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Axl receptor tyrosine kinase expression in human lung cancer cell lines correlates with cellular adhesion

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Abstract

Axl is a receptor tyrosine kinase (RTK) with oncogenic potential and transforming activity. Since Axl bears structural similarities to cell adhesion molecules such as neural cell adhesion molecule (NCAM) (FNIII domains), it is thought that Axl might play a role in adhesion. In this study, we have analysed the expression of the Axl protein and its ligand, Gas6, in human lung cancer cell lines of different histological origin. *Axl* expression occurred in approximately 60% of non-small cell lung cancer (NSCLC) cell lines, which grow adherently, and in normal bronchial epithelial cells (NHBE), but not in cell lines of small cell lung cancer origin (SCLC), which grow in suspension. A number of SCLC sublines, which could be selected spontaneously or after oncogene transfection for adherent growth, all expressed Axl protein. Overexpression of Axl *per se*, however, did not induce any change in the adhesion phenotype. All Axl-expressing cell lines demonstrated a membrane-bound 140 kD form, as well as a soluble 85 kD form, detectable in supernatant, of Axl-RTK. Expression of the Axl ligand Gas6 was detected in approximately 80% of all cell lines investigated. We conclude from these data that loss of Axl expression is a feature of SCLC tumour cells. Axl expression appears to be a consequence of cellular adhesion and possibly influences differentiation in human lung cancers.

Keywords: Axl receptor tyrosine kinase; Gas6; Lung cancer; Cell adhesion

1. Introduction

Axl is a receptor tyrosine kinase which was first isolated during transfection experiments using DNA from patients with myeloproliferative disorders [1,2]. Axl appears to possess oncogenic potential, being able to transform various cell types [3–5]. The association of Axl expression with myeloid leucogenesis (acute myelogenous leukaemia (AML), chronic myelogenous leukaemia (CML) and myelodysplasia) suggests that Axl may play a role in the differentiation pathways of this lineage [3,4,6]. It also appears to be involved in normal mesenchymal and nervous system development and in tumorigenesis [1,6]. In normal tissues, Axl expression is observed predominantly in fibroblasts, myeloid progenitor cells, macrophages, neural tissue, ovarian follicles, cardiac and skeletal muscle, but only rarely in epithelial cells [1].

Axl constitutes a transmembrane protein which can be post-translationally processed by proteolytic cleavage, resulting in the release of a soluble fragment, the extracellular domain (an 85 kD protein), from the membrane-bound portion, which comprises the transmembrane and kinase domains (molecular weight of 55 kD) [7,8]. The soluble extracellular domain has been reported to bind the Growth Arrest Specific Gene-6 (*Gas6*) product, a ligand for Axl [9], and can thereby competitively inhibit the kinase activity of the full length transmembrane receptor. The soluble extracellular Axl domain has also been detected in murine tumours *in vivo* [7,8].

The Axl ligand Gas6 is a vitamin K-dependent protein with structural homology to the anticoagulation

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factor Protein S. Gas6 is a common ligand for the Axl RTK subfamily, activating all members [10,11]. *Gas6* mRNA is detectable in normal lung tissue *in vitro*, among other tissues [12]. In murine cell lines, recombinant human Gas6 protein interacts with the extracellular domain of the Axl-RTK, leading to increased receptor kinase activity and activation of the mitogen-activated protein (MAP) kinase pathway [13,14] or the phosphatidyl-inositol (PI)3-kinase pathway [15,16]. Gas6 thereby exerts mitogenic activity in these cell lines and, furthermore, has been shown to initiate coordinated entry into S phase of the cell cycle when bound to endogenous Axl [14]. It also appears that Gas6 is able to protect serum-starved NIH3T3 cells from apoptosis [13,14]. Myeloid 32D cells, an interleukin(IL)-3 growth-dependent cell line, displays IL-3 independent growth when activated Axl-kinase is expressed, but this is not due to Gas6-Axl interactions [15,16]. These findings suggest that in fibroblasts and in myeloid cells Axl and Gas6 are involved in positive growth control [13,15].

In summary, Axl appears to exert varying effects in different tissues, inducing differentiation in some, mitogenesis or protection from apoptosis in others and bearing homology to important cell adhesion molecules. To determine the potential role of Axl in epithelial cells of the lung, we analysed the expression of Axl and its ligand Gas6 in a panel of small cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC) cell lines. In this study, we report on the histiotypic expression of Axl and its ligand Gas6 in lung cancer cells and their relationship to the adhesion phenotype.

2. Materials and methods

2.1. Lung cancer cell lines and tissue

The human lung carcinoma cell lines analysed comprised the squamous cell carcinoma (SCC) lines EPLC-32M1 and U1752, the large cell carcinoma (LCC) lines LCLC-103H, U1810 and LCLC-97TM1, the adenocarcinoma (ADC) lines NCI-H23, -H125, -H820, -H1573, -H2009, -H2077 and -H2126, and the small cell carcinoma (SCLC) lines NCI-H22, -H24, -H60, -H69, DMS79, NCI-H82, -H146, -H510, -H526, -H841 and N592 and, as a non-tumour control, NHBE (normal human bronchial epithelial cells) [17]. The NIH-H69.5 subclone (*H-ras*/c-*myc* transfected) has been used before as described in Refs. [18,19]. All cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco BRL, Eggenstein, Germany) with 10% heat-inactivated fetal calf serum (Gibco BRL, Eggenstein, Germany), except for NHBE cells which were cultured in BEGM (bronchial epithelial growth medium).

