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# Expression of Functional Very Late Antigen-$\alpha_1$, -$\alpha_2$, -$\alpha_3$ and -$\alpha_6$ Integrins on Ewing's Sarcoma and Primitive Peripheral Neuroectodermal Tumour Cells and Modulation by Interferon-$\gamma$ and Tumour Necrosis Factor-$\alpha$

F. van Valen, H. Hanenberg and H. Jürgens

**Twelve different human primary and metastatic Ewing's sarcoma (ES) and primitive peripheral neuroectodermal tumour (pPNET) cell lines were examined by fluorocytometric analysis for the expression of $\alpha_1$, $\alpha_2$, $\alpha_3$ and $\alpha_6$ very late antigen (VLA) $\beta_1$-integrins. VLA-$\alpha_1$, was abundantly expressed on all typical ES cell lines and pPNET cell lines, while absent from atypical (large cell) ES cells. VLA-$\alpha_2$ was displayed on some ES and pPNET cell lines. In two different pPNET cell lines, derived from the same patient, VLA-$\alpha_2$ expression was considerably higher on primary cells compared with metastatic cells. VLA-$\alpha_3$ was exclusively expressed on pPNET cell lines. Expression of VLA-$\alpha_6$ was higher on metastatic than on primary ES and pPNET cells. Adhesion assays on purified extracellular matrix (ECM) proteins, using monospecific adhesion-blocking antibodies, disclosed VLA-1 ($\alpha_1\beta_1$) on typical ES cells and pPNET cells, and VLA-2 ($\alpha_2\beta_1$) on atypical ES cells, as dual collagen type IV (COIV)/laminin (LM) binding sites, and VLA-6 ($\alpha_6\beta_1$) as a specific LM binding site. Treatment of typical ES cells and pPNET cells for up to 48 h with recombinant human interferon-$\gamma$ (rhIFN$\gamma$) and tumour necrosis factor-$\alpha$ (rhTNF$\alpha$) upregulated $\alpha_1$ and $\beta_1$ expression, concomitant with an increase in cell adhesion to COIV and LM. Alternatively, these cytokines downregulated the expression of $\alpha_2$, $\alpha_6$ and $\beta_1$ on atypical ES cells, concomitant with a decrease in the adhesion to COIV and LM. In conclusion, these findings suggest that the difference in repertory of CO and LM integrin receptors on ES cells and pPNET cells reflects tumour status and degree of differentiation. Furthermore, our data indicate that IFN$\gamma$- and TNF$\alpha$-mediated alteration in the level of expression of distinct VLAs on ES and pPNET cells is correlated with changes in the adhesive behaviour of these tumour cells.**

**Key words: very late antigen, integrins, tumour cell adhesion, collagen, laminin, Ewing's sarcoma cells, primitive peripheral neuroectodermal tumour cells**

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## INTRODUCTION

EWING'S SARCOMA (ES) accounts for 10–20% of all primary bone tumours, and most frequently occurs in children between 10 and 15 years of age. ES is a rapidly disseminating neoplasm, presenting in 20% of patients with pulmonary metastasis, osseous lesions and diffuse bone marrow involvement. Lymphatic spread is seen in less than 10% of cases [1]. Histologically, the cells of conventional ES are homogeneously rounded (about 8–10 μm in size) with scant cytoplasm, indistinct cell boundaries and with dented nuclei containing faint chromatin and inconspicuous nucleoli, and moderate mitotic activity. In addition to this classic or typical ES, a small group of lesions have been classified on the basis of morphology and ultrastructure as atypical or large-cell ES, constituting of large principal cells (15–20 μm in size) exhibiting one or more prominent nucleoli and high mitotic activity [2]. On the basis of common cytogenetic, molecular genetic and biological characteristics, ES is closely related to primitive peripheral neuroectodermal tumours (pPNETs) [3–5]. It has been postulated that ES and pPNET represent different stages of differentiation in a malignancy of neuroectodermal origin, with ES being the less differentiated tumour [6].

Accumulating data suggest that tumour cell interaction with extracellular matrix (ECM) adhesive glycoproteins plays a crucial role in the complex process of tumour invasion and metastasis [7]. This adhesive interaction is mainly mediated by the $\beta_1$-integrin subfamily of cell-surface receptors, the very late antigens (VLAs). They are heterodimeric glycoprotein complexes consisting of a common $\beta$-chain ($\beta_1$) non-covalently linked with different $\alpha$ subunits, and are designated as VLA-1 ($\alpha_1\beta_1$) to

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VLA-8 ($\alpha_8\beta_1$) [8]. The VLA group of $\beta_1$-integrins includes receptors for distinct ECM components of basal lamina and stroma, such as collagen (VLA-1, VLA-2, VLA-3) and laminin (VLA-1, VLA-2, VLA-3, VLA-6). VLA integrins may contribute to the invasive capacity and metastatic behaviour of neoplastic cells as indicated by studies showing that variation in the level of expression and function of distinct VLA receptors affects the metastatic properties of tumour cells [9, 10]. Changes in the level of VLA expression can be triggered by oncogenic and chemical transformation [11, 12]. In addition, cytokines such as transforming growth factor (TGF)-$\beta$ and interleukin (IL)-1$\beta$ can modify VLA receptor expression and adhesive behaviour of tumour cells [13, 14].

