of 26 had serum NSE below the upper limit. Some metastatic sites such as the brain are not correlated with high levels of serum NSE [9, 10], whereas liver and bone metastases are generally associated with very high levels of serum NSE [10, 16]. In our patient population, the highest levels of NSE were observed for patients with liver and bone metastases, and the lowest for cutaneous, peripheral lymph node, choroid and brain metastases as the only extrathoracic disease.

In conclusion, we have shown, in accordance with other authors, that serum NSE reflects to a certain degree the extent of disease in SCLC. Nevertheless, the importance of the overlap between the values for limited and extensive disease prevents its use for determining disease stage with sufficient accuracy.


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*Eur J Cancer, Vol. 29A, No. 16, pp. 2250–2255, 1993.*
*Printed in Great Britain*
*0959–8049/93 $6.00 + 0.00*


# Differential Expression of the Intercellular Adhesion Molecule-1 (ICAM-1) in Lung Cancer Cell Lines of Various Histological Types

**Cordula Schardt, Jochen Heymanns, Christof Schardt, Martin Rotsch and Klaus Havemann**

**Ten small cell lung carcinoma and 12 non-small cell lung carcinoma cell lines of various histological types were studied for constitutive expression of the intercellular adhesion molecule-1 (ICAM-1). ICAM-1 was present in all squamous and large cell carcinoma cell lines whereas two out of five adenocarcinoma and all small cell lung cancer (SCLC) cell lines showed no basal ICAM-1 expression. ICAM-1 expression was upregulated by tumour necrosis factor-α (TNF-α) in a time- and dose-dependent manner in cell lines with basal ICAM-1 expression. Western blot analysis revealed a molecular size of 85 kDa for ICAM-1 in all but one cell line. The TNF-α-induced upregulation of ICAM-1 occurs on the transcriptional level. Adhesion of peripheral blood mononuclear cells to lung tumour cell lines could be inhibited by monoclonal antibodies (MAb) (CD11a;CD18) against the receptor of ICAM-1, the leukocyte function-associated antigen-1 (LFA-1), but not by a MAb (CD54) against ICAM-1 itself.**
*Eur J Cancer*, Vol. 29A, No. 16, pp. 2250–2255, 1993.

## INTRODUCTION

CELL ADHESION molecules (CAM) of the immunoglobulin supergene family are relevant for tumorigenesis and the development of metastatic sites [1, 2]. Important molecules of the immunoglobulin supergene family are the intercellular adhesion molecules 1 and 2 (ICAM-1, ICAM-2). ICAM-1 is a transmembrane glycoprotein with five immunoglobulin-like domains [3]. The molecular weight ranges from 74 to 114 kDa due to the extent of glycosylation [4]. ICAM-1 is constitutively expressed on a variety of haematopoietic and non-haematopoietic cells and tissues, whereas ICAM-2 [5] is restricted to haematopoietic cells and to vascular endothelium. ICAM-1 expression can be upregulated

by inflammatory cytokines [6], phorbolesters and in some cases by interleukin-4 (IL-4) and lipopolysaccharides [7]. ICAM-1 is the ligand for the leukocyte function-associated antigen-1 (LFA-1; CD11a/CD18), which belongs to the integrin family, and thus is critical for adhesion of LFA-1 immunocompetent cells to target cells [1, 2, 8].

ICAM-1 was found on several tumour tissues such as melanomas, lung carcinomas, ovarian carcinomas and others [9–13]. *De novo* expression of ICAM-1 on melanomas has been shown to correlate with metastatic spread and poor prognosis for the patients [9, 14, 15].

We and others have already demonstrated the presence of the neural cell adhesion molecule (NCAM), another member of the immunoglobulin supergene family, on small cell lung cancer (SCLC) and also in 20% of non-SCLC cell lines (NSCLC) [16–19]. Therefore, we assumed that ICAM-1 as an adhesion molecule with known homology to NCAM [20] might be found on certain types of lung cancer cell lines. In addition to flow cytometry and blotting techniques, we performed a cell adhesion assay to investigate possible interactions between ICAM-1-positive tumour cells and LFA-1-bearing leukocytes.