2.2. RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from all cell lines using RNAzol B (AGS, Heidelberg, Germany) as described in the supplier's manual. cDNA synthesis was performed at 37 °C for 120 min in a volume of 30 μl containing 1 μg total RNA and Oligo(d)T as primer. RT-PCR was performed in a volume of 50 μl using 1 μl of the first strand cDNA, 0.1 μg of each primer (forward and reverse), 1.25 μM of each deoxynucleotide triphosphate (dNTP), 1×PCR-buffer (Eurogentec, Seraing, Belgium), 1.5 mM $MgCl_2$ and 1 unit of *Taq* polymerase (Eurogentec, Seraing, Belgium) in the presence of α^{33}P-dCTP. The following primers were used: *Axl* forward (5′-GGT GGC TGT GAA GAC GAT GA-3′) and *Axl* reverse (5′-CTC AGA TAC TCC ATG CCA-3′); *Gas6* forward (5′-CAA TCT CTG TTG AGG AGC TGG-3′) and *Gas6* reverse (5′-CCG TCA CCG AGA AGC GCT-3′); glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*) forward (5′-CGT CTT CAC CAC CAT GGA GA) and *GAPDH* reverse (5′-CGG CCA TCA CGC CAC AGT TT). PCR conditions were a denaturing step at 94 °C for 5 min, an annealing step at 56 °C (*Axl*), 59.5 °C (*Gas6*) or 58 °C (*GAPDH*) for 1 min, and an extension step at 72 °C for 2 min for one cycle, followed by 24 cycles (*Axl* and *Gas6*) or 19 cycles (*GAPDH*) of 94 °C for 1 min, 56 °C, 59.5 or 58 °C for 1 min and 72 °C for 2 min. 10 μl aliquots of the reaction were electrophoresed on 6% polyacrylamide gels, which were then dried and exposed to X-ray films (X-Omat AR, Kodak, Stuttgart, Germany).

2.3. Protein extraction and western blotting

To extract protein from tissue culture supernatants, subconfluent proliferating cells were cultured in serum-free RPMI 1640 for 4 days. Conditioned media were then harvested and proteins were precipitated from 5 ml supernatant overnight in the presence of 7% (w/v) trichloroacetic acid. The resuspended pellets were applied to a 7.5% (w/v) sodium dodecyl sulphate (SDS)-gel. Whole cell extracts were obtained from subconfluently growing cells, washed with 1× phosphate-buffered solution (PBS) and harvested using a cell scraper. Cells were lysed with 1× radioimmuno-precipitation assay (RIPA) buffer containing desoxycholate and genomic DNA was fragmented using a 21-gauge needle. Extracts were incubated for 1 h before centrifugation. Supernatants were quantified and 150 μg of each extract was applied to a 5% (w/v) SDS gel. After electrophoresis, SDS-gels were transferred to nitrocellulose membranes. Blots were reacted with rabbit anti-human Gas6-antiserum or rabbit anti-human Axl-antiserum (diluted 1:100). Immunoreactive bands were identified using biotinylated anti-rabbit antibodies (DAKO, Glostrup, Den-

mark) and stained using the DAB-Kit (Vector Laboratories, Inc., Burlingame, CA, USA).

2.4. Transfection of lung cancer cell lines

Axl- and epidermal growth factor receptor (EGFR)-negative cell lines (the NSCLC cell line NCI-H2077 (Gas6-positive) and the SCLC cell lines NCI-H82 (Gas6-negative) and NCI-H69 (Gas6-positive) were transfected using lipofectamine reagent (Gibco BRL) as described in the supplier's manual using 2 μg of the plasmid. The expression vectors used either contained the wild-type *Axl* gene (pCMV-*neoAxl*; A), a construct encoding an *EGFR/Axl-kinase* chimeric receptor (pCMV-*neoEGFR/Axl*; EA) in which the extracellular and transmembrane region of the human Axl receptor was fused to the kinase domain of the human EGF receptor, and is therefore susceptible to tyrosine phosphorylation after stimulation with EGF [19], or an empty expression vector (pCMV-*neo*; LV) as a control. Likewise, a *fra*-1 expression construct (pMV-*Fra1*) was chosen to generate Fra-1-expressing cell lines. The cells were incubated for 5 h with plasmid and lipofectamine reagent, washed and transfected cells were selected after 24 h using G418. Polyclonal subpopulations were cultured and clones were controlled for positivity by RT-PCR.

2.5. Selection of adherent SCLC subclones

Transfected and parental SCLC cell lines were cultured under normal conditions. A few SCLC cells were noted to sink to the bottom of flasks, becoming adherent and susceptible to removal only by ethylene diamine tetra acetic acid (EDTA)/trypsin solution. These cells were selected and subcultured to create adherent SCLC cell lines.

2.6. Growth kinetics

Transfected and selected cells were preincubated in serum-free medium for 4 days. Serum-starved cells were seeded at a concentration of 12 000 cells/ml into 24-well plates with or without stimulant (Gas6 or EGF, both at 100 ng/ml). Growth rate was measured using a substrate assay (bioreduction of tetrazolium derivate MTS; CellTiter 96 AQ, Promega, Mannheim, Germany) every second day for 14 days. 200 μl of the substrate assay solution was added to each well and incubated for 4 hours at 37 °C. Absorption was measured at 490 nm with cell-free medium including Cell Titer 96 AQ solution as zero. Absorption value was plotted against days with or without stimulant to demonstrate the growth rate. All kinetics were performed with four values for each measuring point and repeated in triplicate.

2.7. Generation of polyclonal antiserum

Anti-Axl amino-terminal antibodies were generated by immunising rabbits with a recombinant protein containing the amino-terminal immunoglobulin domain (AS 31–163). The sequence of this fragment, coding for an extracellular portion of Axl, was cloned into a GST expression vector (pGEX-GST) to generate an Axl-GST fusion protein. Sequencing was performed to confirm the correct reading frame. The GST-Axl protein was synthesised in protease-free bacteria and purified over a glutathione sepharose 4B column. Protein fractions were analysed on a Coomassie stained 10% (w/v) SDS-gel. Fractions containing the fusion protein were injected into rabbits (Eurogentec, Seraing, Belgium) at serial intervals of 4 weeks and serum was collected 2 weeks later. To avoid unspecific binding, the serum was purified over HiTrap-columns (Pharmacia, Uppsala, Sweden) on which the GST-Axl protein was coupled. Western bolt analysis showed that the purified antibody was able to detect 0.002 μg of the fusion protein. Using whole cell extracts, only weak unspecific binding could be detected.