In the present study, we investigated the expression of $\alpha_1$-, $\alpha_2$-, $\alpha_3$- and $\alpha_6$-VLA integrins and their role in adhesion to collagen type IV and laminin of ES and pPNET cell lines derived from primary and metastatic lesions. As ES and pPNET cells exihibit specific interferon (IFN)$\gamma$ and tumour necrosis factor (TNF)$\alpha$ receptors, which when occupied mediate synergistic inhibition of cell proliferation [15], we also examined the potential role of IFN$\gamma$ and TNF$\alpha$ in VLA receptor modulation and tumour cell–ECM protein interaction. Preliminary aspects of part of this study have been presented previously [16].

## MATERIALS AND METHODS

### *Cell lines*

The ES cell lines used in this study were derived from primary tumours (RM-82, WE-68) and a metastatic lesion (VH-64) [15]. An atypical (large-cell) ES cell line, GG-62, was established from a metastatic tumour [17]. The pPNET cell lines were derived from a primary lesion (STA-ET-2.1), metastatic tumours (STA-ET-2.2, STA-ET-3) and a recurrence of a primary lesion (STA-ET-1) [18], and were kindly provided by Dr P. Ambros (Vienna, Austria). Cell lines STA-ET-2.1 and STA-ET-2.2 were obtained from the same patient. A pPNET cell line, JS-73, was established in our laboratory from a primary tumour. The primary ES cell lines, RD-ES and SK-ES-1, and the metastatic pPNET cell line, SK-N-MC, were from ATCC (Rockville, Maryland, U.S.A.). Cell lines were maintained in RPMI 1640 medium supplemented with 2 mmol/l glutamine, antibiotic/antimycotic solution and 10% fetal calf serum (FCS; Gibco, Grand Island, New York, U.S.A.) at 37°C in 5% $CO_2$ [17].

### *Antibodies and adhesive proteins*

The following monoclonal antibodies (MAbs; all of mouse origin unless stated) were used; anti-$\alpha_1$ MAb TS2/7 (Biermann, Bad Nauheim, Germany); anti-$\alpha_2$ MAb Gi9, anti-$\alpha_3$ MAb P1B5, rat anti-$\alpha_6$ MAb GoH3, anti-$\beta_1$ MAb K20 and fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG (Dianova-Immunotech, Hamburg, Germany); FITC-conjugated goat anti-mouse IgG (Bibby-Dunn, Asbach, Germany). Laminin (LM) was purchased from Boehringer (Mannheim, Germany) and collagen type IV (COIV) from Sigma (München, Germany).

### *Cytokine treatment of cell cultures*

Cells were plated in 25-cm$^2$ tissue culture flasks (Falcon Plastics, Oxnard, California, U.S.A.) at $1 \times 10^6$ in 8 ml of 10% FCS-RPMI 1640. After 3 days, culture medium was replaced by 1% FCS-RPMI 1640 and cells were incubated with 20 ng/ml rhIFN$\gamma$ (specific activity $2.5 \times 10^7$ U/mg; Bioferon, Laupheim, Germany) and 20 ng/ml recombinant human TNF (rhTNF)$\alpha$ (specific activity $4 \times 10^7$ U/mg; generously provided by Thomae, Biberach-Riss, Germany) for 48 h at 37°C. Preliminary time-course experiments showed that cytokine-mediated changes in the level of VLA subunit expression were detectable 24 h after the addition of rhIFN$\gamma$ and rhTNF$\alpha$ to the cells, and were maximal by 48 h. Cell viability by trypan blue exclusion was not significantly affected (>90%) by any treatments. After incubation of cultures, treated and untreated cells were tested for VLA-$\alpha$ and -$\beta_1$ subunit expression and ECM protein binding.

### *Fluorocytometric analysis*

Cell cultures were detached in phosphate buffered saline (PBS)/5 mmol/l EDTA (pH 7.0) and centrifuged twice in cold wash buffer PBS/3% fetal calf serum (FCS)/0.02% $NaN_3$ (pH 7.0). Cells ($5 \times 10^5$) in 20-µl aliquots were incubated on ice for 40 min with 20 µl of saturating concentrations of primary antibody or mouse/rat IgG. After washing twice, 20 µl of a 1:40 dilution of FITC-labelled goat anti-mouse or anti-rat IgG was added to the resuspended cells for 30 min on ice. The stained cells were washed twice, resuspended in wash buffer containing propidium iodide (50 µg/ml) for exclusion of cell debris, and analysed by a fluorescence-activated cell sorter (FACScan) using Lysis-II software (Becton Dickinson, Heidelberg, Germany). For each sample, $1 \times 10^4$ cells were collected at a flow rate of $1 \times 10^3$ cells/s. Cells were gated using dot blots with the forward light scatter and the FL-3 characteristics, and were analysed in FL-1 histograms for the expression of integrins. Determinations were performed in duplicate for three separate experiments. Data are presented in fluorescence intensity (MFI) units [19] as calculated by the formula: MFI units = (mean fluorescence channel × % positive cells of experimental sample) − (mean fluorescence channel × % positive cells of control sample).

