

Expression of the Receptor Tyrosine Kinase Axl Promotes Ocular Melanoma Cell Survival

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ABSTRACT

Metastatic tumor cells originating from cancers of a variety of tissues such as breast, skin, and prostate may remain dormant for long periods of time. In the case of uveal melanoma, the principal malignancy of the eye, complete removal of the primary tumor by enucleation can nonetheless be followed by metastatic tumor growth in distant organs months, years, or even decades later. This suggests that tumor cells have already spread to secondary sites at the time of treatment and remain dormant as micrometastases. Identifying factors that govern long-lived survival of metastatic tumor cells is therefore key to decreasing mortality associated with this and other diseases. While investigating factors differentially expressed in melanoma cells and normal melanocytes, we identified the receptor tyrosine kinase Axl and found up-regulation of Axl in uveal melanomas and melanoma cell lines by RNase protection, Western analysis, and immunohistochemistry. Axl has been shown to mediate cell growth and survival through its ligand Gas6 in non-transformed cells. To test whether stimulation of Axl can enhance survival of uveal melanoma cells, we assessed the degree of mitogenesis and cell survival by bromodeoxyuridine incorporation and trypan blue exclusion, respectively, upon stimulation of Mel 290 uveal melanoma cells with Gas6 *in vitro*. We show that Gas6 mediates mitogenesis and cell survival in Mel 290 cells. We further demonstrate that these effects occur specifically through the Axl receptor by modulating the expression of Axl with an antisense construct. cDNA microarray analysis of 12,687 genes then revealed that Gas6 stimulation of Axl in Mel 290 cells results primarily in the down-regulation of Cyr61, a member of the CCN protein family involved in tumor progression. These data show that the Axl pathway mediates increased survival of uveal melanoma cells, potentially advantageous during cancer dormancy, and that Axl may function in part through regulation of Cyr61.

INTRODUCTION

Uveal melanoma is the most common primary ocular tumor in adults with about 1,300–2,000 new cases occurring in the United States each year. Depending on size, traditional treatment of the primary tumor has been enucleation. However, alternative treatments to preserve the eye have been devised including cryotherapy, radioactive therapy, transpupillary thermotherapy, surgical resection, and photocoagulation. Despite these advances in treatment, mortality varies considerably, to >50% in high-risk patients, primarily because of metastasis to the liver (1). Other organs such as lungs, bones, and skin are also invaded frequently (2). Although the incidence of overt metastases is low (2–3%) at the time of presentation of the primary tumor (3, 4), it is hypothesized that micrometastases already have been established at this time (5). These may remain dormant for many

years before enlargement occurs (6–8). The mechanisms by which tumor cells remain confined to micrometastases and what causes their outgrowth are not well understood (reviewed in Ref. 9).

The Axl receptor (also named UFO and Ark) is a receptor tyrosine kinase and was first identified as a transforming gene in chronic myeloid leukemia (10, 11). It is highly expressed in metastatic colon and prostate carcinoma; gastric, uterine endometrial, and certain types of breast cancer; and sarcoma (12–17). Axl is part of a family of receptor tyrosine kinases that includes c-Mer and Sky/Rse and is expressed ubiquitously (10, 18, 19). It contains immunoglobulin-like and fibronectin III repeats similar to cadherin-type adhesion molecules and receptor tyrosine phosphatases. Intracellular signaling is mediated by a multi-substrate docking site (20). Gas6 is the ligand for Axl (21), and it has been shown to act as a growth and/or survival factor for many nontransformed cell types and an Axl-transfected tumor cell line (22–28). These two activities appear independent in most cell systems and may involve distinct pathways (29, 30). Because several metastatic cell types overexpress Axl, another role of the receptor may be played during invasion and metastasis.

In an effort to identify genes that are differentially expressed in uveal melanoma cells as compared with normal uveal melanocytes, we previously used suppression subtractive hybridization (31). High levels of Axl expression were found in the uveal melanoma cell line Mel 290. We demonstrate here that the addition of Gas6 to serum-starved Mel 290 cells partially rescued these cells due to increased mitogenesis and cell survival. Because the result of the addition of Gas6 is no net change in cell number, these data may suggest a possible mechanism for uveal melanoma tumor cells to remain dormant in the form of micrometastases for extended periods of time.

MATERIALS AND METHODS

Cell Culture. Normal uveal melanocytes were isolated from donor eyes and maintained in culture for 4–6 months, doubling every 3–4 days (32). Cells were maintained in F12 medium supplemented with 10% fetal bovine serum, 50 µg/ml gentamicin, 20 ng/ml human recombinant basic fibroblast growth factor (bFGF; Promega, Madison, WI), 0.1 mM isobutylmethylxanthine, and 10 ng/ml cholera toxin.

Uveal melanoma cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 0.01 M HEPES, 100 units/ml penicillin, 100 mg/ml streptomycin, and 0.1% fungizone (BioWhittaker, Walkerville, MD).

RNase Protection Assay. Axl transcript levels were compared between uveal melanocytes and Mel 290 by RNase protection assay using a 250-base antisense Axl riboprobe and a 177-base antisense glyceraldehyde-3-phosphate dehydrogenase (GAPDH) riboprobe as an internal standard. RNase protection assays were performed as described elsewhere (33) with the modification that 750 units/ml of RNase T1 (Boehringer Mannheim Biochemicals, Indianapolis, IN) was used.

Immunoblotting. Proteins from cellular lysates of uveal melanocytes and Mel 290 were resolved on an 8% polyacrylamide gel and transferred to Immobilon as described elsewhere (31). Nonspecific sites were saturated with 5% w/v nonfat dry milk and 5% BSA in TBST [10 mM Tris-Cl (pH 8), 150 mM NaCl, and 0.05% Tween 20] for 1 h at room temperature. Blots were incubated with a rabbit antibody (1:250 dilution) directed against a peptide derived from the human Axl sequence (C-20; Santa Cruz Biotechnology, Santa Cruz, CA).