2.8. Binding assay of Gas6 to Axl-Fc protein/protein interaction analysis

Binding activity was measured using BIAcore TM Instruments biosensor technology on research grade BIAcore CM5 sensor chips. Purified soluble Axl-Fc was immobilised by a covalently coupled primary anti-Fc-antibody according to the manufacturer's instructions. Recombinant Gas6 in a dilution series (in 1× PBS) was passed over the immobilised Axl-Fc fusion protein for 20 min. Binding of the ligand was monitored in real time by the increase in the relative resonance unit (RU) in the sensogram. The Axl-Fc was regenerated by elution with 20 mM HCl (pH 1.7). The rate constants of association (k_a) and dissociation (k_d) and dissociation constant ($K_d = k_d/k_a$) were calculated using software supplied by the manufacturer.

3. Results

3.1. Differential expression of Axl-RTK in the NSCLC versus SCLC cell lines

To investigate the expression of Axl-RTK, a panel of lung cancer cell lines derived from tumours of all the major histological subtypes (2 cell lines of SCC; 3 of LCC; 7 of ADC and 11 of SCLC origin) was screened. Semi-quantitative RT-PCR demonstrated low to abundant expression of *Axl* mRNA in seven of 12 NSCLC cell lines (2 SCC; 2 LCC; 3 ADC) (Fig. 1, panel A), but

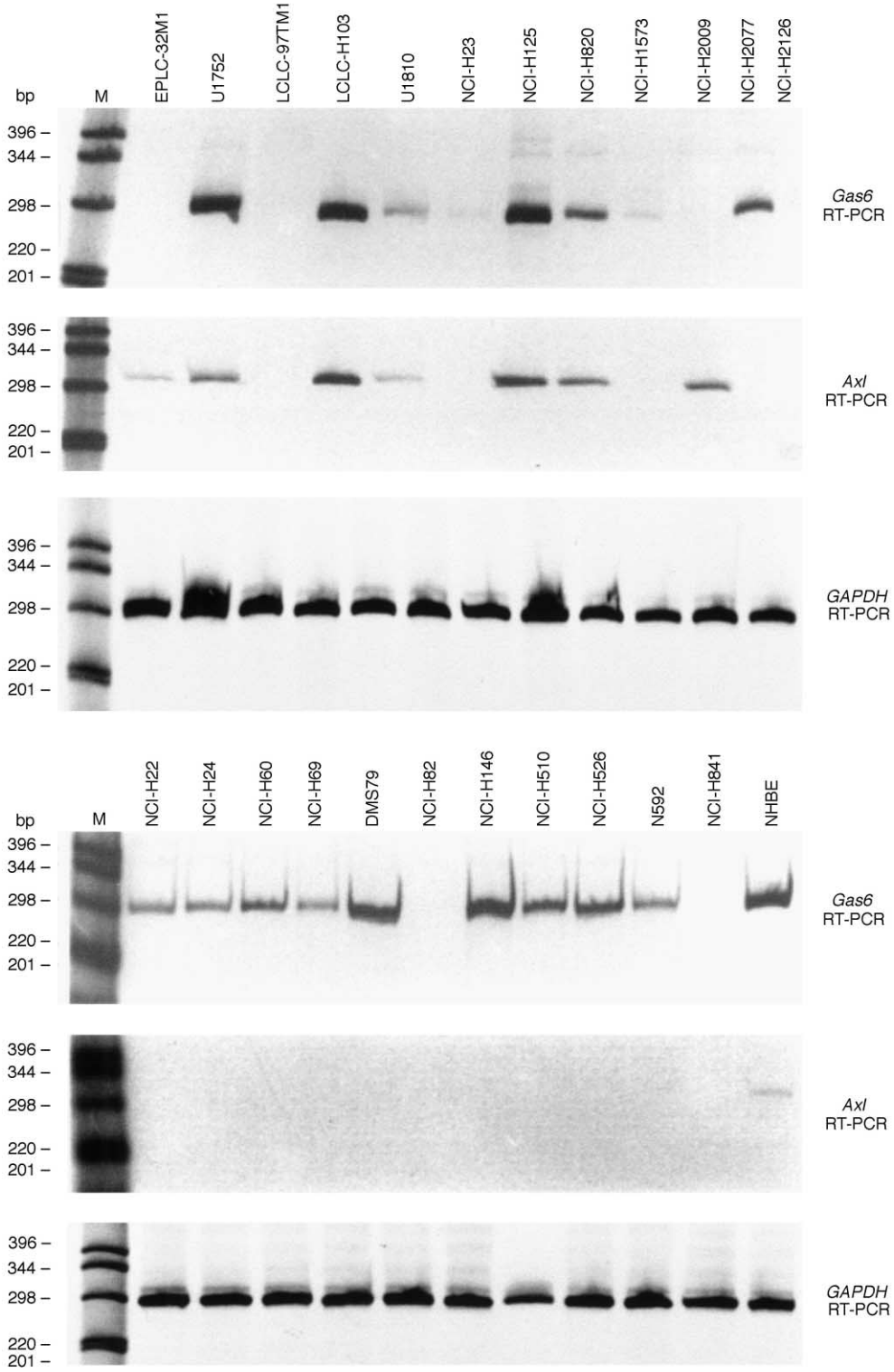

Fig. 1. *Gas6* and *Axl*-RTK expression in lung cancer cell lines and NHBE (normal human bronchial epithelial) cells as shown by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). (A) NSCLC (non-small cell lung cancer); (B) SCLC (small-cell lung cancer) cell lines. *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) serving as a control for equivalent mRNA content. All PCRs were performed using total RNA.