## MATERIAL AND METHODS

*Cell culture and TNF-α treatment*

The human SCLC and NSCLC cell lines were obtained from the National Cancer Institute, Bethesda, Maryland, U.S.A. (NCI-H157, NCI-H125, NCI-H23, NCI-H322, NCI-H596, NCI-H60, NCI-N592, NCI-H69, NCI-H526, NCI-H82, NCI-H841, A-549) [21], from the Department of Pathology, Uppsala, Sweden (U-1810, U-1752) [22] or were established at our laboratory (LCLC-103H, LCLC-97TM1, EPLC-32M1, EPLC-65H, MSTO-211H, SCLC-16HC, SCLC-22H, SCLC-24H, SCLC-86M1) [23, 24]. The cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS; Gibco/BRL, U.S.A.) and cultured at 37°C in a 95% air, 5% $CO_2$ humidified incubator. For tumour necrosis factor-α (TNF-α) treatment, cells were incubated for 16 h with recombinant human TNF-α (Bachem, Heidelberg, Germany) at a final concentration of 10 ng/ml in RPMI 1640 medium and 10% FCS unless otherwise indicated.

*ICAM-1 immunostaining and flow cytometry*

Cell cultures in log growth phase were harvested,using 0.02% EDTA in phosphate buffered saline (PBS) for the adherently growing cell lines (all NSCLC and NCI-H841), washed twice in PBS and adjusted to $1 \times 10^7$ cells/ml. Cell viability, as determined by the trypan blue dye exclusion test, was above 85%.

Direct immunostaining of ICAM-1 on single cell suspensions ($1 \times 10^7$ cells/ml) was performed using the anti-ICAM-1 fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (MAb) clone 84/H10 (CD54; Dianova, Hamburg, Germany, 2 μg/$5 \times 10^{-5}$ cells). The control was performed with a FITC-conjugated non-relevant mouse IgG1 (Dianova). Flow cytometry was performed with a fluorescence-activated cell sorter (FACS) Analyzer (Becton Dickinson, Mountain View, California, U.S.A.). In each probe 5000 cells were analysed for cell volume, right-angle light scatter and green fluorescence. Data were acquired in list mode, collected on a 3 decade log-scale and analysed on a Hewlett Packard model 9000 computer using the software program Consort 30 (Becton Dickinson). In each cell line, cells stained with the antibody were compared with cells incubated with the irrelevant mouse IgG1. Electronic gates were set to exclude 98% of the cells in the isotype control. Cells with fluorescence higher than that of the gated population were counted as positive. Cell lines with a portion of more than 15% positive cells were scored positive for ICAM-1 expression. In addition, mean fluorescence intensity was calculated.

*Western blot analysis*

Cells were harvested and homogenised in 0.25 mol/l sucrose,1 mmol/l EDTA, 5 mmol/l Tris, 1 mmol/l phenylmethylsulfonyl fluoride, and 1000 U/ml Aprotinin pH 8.0 using a glass plunger. The crude homogenate was centrifuged at 1000 *g*. The supernatant was saved and centrifugued for 1 h at 40 000 *g*, 4°C. The resulting pellet was homogenised in 25 mmol/l Hepes pH 7.4 and stored at −80°C.

One hundred micrograms of membrane protein per lane were solubilised in Laemmli buffer pH 6.8, boiled for 3 min and subjected to a 3–15% SDS-PAGE. The discontinuous buffer system described by Laemmli [25] was used. The proteins were transferred onto 0.45 μm nitrocellulose sheets for 2 h at 0.8 A. The sheets were blocked overnight with 3% bovine serum albumin (BSA) and PBS/0.05% Tween-20. The nitrocellulose sheets were overlayed with a 1:10 dilution of anti-ICAM-1 antibody P3.58BA-11 [26] for 2 h followed by an incubation with peroxidase-conjugated goat anti-mouse IgG 1:5000 (Dianova). Each step was followed by extensively washing in PBS/Tween-20 (0.05%). Finally, bound peroxidase was visualised with 4-nitrotetrazolium-chloride-blue.