### *Cell adhesion assay*

ECM protein-coated culture dishes were used to quantitate cell adhesion and the effects of various MAbs on this adhesion. Wells of 24-well culture plates (Costar, Cambridge, Massachusetts, U.S.A.) were coated for 18 h at room temperature with COIV (40 µg/ml in 0.1 N acetic acid; 5 µg/cm$^2$) and LM (20 µg/ml in PBS; 2.5 µg/cm$^2$). Unbound protein was removed by washing, and remaining non-specific binding sites were blocked by incubation for 2 h at 37°C with RPMI 1640 containing 0.2% bovine serum albumin (BSA; adhesion medium). Tumour cells were harvested in PBS/5 mmol/l EDTA solution, washed twice and resuspended at $5 \times 10^5$ cells/ml in adhesion medium. Subsequently, the cells were added to the wells ($1.5 \times 10^5$ cells/well) and allowed to attach for 45 min at 37°C. Thereafter, the non-adherent cells were washed off with PBS, while the adherent cells were detached by trypsinisation and counted in a Coulter ZM counter (Coulter Electronics, Krefeld, Germany). In blocking experiments, cells were pre-incubated for 30 min on ice with 40 µg/ml of the appropriate MAbs prior to seeding into the wells. Determinations were performed in duplicate for three separate experiments. Data are expressed as percentage of cell adhesion in the presence of mouse/rat IgG, used as negative control.

## RESULTS

### *Fluorocytometric analysis*

Expression of the $\alpha_1$, $\alpha_2$, $\alpha_3$, $\alpha_6$ and $\beta_1$ VLA subunits on 12 primary and metastatic ES and pPNET cell lines was assessed by fluorocytometry. As shown in Table 1, all cell lines abundantly expressed the $\beta_1$ subunit of the VLA receptors. However, there was a marked heterogeneity in VLA-$\alpha$ subunit expression among the various cell lines. The $\alpha_1$ subunit appeared highly displayed

*Table 1. Expression of VLA-$\alpha_1$, -$\alpha_2$, -$\alpha_3$, -$\alpha_6$ and -$\beta_1$ subunits on primary and metastatic ES and pPNET cells*

| | MAbs to: | | | | |
|---|---|---|---|---|---|
| | VLA-$\alpha_1$ | VLA-$\alpha_2$ | VLA-$\alpha_3$ | VLA-$\alpha_6$ | VLA-$\beta_1$ |
| Typical ES | | | | | |
| *Primary* | | | | | |
| RM-82 | 2936 ± 132 (59) | 49 ± 12 (3) | 13 ± 4 (2) | 443 ± 34 (16) | 5749 ± 201 (87) |
| WE-68 | 2173 ± 189 (70) | 2661 ± 113 (65) | 40 ± 18 (2) | 152 ± 15 (9) | 4023 ± 321 (81) |
| RD-ES | 2053 ± 178 (81) | 11 ± 5 (3) | 176 ± 17 (8) | 8 ± 3 (2) | 3785 ± 188 (82) |
| SK-ES-1 | 549 ± 32 (51) | 94 ± 29 (9) | 90 ± 18 (5) | 1726 ± 94 (39) | 2120 ± 112 (94) |
| *Metastatic* | | | | | |
| VH-64 | 5104 ± 324 (100) | 81 ± 19 (9) | 52 ± 9 (6) | 4468 ± 351 (94) | 5441 ± 299 (99) |
| Atypical ES | | | | | |
| *Metastatic* | | | | | |
| GG-62 | 8 ± 5 (1) | 2387 ± 122 (72) | 9 ± 5 (1) | 5622 ± 178 (98) | 6918 ± 195 (89) |
| pPNET | | | | | |
| *Primary* | | | | | |
| JS-73 | 4307 ± 251 (67) | 432 ± 25 (13) | 976 ± 78 (39) | 955 ± 82 (31) | 7373 ± 377 (91) |
| STA-ET-1 | 7727 ± 579 (100) | 108 ± 8 (3) | 32 ± 13 (3) | 11 ± 6 (5) | 6416 ± 141 (94) |
| STA-ET-2.1 | 3284 ± 214 (78) | 6531 ± 361 (79) | 3115 ± 216 (52) | 41 ± 21 (4) | 8965 ± 722 (99) |
| *Metastatic* | | | | | |
| STA-ET-2.2 | 1585 ± 79 (32) | 205 ± 16 (15) | 2167 ± 161 (35) | 3668 ± 192 (90) | 3499 ± 233 (70) |
| STA-ET-3 | 6684 ± 361 (93) | 32 ± 10 (3) | 4504 ± 251 (48) | 71 ± 21 (6) | 7592 ± 382 (93) |
| SK-N-MC | 4244 ± 208 (85) | 66 ± 13 (8) | 11 ± 9 (2) | 4295 ± 119 (81) | 2589 ± 256 (97) |