Table 1
Histiotype-related expression profile of *Axl* and its ligand *Gas6* in lung cancer cell lines

	NSCLC		SCLC		NHBE
Axl	7/12	58%	0/10	0%	Yes
Gas6	10/12	83%	8/10	80%	Yes
Axl and *Gas6*	5/12	42%	0/10	0%	Yes

NSCLC, non-small cell lung cancer; SCLC, small-cell lung cancer; NHBE, normal human bronchial epithelial cells.

in none of the 11 SCLC cell lines (Fig. 1, panel B). Low expression of Axl was also noted in the non-transformed NHBE cells (Fig. 1, panel B).

3.2. Expression of Gas6

mRNA for the Axl ligand, *Gas6*, was detected in seven of 12 NSCLC (1 SCC; 2 LCC; 4 ADC) (Fig. 1, panel A) and in nine of 11 SCLC cell lines (Fig. 1, panel B). Co-expression of *Axl* and *Gas6* was observed in 5 NSCLC lines and in the NHBE cells (Table 1) but, given their lack of *Axl* expression, in none of the SCLC cell lines.

3.3. Protein synthesis and post-translational modification of Axl and secretion of Gas6

In order to investigate whether *Axl* and *Gas6* mRNAs are translated and then processed or secreted in these cell lines, we analysed whole cell extracts and conditioned supernatants by Western blot using Axl polyclonal rabbit antiserum or Gas6 antiserum. The former antiserum detected specific bands of immunoreactive protein migrating at the expected sizes of 140 kD (full length Axl-RTK) and 85 kD (processed soluble extracellular Axl) and the latter antiserum Gas6 at 70 kD.

Western blot analysis of whole cell extracts detected full length Axl protein in the majority of NSCLC cell lines and NHBE cells, but no Axl protein in the SCLC cell lines (Fig. 2, panels A and B), confirming the results obtained by RT-PCR.

Analysis of the tissue culture supernatant demonstrated the soluble Axl form migrating at 85 kD in some of the supernatants of the NSCLC cell lines and NHBE only (Fig. 3 panels A and B), indicating that proteolytic cleavage of Axl had occurred in these cell lines. To investigate whether the Axl ligand, Gas6, is also secreted into the tissue culture supernatant, western blot analysis was performed on the protein extract from serum-free supernatants. Gas6 protein was detected in supernatants of NSCLC (9/12) (Fig. 2 panel A), SCLC (7/10) cell lines and NHBE cells (Fig. 2 panel B), in equivalently loaded quantities.

3.4. In vitro interaction of Gas6 with Axl

To establish whether recombinant Gas6 would indeed bind to Axl protein and to measure the affinity of this reaction, we investigated the kinetics of its interaction with Axl-Fc immobilised on a BIA-core sensor chip. Injection of purified human recombinant Gas6 over the Axl-Fc chip resulted in rapid association, but only minimal dissociation. The on- and off- rates and dissociation constant for Gas6 interaction with Axl-Fc were calculated from the sensograms (Table 2). The K_d for Gas6 binding to Axl-Fc (equilibrium dissociation constant) was highly specific (1.78×10^{-10} M) and lower than the one reported previously for the binding of Gas6 to the extracellular domain of Axl [9,11].

3.5. Biological activity of Axl in lung cancer cells

In order to investigate the biological effects of Axl, we transfected three Axl- and EGFR-negative cell lines with expression vectors containing wildtype *Axl*, an *EGFR/Axl*-kinase chimeric receptor construct (the product is susceptible to tyrosine phosphorylation) or an empty expression vector as a control. Appropriate cell lines and constructs were chosen to allow monitoring of Axl-mediated effects with respect to phenotypic background, cellular adherence, presence or absence of endogenous Gas6 ligand and external manipulation of chimeric EGFR-Axl receptor by EGF foster ligand. G418-resistant cell populations emerging from transfections were subjected to RT-PCR analysis, using primers for the extracellular and intracellular domains of Axl, confirming the expression of the appropriate constructs. None of the transfected cell lines showed any morphological variation from the parental cell lines. Irrespective of the presence or absence of endogenous Gas6, neither of the *axl*-transfected SCLC cell lines showed an altered cell adhesion phenotype in comparison to the parental lines.

The effect of Axl-kinase activation was investigated by EGF treatment of NCI-H2077, NCI-H69 and NCI-H82 transfected with the chimeric *Axl-EGFR* construct, which permitted pure activation of Axl-kinase without

Table 2
Kinetic and dissociation constants of binding interactions between Gas6 and Axl-Fc fusion protein

Receptor-Fc	Ligand	Association constant (k_a) $M^{-1}s^{-1}$	Dissociation constant (k_d) s^{-1}	Equilibrium dissociation constant (K_d) M	Affinity constant (K_a) M^{-1}
Human Axl-Fc	Human Gas6	7.41×10^{5}	1.32×10^{-4}	1.78×10^{-10}	5.61×10^{9}

interference from the remaining Axl family members, Tyro3 and cMer, both of which are expressed in these cell lines and share Gas6 as ligand. Furthermore, the use of this construct with the foster ligand EGF avoided the requirement of pp190 protein to stimulate Axl receptor kinase activity [20].

EGF treatment of the serum-starved cells did not alter the cell growth kinetics. Neither was any effect on the survival of the starved cells after EGF treatment observed (Fig. 4A). In order to determine whether wild-type Axl protein itself stimulates the proliferation of lung cancer cells, the growth kinetics of wild-type *Axl*-transfected cell lines in the absence of serum were analysed. No growth occurred in any of these serum-starved transfected cell lines, despite the expression of exogenous Axl and, in the case of cell lines NCI-2077 and NCI-H69, endogenous Gas6 secretion (Fig. 4B). Treatment of wild-type Axl-transfected cell lines with increasing concentrations of recombinant human Gas6 likewise showed no effect on the cellular proliferation index (Fig. 4C). Nor did Gas6 treatment alter the morphology or adhesion properties of these cells.