*Northern blot analysis*

RNA was extracted as previously described [27]. Ten micrograms of RNA per lane were loaded and size-fractionated by electrophoresis through a 1% agarose–formaldehyde gel and blotted onto nylon membranes (Gene screen Plus, DuPont, Germany). Blots were hybridised with a random primer extended cDNA probe for ICAM-1 [26] that had a specific activity of $2–5 \times 10^9$ cpm/μg DNA. After hybridisation, high stringency conditions were used for washing. The filters were exposed to Kodak XAR-5 film (Kodak, Rochester, New York, U.S.A.) for 18 h at −70°C using intensifying screens.

*Adhesion assay*

Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors by Ficoll-Isopaque gradient centrifugation. Monocytes and marcrophages were depleted by plastic adherence for 1 h in RPMI 1640 medium and 10% FCS. Remaining cells ($1 \times 10^7$/ml) were labelled overnight with 150 μCi [$^{35}$S]-methionine in methionine-free RPMI 1640 medium and 10% FCS.

NSCLC cells were seeded in 96-well tissue culture plates ($5 \times 10^4$/well) and cultivated for 24 h. Then half of the wells were incubated with TNF-α 10 ng/ml for 16 h.

Labelled PBMC were washed three times, adjusted to 250 000 cells/100 μl and incubated for 1 h at 20°C either in pure medium or in medium supplemented with 5 μg/ml CD11a (clone MHM24), 5 μg/ml CD18 (clone MHM23; both from Dako Diagnostics, Heidelberg, Germany), 10 μg/ml CD54(clone 84/H10) or an irrelevant mouse IgG 5 μg/ml. Then PBMC were

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Revised 13 Apr. 1993; accepted 21 May 1993.

added to the microtitre plates containing NSCLC cells and incubated for 1 h at 37°C. Total number of PBMC added to the lung cancer cells represents "total activity". After 1 h of incubation microtitre plates were gently washed six times with 100 μl PBS to remove non-adherent PBMC. Remaining adherent PBMC were solubilised in $H_2O$ with 5% Triton-X100 and counted in a LKB liquid scintillation counter. These cells represent the "bound activity". The assays were performed in triplicate and repeated with PBMC from different donors. The extent of PBMC adhesion to tumour cells was calculated as bound activity (in the absence or presence of an antibody)/total activity × 100%. Values were not corrected for background binding. Significance of differences between bound PBMC preincubated in pure medium or in the presence of one of the mentioned antibodies was calculated with the *t*-test for paired samples.

## RESULTS

*Flow cytometric analysis*

Twenty-two established lung carcinoma cell lines were investigated for ICAM-1-expression by flow cytometry. Basal ICAM-1 expression under normal growth conditions was found on most NSCLC cell lines (10 out of 12) as shown in Table 1. Two out of three adenocarcinoma cell lines and all SCLC cell lines were classified as ICAM-1-negative under normal cell culture conditions. ICAM-1 expression could be induced by a factor of 1.5 to 4.5 after treatment with TNF-α for 16 h, as determined by the increase of the mean fluorescence intensity or the percentage of positive cells in those cell lines with basal ICAM-1 expression (Table 1). Cell line U-1810 had the highest basal ICAM-1 expression and was used for experiments to analyse the influence of TNF-α on ICAM-1 expression in several ways. (i) Dose-dependency: 0.1–10.0 ng/ml TNF-α in the culture medium upregulated ICAM-1 in a dose-dependent manner. One hundred nanograms TNF-α/ml did not increase ICAM-1 expression further. (ii) Culture conditions: withdrawal of FCS did not influence basal ICAM-1 expression nor its inducibility by TNF-α. (iii) Time-dependency: incubation of cell line U-1810 with TNF-α 10 ng/ml for a period of up to 96 h revealed an increase of ICAM-1 expression already after 2 h of incubation, reaching a maximum after 16 h. After 24 h of exposure to TNF-α, ICAM-1 expression started to decline slowly, but did not reach the basal ICAM-1 level after an incubation period of over 96 h (Fig. 1).