Cells were incubated with TS2/7 anti-$\alpha_1$, Gi9 anti-$\alpha_2$, P1B5 anti-$\alpha_3$, GoH3 anti-$\alpha_6$ and K20 anti-$\beta_1$ MAbs and analysed by fluorocytometry. Data are expressed in MFI units ± S.D. Percentage of positive cells is shown in parentheses.

in nearly all typical ES and pPNET cell lines, but was absent from atypical ES cells. The $\alpha_2$ subunit was detected on 1/5 typical ES cell lines, 3/6 pPNET cell lines and on the atypical ES cell line. Interestingly, in the two different pPNET cell lines established from the same patient, $\alpha_2$ was highly expressed on primary STA-ET-2.1 cells and poorly expressed on metastatic STA-ET-2.2 cells. Expression of $\alpha_3$ was moderate to high on 4/6 primary and metastatic pPNET cell lines and essentially undetectable (<10%) on the ES cell lines. The $\alpha_6$ subunit was highly displayed on almost all metastatic ES and pPNET cell lines, whereas cell lines established from primary tumours showed moderate $\alpha_6$ expression or none at all.

*Tumour cell adhesion to ECM proteins*

To establish the function of $\alpha_1$-, $\alpha_2$-, $\alpha_3$- and $\alpha_6$-VLA integrins, antibody-blocking studies on COIV and LM were performed. The VLA receptor characteristics of five cell lines were examined: RM-82 (primary ES), VH-64 (metastatic ES), GG-62 (metastatic large-cell ES), STA-ET-2.1 (primary pPNET) and STA-ET-2.2 (metastatic pPNET) (Figure 1). Anti-$\alpha_1$ MAb TS2/7 potently inhibited adhesion of RM-82, VH-64, STA-ET-2.1 and STA-ET-2.2 to COIV by 35% to 89%. Similarly, the anti-$\alpha_1$ MAb blocked adhesion of these cell lines to LM by 40% to 77%. Anti-$\alpha_2$ MAb Gi9 completely blocked adhesion of GG-62 to COIV while adhesion to LM was decreased by 64%. Anti-$\alpha_2$ MAb Gi9 partially reduced adhesion of STA-ET-2.1 to COIV by 32%, while adhesion to LM was not impaired. Anti-$\alpha_3$ MAb P1B5 was found to be inactive in affecting adhesion of STA-ET-2.1 and STA-ET-2.2 to COIV and LM. Anti-$\alpha_6$ MAb GoH3 blocked adhesion of VH-64, GG-62 and STA-ET-2.2 to LM by 35% to 85% without affecting their adhesion to COIV. Anti-$\beta_1$ MAb K20 completely blocked adhesion of RM-82, VH-64, GG-62, STA-ET-2.1 and STA-ET-2.2 to COIV and LM.

*Influence of IFNγ and TNFα on VLA subunit expression and adhesion of tumour cells to ECM proteins*

Treatment of tumour cell cultures for 48 h with a combination of 20 ng/ml rhIFNγ and 20 ng/ml rhTNFα stimulated the level of expression for $\alpha_1$ and $\beta_1$ on almost all typical ES cell lines and pPNET cell lines without affecting the expression of $\alpha_2$, $\alpha_3$ and $\alpha_6$ (Table 2). Of the cell lines, VH-64 was most responsive to the combination of rhIFNγ and rhTNFα, increasing the mean fluorescence intensity for $\alpha_1$ by 3.16-fold and for $\beta_1$ by 2-fold. By contrast, in atypical ES GG-62 cells, rhIFNγ plus rhTNFα did not influence $\alpha_1$ expression but caused a marked decrease in the mean fluorescence intensity for $\alpha_2$ (≈62%), $\alpha_6$ (≈75%) and $\beta_1$ (≈60%). Subsequently, VH-64 and GG-62 cells were used for further characterisation of cytokine action and evaluation of the possible consequences of VLA modulation on cell adhesive behaviour. As illustrated in Figure 2a, rhIFNγ and rhTNFα were synergistic in upregulating VLA-$\alpha_1$ and -$\beta_1$ subunit expression in VH-64 cells, partially resistant to both agents used alone. The combined treatment of VH-64 cells with rhIFNγ and rhTNFα strongly increased the attachment of the cells to COIV and LM (Figure 2b). Maximal adhesion of cytokine-treated VH-64 cells occurred after 10–20 min, while untreated cells adhered maximally after 30–40 min. The GG-62 cell line also appeared fairly resistant to the VLA modifying effects of rhIFNγ and rhTNFα used alone, while pronounced reduction in VLA-$\alpha_2$, -$\alpha_6$ and -$\beta_1$ expression was observed by the combined treatment (Figure 3a). As demonstrated in Figure 3b, the cytokine combination caused a considerable decrease in GG-62 cell adherence