3.6. Adherent SCLC clones

From each of the transfected SCLC cell lines and non-transfected parental cell lines, adherent clones could be established. These adherent cells appeared

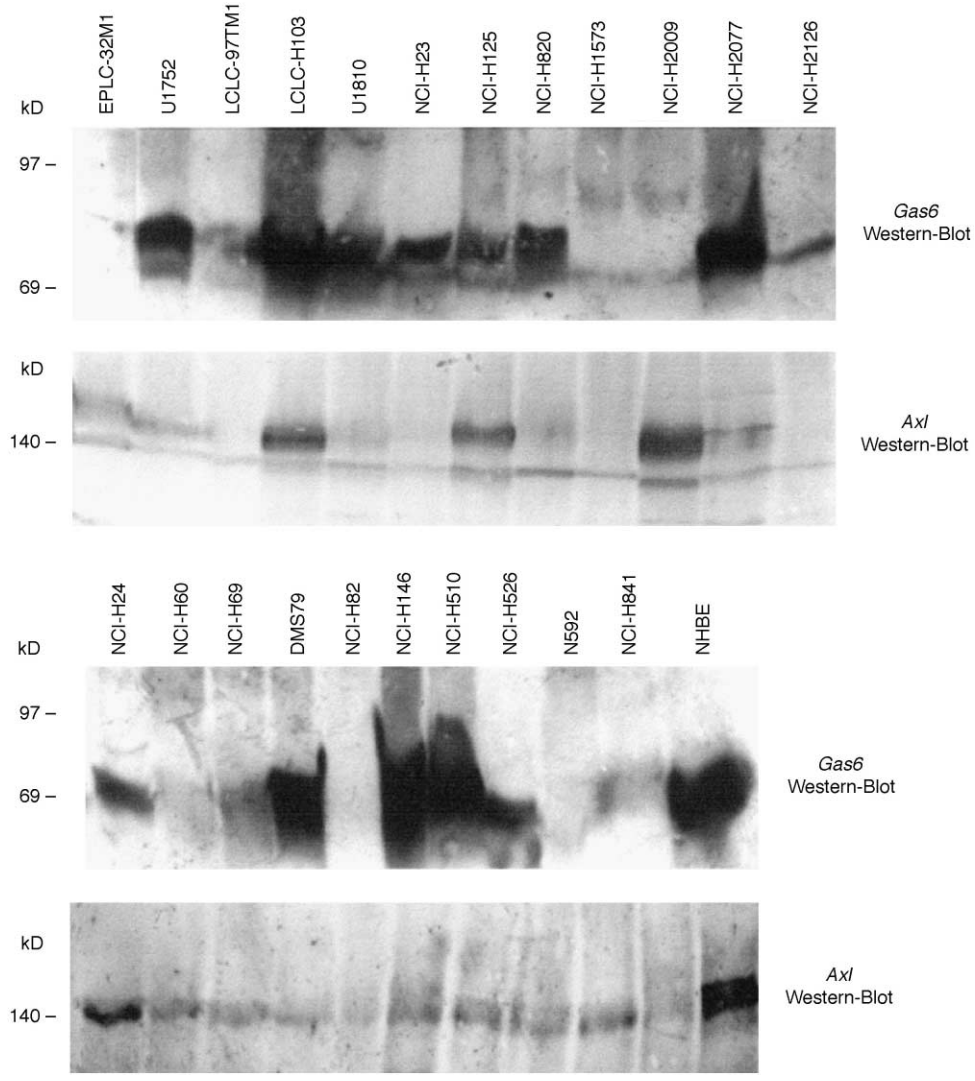

Fig. 2. Western blot analysis of Gas6 and Axl expression in lung cancer cells: (A) NSCLC; (B) SCLC cell lines. For western blot analysis of Gas6 expression, tissue culture supernatant from serum-deprived lung cancer cell lines was used, proteins were concentrated by immunoprecipitated and, after redissoving, separated by SDS (sodium dodecyl sulphate)-PAGE (polyacrylamide gel electrophoresis) and transferred to nylon membranes. Detectable protein migrated at approximately 70 kD. For the detection of Axl protein, whole cell extracts harvested from lung cancer cell lines were prepared and 150 μg of protein was subjected to SDS-PAGE analysis. Detectable protein migrated at approximately 140 kD (below: faint band of immunoreactivity visible in all lanes represents unspecific antibody absorption, especially visible in lower panel, part B due to largely extended DAB-staining, while top band of doublet in lane NHBE represents specific Axl protein).

morphologically to be similar to NSCLC cell lines. RT-PCR for the SCLC marker, *c-kit*, and NSCLC marker, *CD44*, demonstrated that the adherent SCLC clones attained a marker expression pattern typical of NSCLC cells (*CD44*-positive and c-*kit*-negative) (data not shown). Notably, the adherent SCLC clones from NCI-H69 and NCI-H82 each demonstrated expression of *Axl*, a feature not found within their respective parental suspension cultures and otherwise confined to the NSCLC group (Fig. 5).

4. Discussion

Our results show that Axl is expressed in approximately 60% of non-small cell lung cancer (NSCLC) cell lines and in normal bronchial epithelial cells (NHBE), but not in cell lines of small cell lung cancer origin (SCLC). Expression of the Axl ligand, Gas6, was detected in approximately 80% of all cell lines investigated. The expression and synthesis of Axl in NSCLC, but not SCLC, cell lines indicates that Axl might serve as a histiotypic marker for the NSCLC group of lung cancers. Since normal bronchial epithelium (NHBE) also expresses Axl, we suggest that the observed absence of Axl expression in SCLC tumour cells is an aberrant feature.