ICAM-1 expression also decreased when TNF-α was removed after an initial incubation for 16 h and fresh medium without TNF-α was added. The decrease was first detectable after 2 h. Even after 96 h of incubation in TNF-α-free medium ICAM-1 expression had not reached base line expression (results not shown).

*Table 1. Expression of ICAM-1 on lung cancer cell lines under standard growth conditions (basal expression; no TNF-α) and after 16 h incubation with TNF-α 10 ng/ml (stimulated expression). Mean fluorescence intensity of immunostained ICAM-1-positive cells and percentage of ICAM-1-positive cells which had a fluorescence beyond 98% of the isotype control*

| Cell line | Mean fluroescence (% of positive cells) Basal ICAM-1 expression without TNF-α | | Stimulated ICAM-1 expression with TNF-α | |
|---|---|---|---|---|
| Large cell carcinoma | | | | |
| LCLC-103H | 34 | (94) | 66 | (100) |
| LCLC-97TM1 | 40 | (92) | 67 | (99) |
| U-1810 | 133 | (92) | 575 | (100) |
| NCI-H157 | 10 | (72) | 41 | (97) |
| Squamous carcinoma | | | | |
| EPLC-32M1 | 8 | (28) | 16 | (59) |
| U-1752 | 27 | (68) | 41 | (96) |
| Adenosquamous carcinoma | | | | |
| NCI-H125 | 24 | (71) | 101 | 98 |
| NCI-H596 | 22 | (98) | 39 | 100 |
| Adenocarcinoma | | | | |
| A-549 | 12 | (13) | 12 | (14) |
| NCI-H23 | 11 | (20) | 14 | (40) |
| NCI-H322 | 8 | (15) | 10 | (12) |
| Mesothelioma | | | | |
| MSTO-211H | 13 | (16) | 15 | (65) |
| SCLC-Classic | | | | |
| SCLC-16HC | 6 | (7) | 9 | (8) |
| SCLC-22H | — | (4) | — | (—) |
| SCLC-24H | — | (3) | — | (—) |
| SCLC-86M1 | — | (5) | — | (—) |
| NCI-H60 | 20 | (9) | 13 | (13) |
| NCI-N592 | — | (7) | — | (—) |
| NCI-H69 | — | (6) | — | (—) |
| SCLC-Variant | | | | |
| NCI-H526 | — | (4) | — | (—) |
| NCI-H82 | 25 | (9) | 25 | (7) |
| NCI-H841 | 16 | (9) | 14 | (10) |

—, not done.

*Western blot analysis*

The influence of TNF-α treatment on the level of ICAM-1 expression was also monitored by western blot analysis. The amount of ICAM-1 protein stained with P3.58Ba-11 was maximal after 16–24 h of incubation with TNF-α (Fig. 2). This result was in accordance to the data obtained by flow cytometry.

Western blots performed with several NSCLC cell lines revealed that P3.58Ba-11 stained ICAM-1 proteins with a molecular weight of 85 kDa except cell line LCLC-97TM1 in which a slightly smaller ICAM-1 protein was found (Fig. 3). In SCLC cell lines no ICAM-1 protein was detected either in untreated or in TNF-α-stimulated cells.