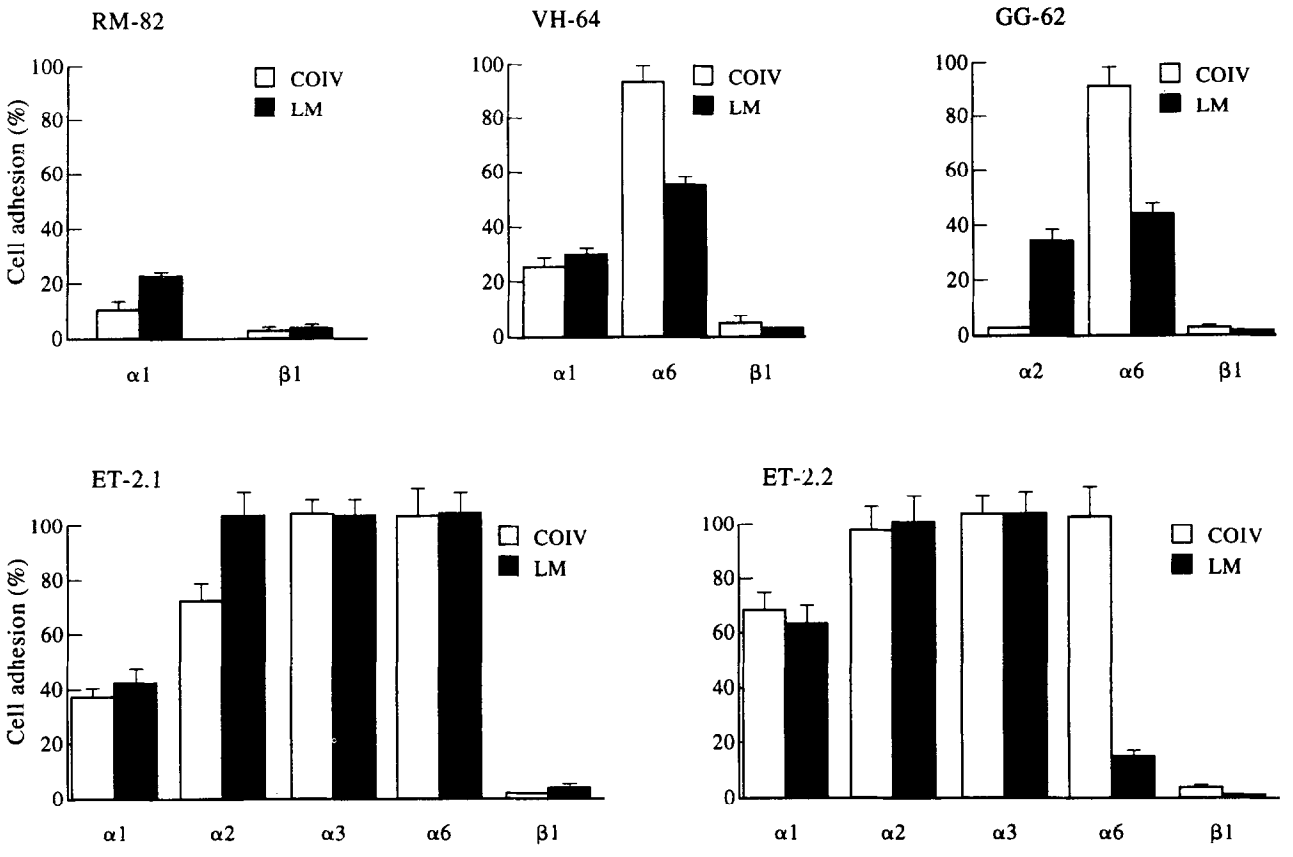


**Figure 1. Effect of MAbs on the adhesion of ES and pPNET cell lines. Cells in 0.2% BSA-RPMI 1640 medium were pre-incubated for 30 min with MAb against $\alpha_1$ (TS2/7), $\alpha_2$ (Gi9), $\alpha_3$ (P1B5), $\alpha_6$ (GoH3) and $\beta_1$ (K20) before seeding into the COIV- and LM-coated wells. Data are mean ± S.D. of duplicate determinations from three separate experiments.**

with ECM proteins. Exposure of GG-62 cells for 48 h to 20 ng/ml rhIFNγ and 20 ng/ml rhTNFα inhibited their subsequent attachment to COIV and LM by approximately 85% in each case. In addition to cell lines VH-64 and GG-62, a synergistic action of rhIFNγ and rhTNFα on VLA subunit expression was observed in all other ES and pPNET cell lines, except for three cell lines (RM-82, STA-ET-3, SK-N-MC) in which the VLA-modifying effects of rhIFNγ and rhTNFα were additive (data not shown).

## DISCUSSION

This study documents a distinct repertory of CO and LM integrin receptors in primary and metastatic ES and pPNET cells. In addition, our results demonstrate that rhIFNγ and rhTNFα selectively alter the expression and function of specific VLA receptors on these tumour cells. On the basis of fluorocytometric and functional data, typical ES cells and pPNET cells display VLA-1 ($\alpha_1\beta_1$) as the most predominant VLA integrin receptor mediating adhesion to both COIV and LM. The $\alpha_1\beta_1$ integrin has been shown to be a dual LM/CO receptor on haematopoietic cells and, in particular, on neural cells [20], where it functions in neurite outgrowth *in vitro* [21] and nerve regeneration *in vivo* [22]. These observations and those presented here are of interest in view of the histogenesis of ES. ES and pPNET share common characteristics, including the chromosomal reciprocal translocation t(11;22)(q24;q12) [3] and high expression of the MIC2/HBA71 antigen [23]. Hence, the expression of high levels of the VLA-1 dual CO/LM receptor on ES and pPNET strengthens the concept that ES is of neuroectodermal origin. Since LM and CO play an important role in sustaining neural crest cell migration [24], VLA-1 receptor expression on ES and pPNET might be a reflection of their embryonic origin.