The finding of co-expression of the Axl receptor and Gas6 ligand within NSCLC cells and NHBE is intriguing and suggests that Axl-Gas6 binding might represent a novel autocrine receptor-ligand system. The significance of such an autocrine mechanism, however, is far from clear since the cellular function of Axl is, as yet, undetermined.

In our series of human lung cancer cell lines expression of *Axl* mRNA was found only in those cells growing adherently, namely cells of NSCLC origin and, as will be discussed later, adherent clones of SCLC. None of the SCLC cell lines growing in suspension expressed *Axl* mRNA. This striking histio- and pheno- typic expression of Axl indicates that Axl plays a role in determining the differentiation of lung cancer cells.

A marked homology exists between the extracellular domains of Axl and a number of cell adhesion molecules, including neural cell adhesion molecule (NCAM). This

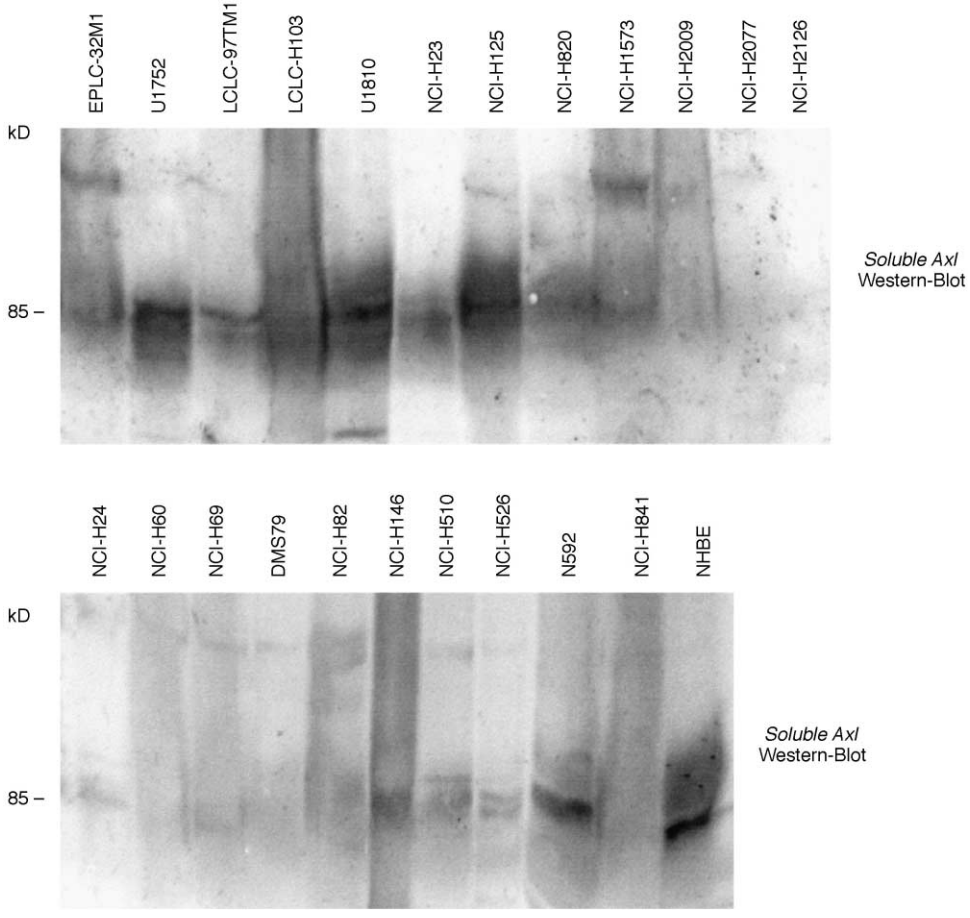

Fig. 3. Western blot analysis of the soluble extracellular Axl fragment in tissue culture supernatant harvested from serum-starved lung cancer cell lines: (A) NSCLC; (B) SCLC cell lines. Proteins were immunoprecipitated and processed as described in the legend to Fig. 2. Detectable protein visible as a broad band of immunoreactivity migrating at approximately 85 kD (distortion due to (salt-)components present in larger quantities of conditioned medium).

raises the possibility that Axl might be a novel cell adhesion molecule, perhaps playing a role in cell-cell or cell matrix interactions (13,15,21). In support of this hypothesis, Gas6 stimulation of Axl has been reported to promote the aggregation of an Axl-expressing myeloid cell line [15,22]. Furthermore, treatment with Gas6 has been reported to induce cell adhesion in Axl-expressing cell lines [15,23]. On the other hand, high amounts of Gas6 have been reported to exert the opposite effect, namely to inhibit the binding of leucocytes to endothelial cells [21]. To investigate whether Axl itself is able to induce adherent growth in lung cancer and to analyse the effect of Gas6, we transfected two SCLC cell lines with a wild-type *Axl* expression vector. Neither of these transfected SCLC cell lines, however, showed any change in their cellular behaviour and continued to grow in suspension, irrespective of the presence of endogenous Gas6 or treatment with recombinant human Gas6. These results demonstrate that Axl activity *per se* is not sufficient to induce cell adhesion in SCLC cell lines.