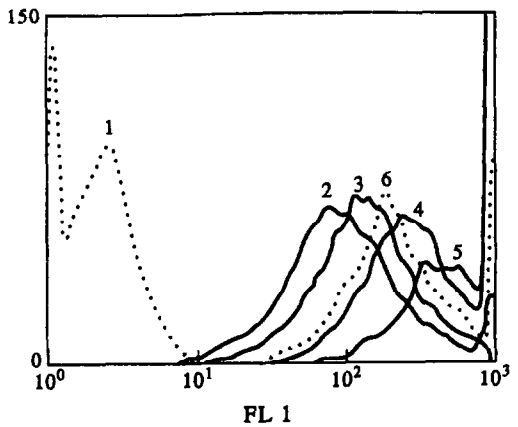


**Fig. 1. Expression of ICAM-1 on cell line U-1810 treated for different intervals (4–96 h) with TNF-α 10 ng/ml. (1) Control performed with a non-relevant antibody, (2) basal ICAM-1 expression, (3) 4 h TNF-α, (4) 8 h TNF-α, (5) 16 h TNF-α, (6) 96 h TNF-α. Ordinate: number of cells/channel, abscissa: relative fluorescence intensity.**

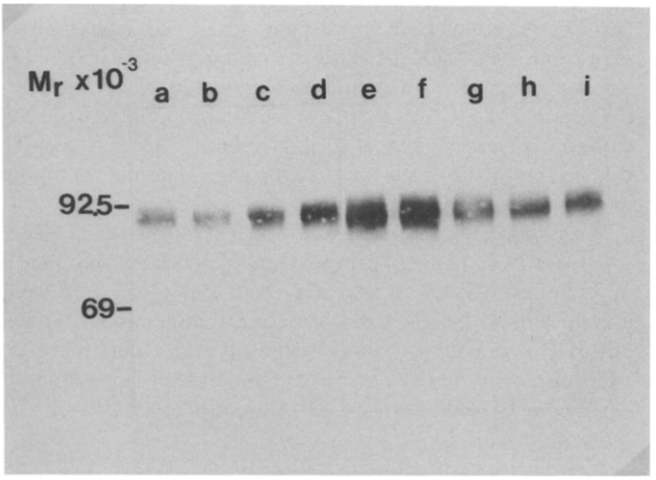


**Fig. 2. Induction of ICAM-1 in U-1810 treated for different intervals (4–96 h) with TNF-α 10 ng/ml. (a) Untreated U-1810 cells, (b) 2 h TNF-α, (c) 4 h TNF-α, (d) 8 h TNF-α, (e) 16 h TNF-α, (f) 24 h TNF-α, (g) 48 h TNF-α, (h) 72 h TNF-α, (i) 96 h TNF-α.**

*Northern blot analysis*

ICAM-1 induction by TNF-α occurred on the transcriptional level as demonstrated by northern blot analysis in cell line U-1810. A faint signal was detectable in unstimulated cells. Maximal induction of both the 3.3 kb and the 2.4 kb transcript was found after 8–16 h of treatment with TNF-α. This indicates that maximal ICAM-1 RNA expression preceded maximal ICAM-1 protein expression for about 4 h (Fig. 4).

The decline of ICAM-1 expression in experiments with long incubation times was probably caused by the inactivation or degradation of TNF-α. Refeeding the cell cultures every 24 h with fresh medium containing TNF-α 10 ng/ml for up to 96 h revealed a constant high ICAM-1 expression on protein and RNA levels (results not shown).

*Cell adhesion assays*

PBMC were seeded onto U-1810 and LCLC-103H cells to evaluate the putative role of ICAM-1/LFA-1 for the adherence of PBMC to tumour cells. Pre-incubation of PBMC with CD11a and CD18 reduced significantly the amount of PBMC bound to tumour cells. In contrast, the anti-ICAM-1 antibodies 84/H10 and P3.58Ba-11 did not reduce PBMC adhesion significantly. Data for cell line U-1810 are shown, similar data were obtained for LCLC-103H (not shown). The activation of PBMC with phorbolester $10^{-8}$ mol/l prior to the seeding onto the tumour monolayer augmented the portion of bound PBMC. The adhesion of the activated or non-activated PBMC occurred via LFA-1, as demonstrated in Fig. 5. ICAM-1 was not involved in PBMC adherence. Upregulation of ICAM-1 by TNF-α led to a

