9^	34.0±4.9^

TS/A cells obtained as reported in Figure 1 were analysed by flow cytometry based on two distinct size-cells (FS/SSlog); region I = small cells, region H = large cells. For phenotypic analysis $1x10^6$ cells were stained with CD54-FITC (4 µg), CD71-FITC (1 µg) or E-cadherin (5 µg) antibodies, or PI solution for cell cycle analysis. Each value represents mean±S.E. of samples performed in triplicate of cytometry analysis collecting at least 10,000 events per sample. *p=0.019 statistically different from TS/A (region I), unpaired t test; [a]: p<0.001 values of region H statistically different from region I, ^: p<0.05 values of TS/A different from TS/A-IL4, Tukey-Kramer Multiple comparison test.

Table 2.* Effects of IL4 on TS/A cells *in vitro

	Conditioned medium	*IL4°* *pg/ml*	*CD54§* *% positive*	*mean channel*
TS/A	–	0	13.6±1.4	0.9±0.05
TS/A	+	169±19	42.1±6.6*	1.8±0.19*
TS/A–IL4	–	192±16	14.4±0.8	0.8±0.04
TS/A-IL4	+	334±17*	24.6±5.1	1.05±0.2

° IL4 was measured by ELISA in supernatants of 72 h cell cultures, which were added with conditioned medium obtained from a 96 h culture of TS/A-IL4 cell line, containing 392 pg/ml IL4.
* p<0.001, statistically different from the corresponding cell lines, without conditioned medium. Student-Newman Keuls test.
§ analysis referred to positive cells in H region (large cells) For phenotypic analysis 1x10^{6} cells were stained with CD54-FITC (4 µg) antibody. Each value represents mean±S.E. of samples performed in triplicate of cytometry analysis collecting at least 10,000 events per sample.

Results

Analysis of cell morphology

The cytologic examination of TS/A and TS/A-IL4 cell lines, following cultivation for 48 h on plastic plates, shows the presence of two cell types, one with a fibroblast shape (I) and the other with a spheroid-epithelial shape (H) *(Figure 1,* upper pictures). The two cell types are differently represented between TS/A (A) and TS/A-IL4 (B) cell lines, with a predominant representation of the fibroblastoid cells over the epitheloid cells in the transfected line. The two cell types, analysed by flow cytometry at 72 h culture, represented two distinct populations (H and I), both with the same values of SS (side scatter – cell complexity), but with a markedly different FS (forward light scatter – cell size) (see histograms of *Figure 1).*

Analysis of cell cycle and phenotype

Cell cycle and phenotype, of the two distinct cell populations characterising TS/A and TS/A-IL4 cell lines, were studied on cells harvested from plastic plates after 72 h cultivation, and is reported in *Table 1*. Fibroblastoid cells (region I of *Figure 1)* are characterised by a cell cycle in which about 75% of the total cells are in G_0/G_1 phase; these cells are almost completely positive for CD71, and are mostly positive for CD54 antigen. No significant differences were observed by comparing cell cycle parameters of the fibroblastoid (I) TS/A and TS/A-IL4 cells. A similar comparison of the epitheloid cells (region H of *Figure 1*) shows that almost all cells of this cell type are in the S+G_2-M phases; however, TS/A-IL4 cells, unlike TS/A ones, are more accumulated in S phase, than in G_2M. This cell type is virtually negative for CD54 but positive for CD71 antigen. E-cadherin is expressed at low level in the epitheloid TS/A cells and is almost completely lost in the corresponding TS/A-IL-4 cell population. For TS/A-IL4 cells, phenotype and cell cycle characteristics of small- and large-size cells are comparable to those reported above for the TS/A cell line, with the exception of E-cadherin which is significantly decreased in both TS/A-IL-4 cell subtypes.

Treatment of TS/A cells with conditioned medium of TS/A-IL4 cells, containing about 170 pg/ml IL4, increases CD54 positivity from about 14 to about 42% *(Table 2).* The total number of TS/A cells harvested from the plastic plates 72 h following challenge with conditioned medium is reduced by 25% (data not shown). The S phase fraction of these treated cells is also reduced by 27% whereas a 20-fold increase in the G_2M fraction is observed compared to the control TS/A cells (data not shown). The same treatment performed on TS/A-IL4 cells increased the expression of CD54 similar to the case of TS/A cells *(Table 2).*

In vivo tumor growth

In vivo transplantation of 10^5 tumor cells, obtained from confluent *in vitro* monolayers of TS/A tumor cells, produces 100% take-rate in Balb/c mice compared to 50% in case of the TS/A-IL4 cells, the tumors of which are smaller in size, grow very slowly and appear macroscopically rejected by 4 weeks from implantation. The co-injection of 10^5 TS/A and 10^5 TS/A-IL4 cells induces the growth of a tumor in all the injected mice, but they