Alternatively, Axl expression might result from the acquisition of adherent cell behaviour. Intriguingly, we

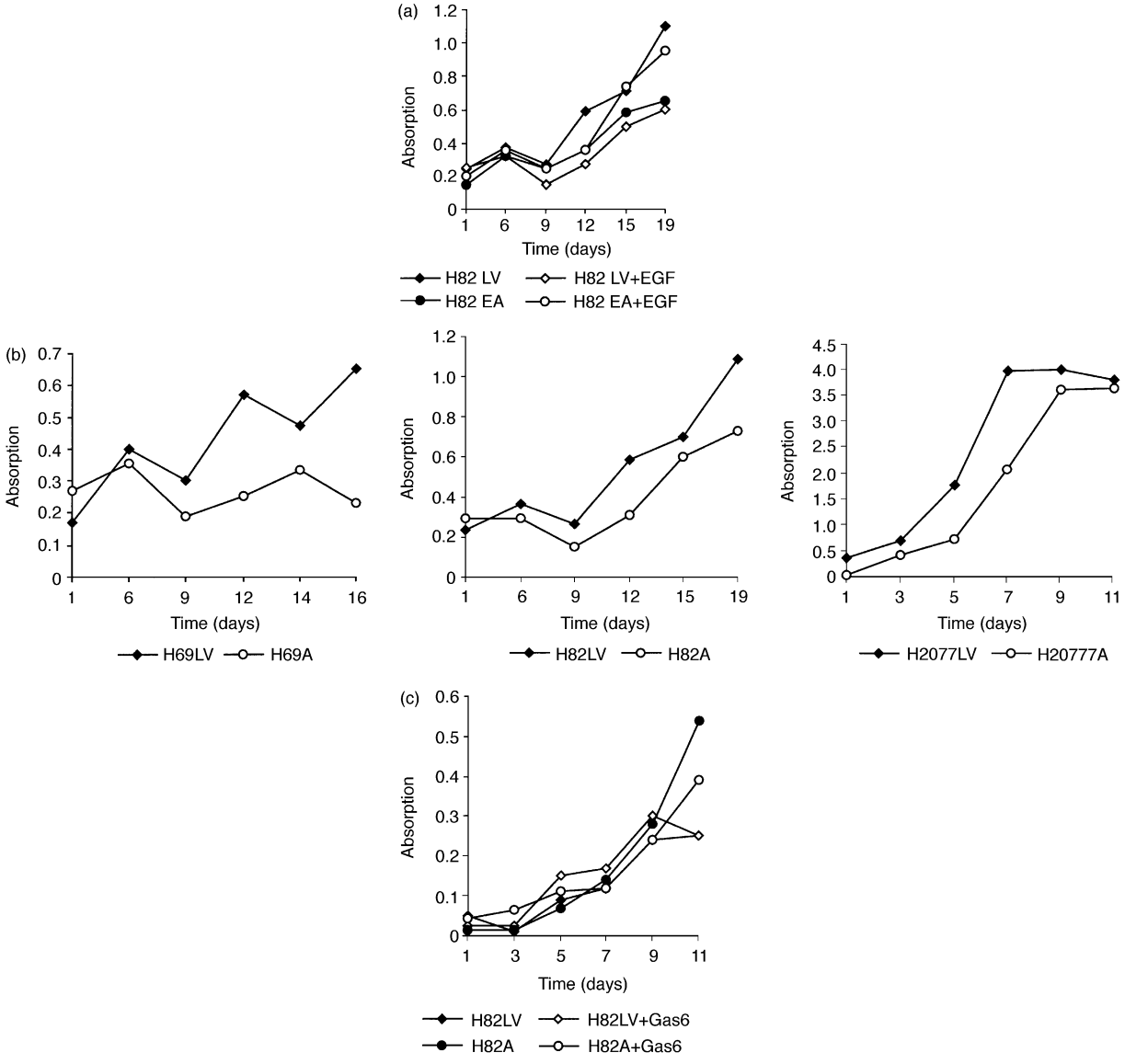

Fig. 4. Cell proliferation of transfected lung cancer cell lines in response to stimulation with foster ligand. Cells were pre-starved for 4 days and seeded in Roswell Park Memorial Institute (RPMI) 1640 medium in the presence or absence of the respective ligand. Proliferating cells were counted every second day as described in Materials and methods. (a) EGF (epidermal growth factor) response curve of lung cancer cell lines transfected with an EGFR (epidermal growth factor receptor)/Axl-kinase chimeric receptor construct (EA) or vector control (LV) (NCI-H82 is shown as an example for all of the cell lines). (b) Cell proliferation assay of *Axl*-wild-type-transfected cell lines (A) or vector control (LV). Cell lines NCI-H69 and NCI-H2077 express endogenous Gas6 ligand, while NCI-H82 does not. (c) Proliferation assay of lung cancer cell lines transfected with a wild-type *Axl*-RTK expression construct (A) or vector control transfected cells (LV) in the presence or absence of recombinant human Gas6 (NCI-H82 is shown as an example for all of the cell lines).

managed to establish adherent clones from each of the suspension parental and transfected SCLC cell lines. All these adherent clones showed Axl expression, in stark contrast to the Axl-negative suspension cultures from which they were derived. This does suggest that Axl expression is a consequence, rather than an inducer, of cell adhesion. It also raises the question of whether Axl influences the process of cellular differentiation. We have demonstrated a notable absence of Axl expression in SCLC cell lines. SCLC cells are characterised by their undifferentiated status. Perhaps the absence of Axl in SCLC cells reflects the putative origin of lung cancer cells from undifferentiated stem cells [24]. Triggering of cell adhesion, by an unidentified mechanism, in Axl-negative undifferentiated SCLC cells might then induce the expression of Axl and resultant cellular differentiation towards the NSCLC phenotype. Our RT-PCR results have verified that adherent SCLC clones do acquire a typical gene expression profile of NSCLC cells (*CD44*-positive, *fra*-1-positive [17,18], unpublished observation), c-*kit*-negative [25]).

Numerous mitogens have been shown to play critical roles in tumorigenesis and the progression of cancer. Endogenous Axl has been shown to exert a mitogenic effect dependent on its Axl kinase activity in serum-starved NIH 3T3 cells [14] and to drive cellular proliferation in murine cell lines [5,9]. To evaluate whether Axl acts as a mitogen in human lung cancer cells, we transfected three Axl-negative lung cancer cell lines of both SCLC and NSCLC origin with different *Axl* receptor constructs (wild-type *Axl*, chimeric human *EGFR*/*Axl* kinase construct and an empty vector control). Growth kinetic studies showed that the proliferation rate of these cells was not influenced by the *de novo* expression of Axl, even when endogenous Gas6 was present. Furthermore, stimulation of exogenous wild-type *Axl* or the EGFR/Axl kinase chimera with recombinant human Gas6 or EGF, respectively, did not induce proliferation. We conclude that, despite the presence of its ligand, neither *de novo* expression nor overexpression of Axl influences mitogenic activity in the investigated lung cancer cell lines.