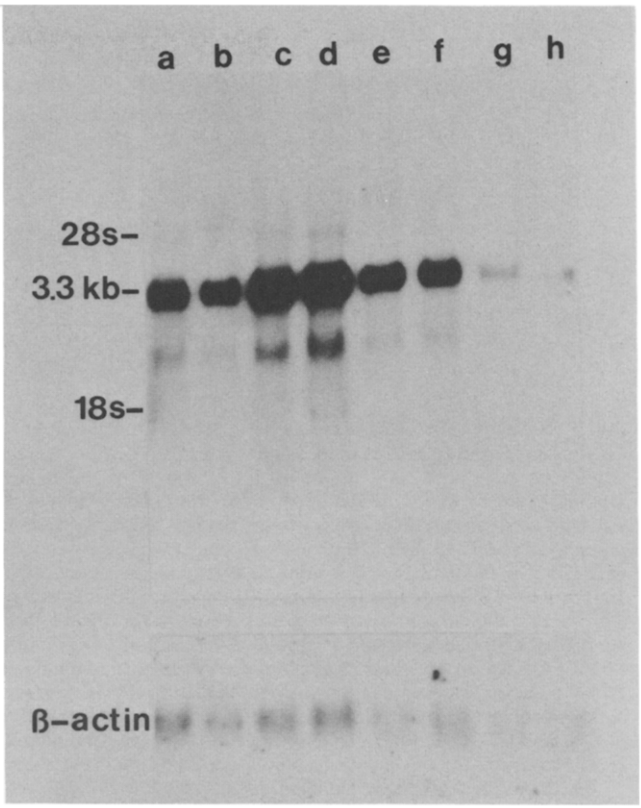


**Fig. 4. Northern blot analysis of ICAM-1 induction in cell line U-1810 after treatment with TNF-α 10 ng/ml for various periods. (a) 48 h TNF-α, (b) 24 h TNF-α, (c) 16 h TNF-α, (d) 8 h TNF-α, (e) 4 h TNF-α, (f) 2 h TNF-α, (g) 1 h TNF-α, (h) control. Ten micrograms of RNA were loaded per lane and the blot was also hybridised with β-actin as internal control.**