Expression of the VLA-2 ($\alpha_2\beta_1$) receptor is scarce among the different ES and pPNET cell lines. However, VLA-2 acting as a CO receptor, is highly displayed in the primary pPNET STA-ET-2.1 cell line and is absent from the metastatic pPNET STA-ET-2.2 cell line derived from the same patient. These data suggest that, in some cases of pPNET, loss of VLA-2 expression may be involved in tumour metastasis. Decreased expression of VLA-2 receptors has been reported for the malignant phenotype of adenocarcinomas of the breast [25] and colon [26]. Conversely, the VLA-$\alpha_2$ integrin has been shown to markedly enhance spontaneous and experimental metastasis of rhabdomyosarcoma cells [9]. VLA-2 is strongly expressed on the metastatic atypical ES cell line GG-62 in which it functions as a dual CO/LM receptor. It remains unknown, at present, whether the expression of VLA-2 in primary atypical ES cells might be higher (or lower) as compared to its metastatic counterpart.

The VLA-$\alpha_3$ integrin subunit was not detected on ES cells, whereas it is highly expressed on most pPNET cell lines. This finding suggests an additional capacity of VLA-3-positive pPNET cells to interact with ECM proteins, and may reflect the higher degree of differentiation of pPNET cells as compared with ES cells. The lack of inhibition of adhesion to COIV and LM by the anti-$\alpha_3$ MAb P1B5 might be explained by the possibility that P1B5 recognises an epitope on the $\alpha_3$-subunit of pPNET cells that is not entailed in cell adherence with COIV and LM. Alternatively, a role for VLA-3 in the adhesion of pPNET cells to COIV and LM may be subordinate due to the

*Table 2. Influence of rhIFNγ combined with rhTNFα on the expression of VLA-α and -$\beta_1$ subunits on primary and metastatic ES and pPNET cells*

| | MAbs to: | | | | |
|---|---|---|---|---|---|
| | VLA-$\alpha_1$ | VLA-$\alpha_2$ | VLA-$\alpha_3$ | VLA-$\alpha_6$ | VLA-$\beta_1$ |
| Typical ES | | | | | |
| *Primary* | | | | | |
| RM-82 | +2814 (+5) | +11 (0) | +2 (0) | −8 (0) | +207 (+1) |
| WE-68 | +2363 (+18) | +10 (+1) | +1 (+1) | −45 (0) | +3098 (+7) |
| RD-ES | +2615 (+6) | +6 (+1) | +4 (+1) | −1 (0) | +1962 (+7) |
| SK-ES-1 | +4015 (+46) | +19 (0) | +19 (0) | 0 (−1) | +1540 (+4) |
| *Metastatic* | | | | | |
| VH-64 | +11030 (0) | 0 (0) | +11 (0) | −88 (−6) | +5412 (0) |
| Atypical ES | | | | | |
| *Metastatic* | | | | | |
| GG-62 | +4 (+1) | −1487 (−46) | +1 (0) | −4199 (−63) | −4144 (−52) |
| pPNET | | | | | |
| *Primary* | | | | | |
| JS-73 | +911 (+9) | +49 (+1) | +88 (+1) | −73 (−6) | +748 (+9) |
| STA-ET-1 | +2018 (0) | +192 (0) | +7 (+1) | −28 (0) | +5767 (+6) |
| STA-ET-2.1 | +102 (+2) | +121 (+3) | +101 (+2) | −11 (−1) | +98 (0) |
| *Metastatic* | | | | | |
| STA-ET-2.2 | +107 (+3) | +18 (0) | +121 (0) | −19 (−4) | +37 (0) |
| STA-ET-3 | +2569 (+5) | +79 (+1) | +101 (+1) | −44 (−3) | +5026 (+7) |
| SK-N-MC | +2175 (+11) | +22 (0) | +3 (0) | −155 (−4) | +30 (+2) |

Cells were incubated for 48 h with a combination of rhIFNγ (20 ng/ml) and rhTNFα (20 ng/ml) and then analysed by fluorocytometry for the expression of VLA-α and -$\beta_1$ subunits recognised by the MAbs listed in Table 1. Data are expressed as difference in MFI units (treated − untreated cells). Difference in percentage of positive cells (treated − untreated cells) is shown in parentheses.