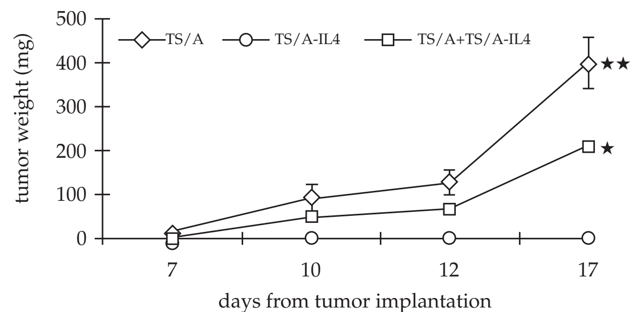


Figure 2. *In vivo primary tumor growth.* 10^5 *viable cells of TS/A (◇),* 10^5 *TS/A-IL4 (○) or* 10^5 *TS/A +* 10^5 *TS/A-IL4 lines (□) were s.c. injected into Balb/c female mice on day 0. Each point represents mean±S.E.M.. obtained in groups of 5 animals. **p<0.01, statistically different vs TS/A-IL4; *p<0.05, statistically different vs TS/A-IL4 and TS/A (Student-Newmann-Keuls multiple comparison test).*

are significantly smaller than that obtained with TS/A alone *(Figure 2).*

The architecture of the tumor mimics the glandular origin of this tumor line with several mammary ducts, which in the TS/A-IL4 line, appear endowed with a better defined basal layer. In these conditions only low amounts of inflammatory cells (eosinophils, neutrophils or lymphocytes) were found in both tumors. The histological examination of these tumors performed on days 19 and 24 from tumor implantation, highlights the reduced tumor mass of the transfected line (data not shown). The main difference between TS/A and TS/A-IL4 line concerns the increased amount of peritumoral connective tissue in TS/A-IL4 line, evidenced by the green-blue colour following Cajal-Gallego staining *(Figure 3,* Panel B) compared to the parental TS/A line *(Figure 3*, Panel A). TS/A-IL4 tumors, unlike the parent line, contained frequently mast cells scattered in the tumor parenchyma *(Figure 3,* Panel C). At a higher magnification, these mast cells appear degranulated, particularly in the areas in which regression phenomena are more evident *(Figure 3,* Panel D).

Discussion

Cell engineering by gene transfer appears to be a new approach providing means of interacting with the para- and autocrine signals that modulate cancer and metastasis growth.[17]

This paper provides evidence that the result of genetic engineering with IL4 does not simply contribute to the enhancement of an immunotherapeutic effect, but demonstrates an autocrine/paracrine effect of IL4 on the tumor cells.

The experiments performed with TS/A mammary adenocarcinoma, show that the IL4 transfected cell line, similarly to the previous report,[2] is completely rejected when implanted in the syngenic Balb/c host. Besides the cytotoxic activity of eosinophils recruited by IL4 release,[2] our data show that tumor rejection is accompanied by other effects related to the acquisition of IL4 release, namely a marked increase in collagen and other extracellular matrix proteins and an increased recruitment and degranulation of mast cells.[4,10] The fact that eosinophils, stimulated by IL4, are not fully responsible for the tumor rejection, is sup-