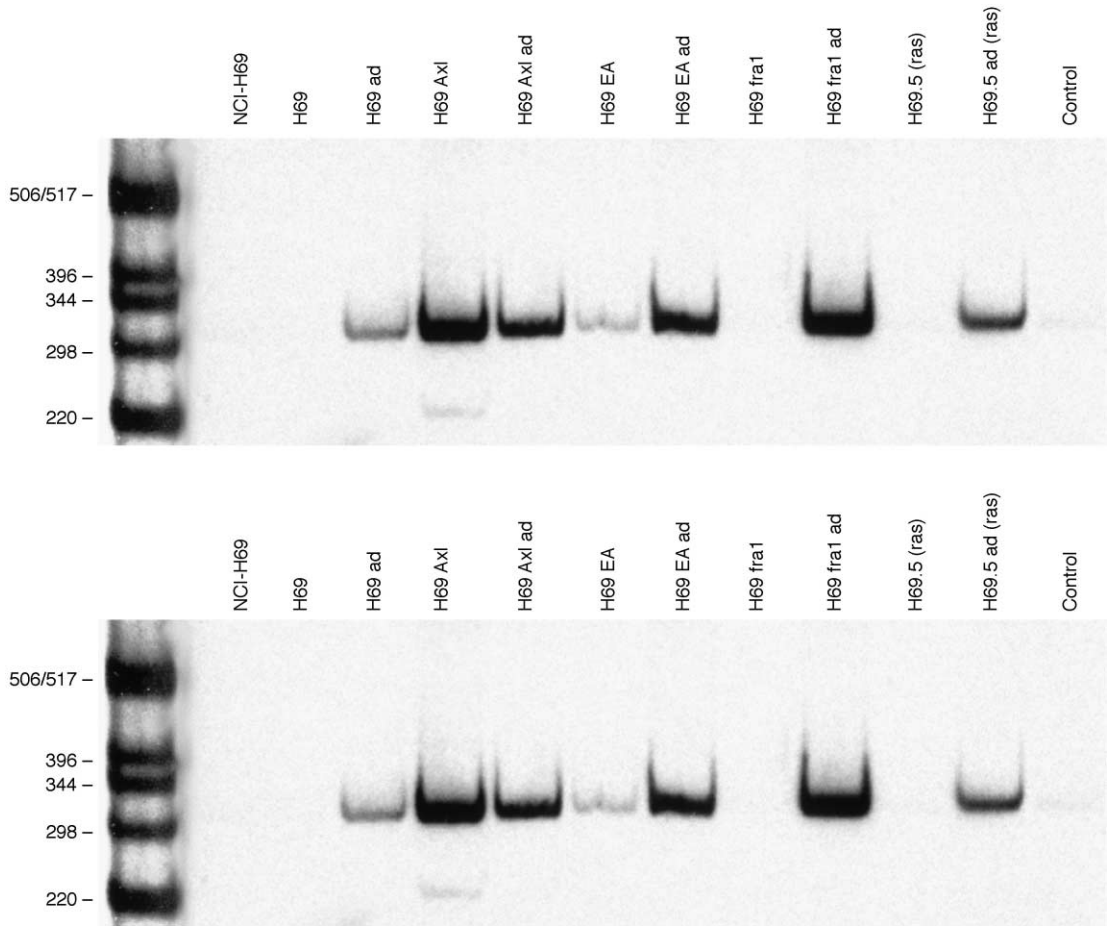

Fig. 5. *Axl*-RTK expression profile in parental and oncogene-transfected NCI-H69 cell clones (*axl*, *fra*-1, H-*ras*) growing in suspension or, alternatively, selected for adherence. Expression analysis was performed by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). All PCRs were performed using total RNA. ad, adherent; EA, *EGFR*/*Axl* kinase chimeric receptor construct; control, PCR reaction without reverse transcriptase.

In agreement with our data for human lung cancer cells, Gas6 has no mitogenic effect in human haematopoietic tissues [26] or human aortic smooth muscle cells [27]. While Axl stimulation by Gas6 has been reported to exert a weak proliferative effect [5,14,27,28], all such experiments were performed in murine cells expressing either endogenous Axl or mixed-species Axl constructs and using recombinant human Gas6. Our study, on the other hand, was designed to investigate the mitogenic activity of Axl in a human cell system using *Axl* constructs of purely human derivation.

A phenomenon recognised to play a role in the regulation of several RTKs is the generation of soluble ligand binding domains by proteolytic cleavage of the full length receptor. For example, c-erbB-2 and CSF-1R are each cleaved by as yet unidentified protease(s) to release their extracellular ligand binding domains as soluble fragments [29,30]. In human lung cancer cell lines a proteolytically cleaved form of Axl has been identified in conditioned media of A549 NSCLC cells [8]. In the supernatants from the NSCLC cell lines and NHBE cells analysed in our study, we could also detect the presence of the Axl extracellular domain, indicating that proteolytic cleavage had occurred in these cells. Failure to detect the Axl extracellular domain in the SCLC supernatants corroborated our findings that Axl was not expressed in the SCLC cell lines. Although the exact role *in vivo* of soluble extracellular domains is unclear, proteolytic cleavage is speculated to provide a means of regulating RTK function. Competitive ligand binding by the soluble extracellular domain is postulated to downregulate signal transduction from the intact membrane-bound receptor [7,8].

In summary, we have shown that Axl expression is a feature of adherent NSCLC cell lines, but is absent in SCLC cell lines growing in suspension. Axl expression appears to be a consequence of cellular adhesion and possibly influences differentiation in human lung cancers.

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