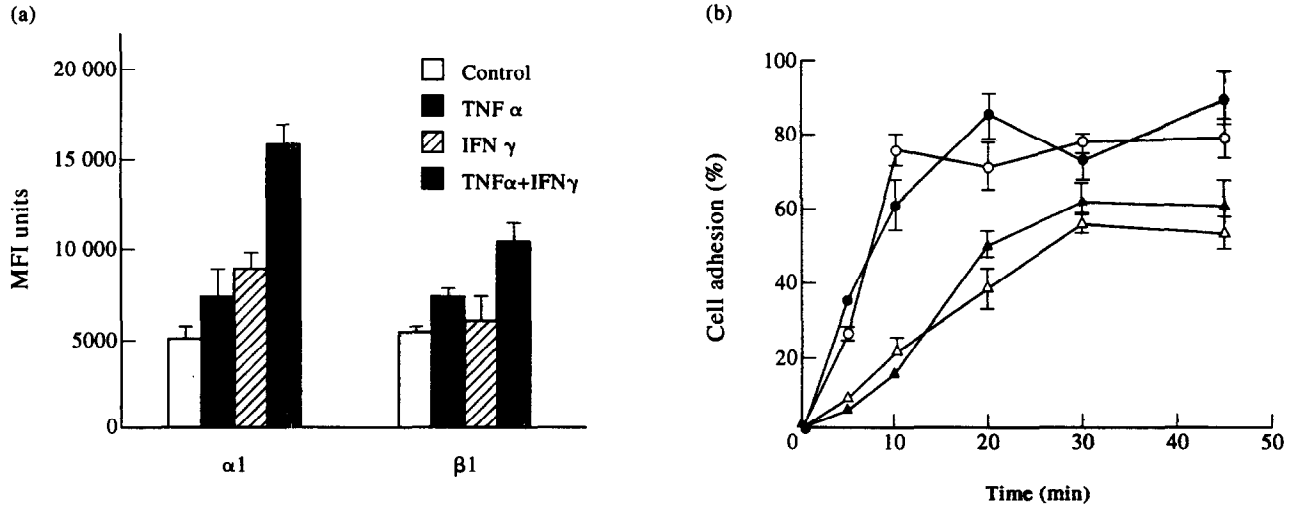


**Figure 2. (a) Effect of rhIFNγ (20 ng/ml) and rhTNFα (20 ng/ml) alone and in combination on the expression of VLA-$\alpha_1$ and -$\beta_1$ in VH-64 cells. (b) Time-dependence of adhesion to COIV (open symbols) and LM (closed symbols) by VH-64 cells incubated in the absence (△ ▲) and presence (○ ●) of rhIFNγ (20 ng/ml) plus rhTNFα (20 ng/ml). Data are mean ± S.D. of duplicate determinations from three separate experiments.**

relative overexpression of functional VLA-1 binding sites on these tumour cells. Alternatively, recent studies have indicated that the VLA-3 integrin could be a receptor for a novel basement membrane component, epiligrin [27].

VLA-6 ($\alpha_6\beta_1$) is preferentially displayed on the surface of metastatic ES and pPNET cells as compared with primary tumour cells, and acts as a specific LM binding site as shown by the ability of an anti-$\alpha_6$ MAb (GoH3) to attenuate tumour cell interaction with LM. These observations imply that an increased VLA-6 expression may play a role in ES and pPNET cell invasion and/or metastasis. Our results are in accordance with the findings of Mortarini and colleagues [28] reporting VLA-$\alpha_6$