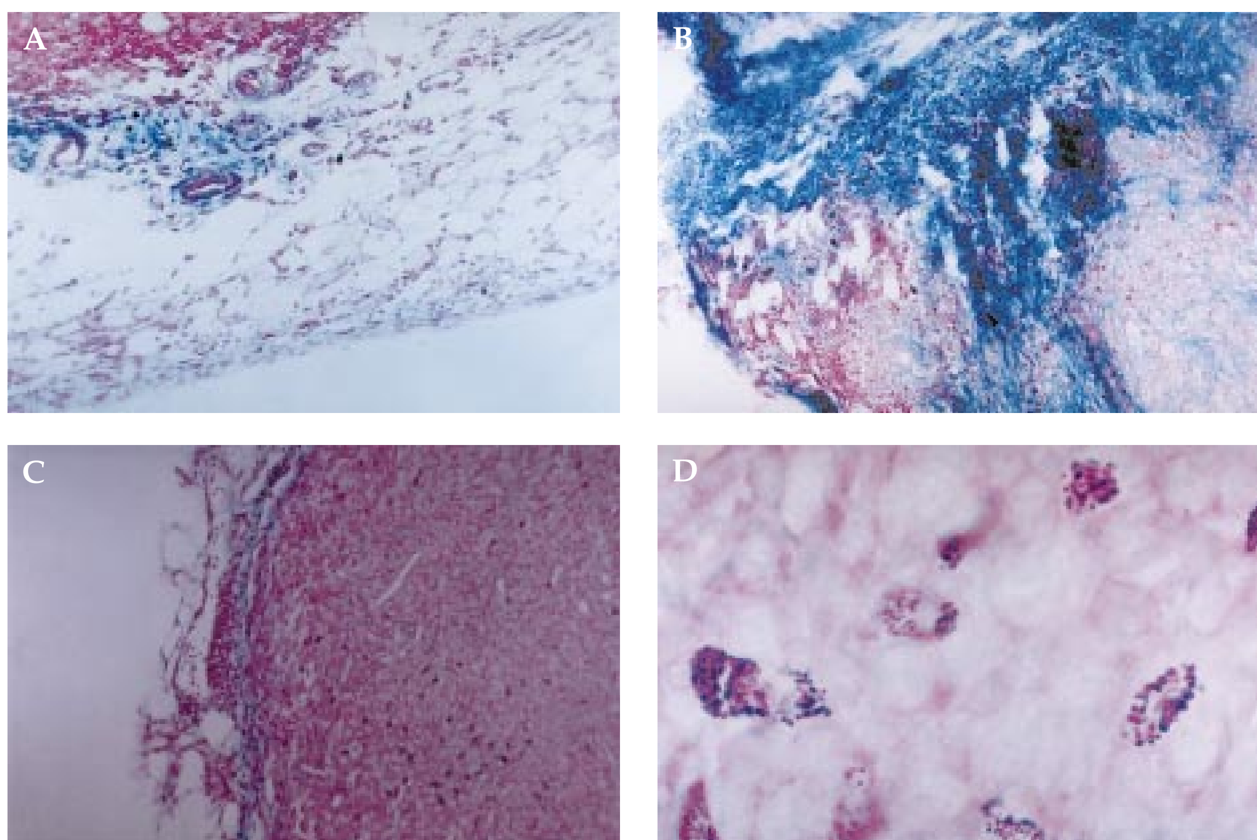


Figure 3. *Histological analysis of primary s.c. tumors. Primary tumors from mice of the experiment of in Figure 2, were excised on days 19 or 24 and processed for histology examination with Cajal-Gallego staining.* ***A****: TS/A 24 days after implantation (125x);* ***B****: TS/A-IL4 24 days after implantation, the blue colour marks connective tissues (125x);* ***C****: TS/A-IL4, mast cells in tumor parenchyma 19 days after implantation (125x);* ***D****: TS/A-IL4, detail of degranulated mast cells (500x).*

ported by other observations. The strong activation and proliferation of eosinophils, induced by TS/A cells transfected with IL5 is devoid of any significant effect on subsequent *in vivo* cell growth,[18] whereas neutrophils were shown to be responsible for the inflammatory rejection of IL-4 secreting tumors *in vivo*.[19]

TS/A cell line appears to be the mixture of cells of two distinct shapes, an epithelioid and a fibroblastoid one. Genetic engineering of this cell line seems to modify quantitatively and qualitatively the balance between these two phenotypes. The overall result is that following IL4 transfection the resulting tumor line appears to growth *in vitro* at a slightly slower rate than the parental line. The slower growth of TS/A-IL4 cell line has to be attributed to the loss of the highly proliferative epithelial. Interestingly, IL4-transfection into TS/A cells resulted in a significant decrease in E-cadherin expression.

Recent studies suggest that cell adhesion molecules (CAMs) can function to stabilize cell-to-cell interaction and/or to provide co-stimulatory signals that are crucial for T cell activation; moreover the adhesion molecules also function as costimulator in NK cell-mediated cytotoxicity.[20] CAMs are also relevant for tumorigenesis and metastasis; ICAM-1 in particular was found on several tumor tissues such as melanomas, lung-, ovarian- and renal carcinomas.[21-24] However, the biological message appears controversial; some author found the ability of NK cells to be significantly enhanced in the presence of ICAM-1 transfected cells,[20] other authors said that the acquisition of ICAM-1 during the process of tumor progression might contribute to the development of metastasis in melanoma,[25] and anti-CD54/ICAM-1 antibodies develop anti-tumor activity against human myeloma.[26]

IL-4 released by TS/A-IL4 cells upregulates CD54 expression *in vitro* on the parental TS/A cell line, and this effect may contribute to the “normalisation” of this cell line. The appearance of CD54 positive cells in a low expressor-population *(Figure 1 and Table 2)* has to be attributed to the induced expression of receptors normally contained inside the cells. These intracytoplasmic receptors can be detected in permeabilised cells and such test showed that 100% of TS/A cells are positive for CD54 receptor (data not shown). The upregulation of CD54 expression, observed *in vitro* using conditioned media, might suggest that the slower growth of primary tumors observed *in vivo,* following co-injection of TS/A and TS/A-IL4 cells, is at least partially due to a paracrine effect of IL-4 on the parental TS/A cells.

The results of the present investigation point out some interesting aspects of this tumor line as a model system for studies on the pharmacological control of solid tumor metastasis. One advantage is given by the different tumorigenic potential of the two cell populations representing this cell line. A more detailed study aimed at evaluating the equilibrium between these two cell populations and the tumor take, growth and metastasis formation. The modulated microenvironment in which they grow may lead us to define new mechanisms of metastasis formation and control.

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