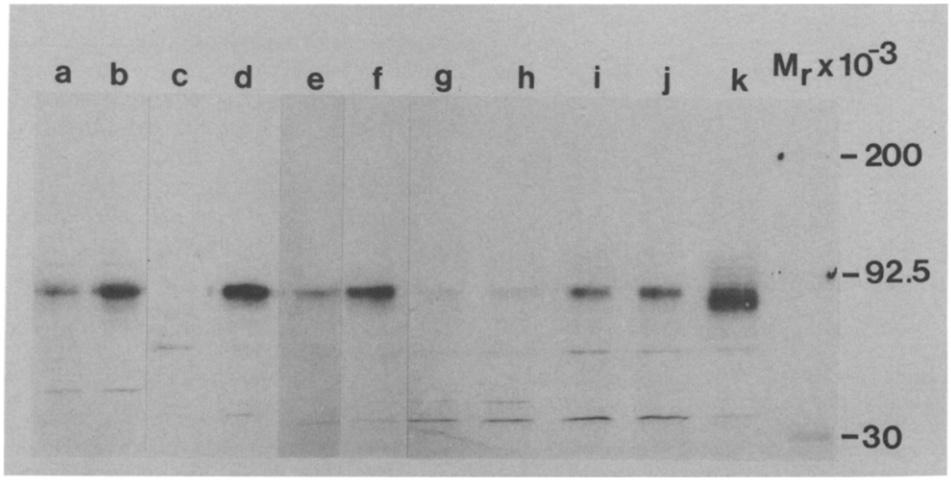


**Fig. 3. Western blot analysis of different NSCLC cell lines expressing ICAM-1. (a) NCI-H157 untreated, (b) TNF-α treatment for 16 h 10 ng/ml, (c) MSTO-211H untreated, (d) TNF-α treatment for 16 h 10 ng/ml, (e) NCI-H125 untreated, (f) TNF-α treatment for 16 h 10 ng/ml, (g) LCLC-H103 untreated, (h) TNF-α treatment for 16 h 10 ng/ml, (i) U-1752 untreated, (j) TNF-α treatment for 16 h 10 ng/ml, (k) LCLC-97TMI untreated.**

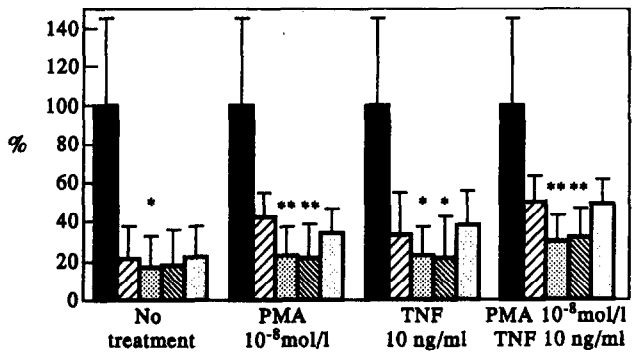


**Fig. 5. Summary of adhesion assays performed with eight different PBMC donors and U-1810 as tumour monolayer. Four different assay conditions are shown. No treatment: no treatment of the tumour monolayer or the PBMC. PMA $10^{-8}$ mol/l: phorbolester $10^{-8}$ mol/l was added to the PBMC prior to the seeding onto the tumour monolayer. TNF 10 ng/ml: the tumour monolayer was stimulated with TNF-α 10 ng/ml for 16 h prior to the adhesion assay. PMA $10^{-8}$ mol/l and TNF 10 ng/ml: tumour monolayer was stimulated with TNF-α 10 ng/ml 16 h prior to the adhesion assay and PBMC were stimulated with phorbolester $10^{-8}$ mol/l. ■ Amount of totally added PBMC. ▨ Amount of bound PBMC in the absence of an antibody. ▩ PBMC adherent in the presence of 5 μg/ml CD11a. ▧ PBMC adherent in the presence of 5 μg/ml CD18. ▦ PBMC adherent in the presence of 10 μg/ml CD54. Significance of differences between total binding ▨ vs. binding in the presence of a blocking antibody was calculated with the t-test for paired samples. $^*P < 0.05$, $^{**}P < 0.005$.**

slight but not significant increase of PBMC adherent to the tumour monolayer. Both anti-ICAM-1 antibodies (84/H10 and P3.58Ba-11) failed to inhibit binding of PBMC to both investigated cell lines U-1810 and LCLC-103H under the mentioned assay conditions in Fig. 6. A mouse IgG isotype control was also without effect (data not shown).

## DISCUSSION

In lung cancer little is known about the expression and the relevance of cell adhesion molecules, their role in metastasis and their interactions with the immune system. SCLC and NSCLC cell lines have not been investigated for ICAM-1 or ICAM-2 expression so far. ICAM-1 expression was only tested in NSCLC tumour specimens with controversial results [9–11]. Natali *et al.* [9] reported one ICAM-1-positive NSCLC out of 15 tested lung tumours whereas Vanky *et al.* [10] found 9 ICAM-1-positive NSCLC out of 12 tested lung carcinomas. Their data are more in accordance with our own.

We assumed that despite NCAM, which was found on SCLC [16–19], ICAM-1 might be present on SCLC cell lines. But in all 10 examined SCLC cell lines, less than 15% of the cells were stained with MAb 84/H10 and, therefore, the lines were classified ICAM-1 negative. As far as tested, ICAM-1 could not be upregulated by TNF-α in SCLC, but little is known about whether SCLC cell lines are TNF-α sensitive and express TNF-α receptors [28].