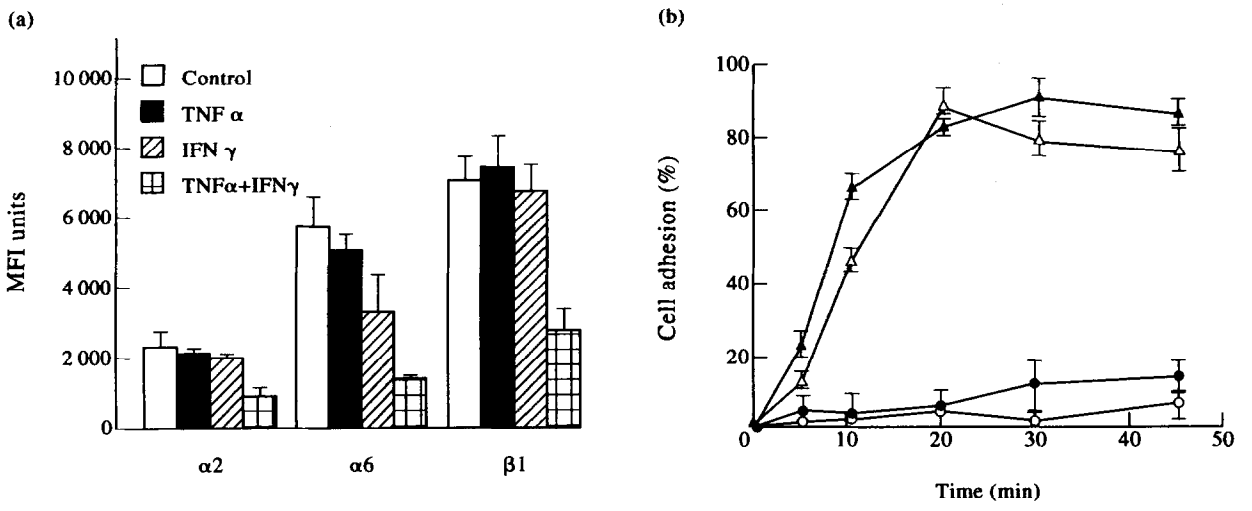


**Figure 3. (a) Effect of rhIFNγ (20 ng/ml) and rhTNFα (20 ng/ml) alone and in combination on the expression of VLA-$\alpha_2$, -$\alpha_6$ and -$\beta_1$ in GG-62 cells. (b) Time-dependence of adhesion to COIV (open symbols) and LM (closed symbols) by GG-62 cells incubated in the absence (△ ▲) and presence (○ ●) of rhIFNγ (20 ng/ml) plus rhTNFα (20 ng/ml). Data are mean ± S.D. of duplicate determinations from three separate experiments.**

overexpression in metastatic human melanoma cells, but not in primary ones.

Exposure of typical ES cells and pPNET cells to rhIFNγ and rhTNFα results in upregulation in the level of expression of the VLA-$\alpha_1$ subunit and its associated $\beta_1$ subunit. This response appears to be selective for the $\alpha_1$ subunit since the level of expression of the $\alpha_2$, $\alpha_3$ and $\alpha_6$ subunits is not altered upon cytokine treatment of these cells. The enhanced display of $\alpha_1\beta_1$ on cytokine-treated cells caused an increase in the attachment of these cells to COIV and LM. Previous studies have shown that rhIFNγ in combination with rhTNFα exert a profound inhibitory influence on ES cell proliferation [15]. Apparently, rhIFNγ and rhTNFα enhance differentiation of typical ES cells and pPNET cells, and the first signs in this process are inhibition of cell growth and stimulation of cell-CO/LM interaction by increased expression of specific CO/LM integrin receptors. Alterations in the expression and function of individual integrins have also been noted in response to oncogenic transformation, leading to a decreased expression of various adhesion receptors, including $\alpha_1\beta_1$ [11]. Thus, our results could be interpreted to mean that rhIFNγ and rhTNFα may help to reverse the less differentiated phenotype of typical ES cells and pPNET cells into a more differentiated state. This consideration, however, does not apply to the observed effects of rhIFNγ and rhTNFα in the atypical ES GG-62 cell line. In these cells, the combination of rhIFNγ and rhTNFα potently decreased the level of expression of the VLA-$\alpha_2$, -$\alpha_6$ and -$\beta_1$ subunits, accompanied by an impaired adhesion capacity of the cytokine-treated cells to COIV and LM. Inasmuch as an increased expression of LM and CO receptors by tumour cells may be implicated in tumour cell invasion and progression [9, 12, 29], our data suggest that the cytokine-mediated downregulation of the expression of LM and CO receptors on metastatic GG-62 cells may render these cells less adhesive to basement membrane components during invasion and metastasis.

Collectively, this study has established the involvement and cytokine-regulation of specific VLA integrins in the adhesion of primary and metastatic ES and pPNET cells to CO and LM. Further studies will be required in order to clarify the *in vivo* role of CO and LM integrin binding sites in the dissemination and metastasis of ES and pPNET.


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# Constitutive Secretion of Bioactive Transforming Growth Factor $\beta_1$ by Small Cell Lung Cancer Cell Lines

**J.R. Fischer, H. Darjes, H. Lahm, M. Schindel, P. Drings and P.H. Krammer**

**We investigated effects of soluble mediators secreted by small cell lung cancer (SCLC) cell lines on modulation of cytokine-induced growth of lymphocytes. We found that interleukin-2 (IL-2)-mediated T-cell growth was inhibited by a cytokine constitutively secreted by the SCLC cell line, NCI-N417. Of several cytokines tested, only transforming growth factor $\beta_1$ (TGF$\beta_1$) severely suppressed IL-2-dependent T-cell growth. Using a specific anti-TGF$\beta_1$ antibody, we found that this antibody blocked the immunosuppressive activity secreted by NCI-N417. Thus, the NCI-N417-derived immunosuppressive molecule was serologically identified as TGF$\beta_1$. Further experiments showed that TGF$\beta_1$ was secreted by four of eight SCLC lines tested. mRNA for TGF$\beta_1$ was expressed in NCI-N417 and in SCLC-22H. Constitutive secretion of biologically active TGF$\beta_1$ by SCLC lines suggests that tumour-derived immunosuppression may have clinical relevance.**

**Key words: TGF$\beta_1$, SCLC, immunosuppression**
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## INTRODUCTION

IN RECENT years, many control mechanisms that regulate activation, proliferation and differentiation of normal lymphocytes by cytokines have been described. In contrast, mechanisms that downregulate immune functions are less well characterised. Interestingly, one factor, transforming growth factor (TGF)$\beta_1$, that was identified as inhibiting immune functions is not only released by lymphocytes, but also by a variety of tumour cells [1–4]. Particularly in patients with malignant tumours, immunosuppression is a common feature [1–4], and it has been speculated that the release of immunosuppressive factors might be an important step in malignant evolution [1]. Thus, tumour