ICAM-1 expression was found on 10 out of 12 NSCLC cell lines. Surprisingly, large cell carcinomas had the highest level of ICAM-1 expression observed. This type of lung cancer is a poorly differentiated carcinoma which lacks characteristics of squamous or glandular maturation but sometimes shows neuroendocrine properties [29].

Treatment with TNF-α revealed maximal ICAM-1 expression after 16–24 h in U-1810. Prolonged incubation periods without refeeding the cells resulted in an ICAM-1 decline. Guarini *et al.* [13] measured ICAM-1 expression after TNF-α treatment on human central nervous system tumours, demonstrating that ICAM-1 expression remained high for more than 96 h. This downregulation might lead to the suggestion that NSCLC, in contrast to brain tumours, are competent in inactivating TNF-α or TNF-α-induced effects.

We have not tested yet whether gamma-interferon, beta-interferon or IL-1 which were reported to stimulate ICAM-1 expression [7], might lead to an upregulation of ICAM-1 in SCLC and NSCLC cell lines.

ICAM-1 on human lung tumour cell lines seems not to be the ligand for LFA-1- and LFA-1-mediated PBMC adherence to these cell lines. Both anti-ICAM-1 antibodies failed to inhibit PBMC binding to the tumour cells although clone 84/H10 but not P3.59Ba-11 was mapped to the domain involved in LFA-1-dependent binding [30]. The upregulation of ICAM-1 by TNF-α (4-fold determined by flow cytometry for U-1810) was not accompanied by a significant increase of adherent PBMC to U-1810 and LCLC-103. We suppose that ICAM-1 regulation in lung cancer cell lines by cytokines is conserved but its functionality concerning lymphocyte attachment is disturbed. In malignant melanoma, *de novo* expression of ICAM-1 correlates with a poorer prognosis, although lymphocytic infiltration was often described.

One reason for the poorer prognosis could be inadequate function of ICAM-1, for example, altered adhesiveness for LFA-1-bearing lymphoide cells [9, 11, 14, 15].

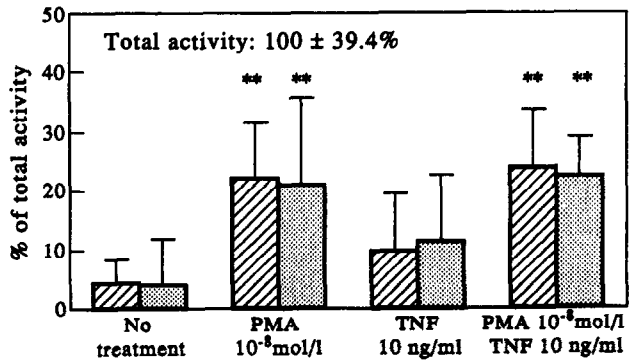


**Fig. 6. U-1810 The percentage of PBMC which can be blocked by CD11a ▨ and CD18 ▧ under assay conditions of no further treatment were compared to assays conditions under stimulation with PMA $10^{-8}$ mol/l or TNF 10 ng/ml or both, as mentioned in Fig. 5. Treatment of the tumour monolayer with TNF-α caused a slight but not significant increase of PBMC bound via ICAM-1/LFA-1. ($P = 0.1$–$0.2$). Treatment of the PBMC with PMA prior to the seeding onto the monolayer increased significantly the amount of PBMC adherent via LFA-1; $^*P < 0.05$, $^{**}P < 0.005$.**

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**Acknowledgements**—We thank Dr J. Johnsons, Institute of Immunology, Munich, Germany for the generous supply of the antibody P3.58Ba-11 and the cDNA probe for ICAM-1. We thank Mr Zoefel for statistical analysis and Mrs D. Lehmann and Mrs E. Wiesing for excellent technical assistance.