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A monoclonal antibody (1:100,000 dilution) against rabbit GAPDH (Biogenesis, Poole, United Kingdom) was used to correct for loading differences. Subsequently, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10000 dilution; Zymed, South San Francisco, CA), and antibody binding was detected by chemiluminescence according to the manufacturer's protocol (Amersham Pharmacia Biotech, Piscataway, NJ).

Immunohistochemistry. Frozen sections (20 μm) of uveal melanoma were incubated with 0.3% methanolic hydrogen peroxide to reduce endogenous peroxidase activity. Slides were blocked with PBS containing 1% w/v BSA and 5% v/v normal goat serum and then incubated overnight with 1 $\mu\text{g}/\text{ml}$ primary antibody [rabbit anti-Axl (17-4 or immunoglobulin-LII, gifts from E. Liu, Genome Institute of Singapore, and L. Fridell, University of Connecticut, Farmington, CT) or normal rabbit IgG (Vector Laboratories, Burlingame, CA)]. Sections were then incubated sequentially with a biotinyl anti-immunoglobulin IgG, 1 $\mu\text{g}/\text{ml}$, (Vector Laboratories) and streptavidin-horseradish peroxidase complex, 1 $\mu\text{g}/\text{ml}$ (Amersham Pharmacia, Inc.). Vector VIP (Vector Laboratories) was used as the substrate to optimize differences between the reaction product (purple) and any background melanin.

Fluorescence *In Situ* Hybridization (FISH) Analysis. To prepare an Axl probe, the bacterial artificial chromosome (BAC) clone CTD219B23 (Research Genetics, Huntsville, AL) containing the *Axl* gene was labeled by nick translation for 90 min at 15°C with digoxigenin-11-dUTP (Roche Molecular Biochemicals, Basel, Switzerland) using a nick translation kit (Roche). Labeled DNA was precipitated in ethanol and rehydrated in Hybrisol VII (Ventana Medical Systems, Tucson, AZ) at 0.01 ng/ml. The probe was denatured for 10 min at 95°C and placed on a metaphase slide of Mel 290 cells (prepared by standard cytogenetic methods), which had been denatured at 72°C in 70% formamide for 2 min and dehydrated in a graded series of alcohol (70, 80, 90, and 100%). The slide was sealed and incubated overnight at 37°C. The slide was washed at 45°C for 3 \times 5 min in 50% formamide in 2 \times SSC (0.03 M NaCl, 0.3 M sodium citrate), followed by 2 \times 5 min in 2 \times SSC. The probe was detected with fluorescein-conjugated anti-digoxigenin (Ventana) for 20 min at 40°C and counterstained with 4',6-diamidino-2-phenylindole.

Reverse Transcription-PCR (RT-PCR): Fresh Tissue. A fresh tumor tissue sample was obtained through the Oculoplastics Service of the Department of Ophthalmology and Visual Sciences, University of Wisconsin Hospital, for isolation of RNA. Total RNA was isolated using a guanidinium isothiocyanate-phenol solution (34). The RNA was treated with DNase I (Promega), followed by reverse transcription with oligo(dT) primers using Moloney murine leukemia virus reverse transcriptase (Promega). First-strand cDNA was treated with RNase H and followed by PCR amplification of a 105-bp Axl fragment using specific primers (sense, 5'-GAGGATGAACAGGATGACTGG-3'; antisense, 5'-ACGAAGGTCTGATGTCCAGGA-3') for 40 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 40 s. A 123-bp GAPDH fragment also was amplified using specific primers (sense, 5'-ACCATGGGGAAGGTGAAGG-3'; antisense, 5'-CATTGATGGCAACAATATCAAC-3') for 40 cycles under the same conditions.

RT-PCR: Archival Tissue. Sections from six different uveal melanomas were obtained from various clinical sources. RNA was isolated from these formalin-fixed, paraffin-embedded sections as described by Stanta *et al.* (35) with some modifications. Briefly, tumor tissue was excised from 6- μm paraffin sections using a scalpel and an alternate H&E-stained tissue section for orientation. Sections were agitated in 1 ml of xylene, centrifuged, and washed with ethanol. The pellet was treated with 6 mg/ml proteinase K, 1 M guanidinium isothiocyanate, 25 mM 2-mercaptoethanol, 0.5% sarcosyl, and 20 mM Tris-Cl (pH 7.8) overnight at 45°C. The solution was extracted with phenol-chloroform and precipitated in isopropanol and glycogen. The ethanol-washed pellet was resuspended in water and added to a solution containing first-strand buffer, 10 mM DTT, 0.8 units/ μl RNasin (Promega) and 1.3 units/ μl DNase I for 30 min at 37°C. After phenol-chloroform extraction, 45.5 pmol of random hexamers were added. Samples were precipitated with sodium acetate and ethanol and resuspended in 10 μl of water. The RNA was denatured for 3 min at 68°C and reverse transcribed with avian myeloblastosis virus reverse transcriptase (Promega) for 1 h at 42°C. The reverse transcriptase was inactivated at 95°C for 2 min, followed by PCR amplification with Axl primers (already described).

Gas6 Assay. Mel 290 cells were grown on 24-well plates for 2 days until roughly 60% confluent in 10% FCS-containing medium. The medium was then

changed to 0.5% serum for 2 days, after which all serum was removed. Gas6 then was added to cells at 500 ng/ml, and after 3 days these cultures were compared with cultures grown in the same medium in the absence of Gas6 by bromodeoxyuridine (BrdUrd) incorporation and immunostaining (see below). Cells were counterstained with Hoechst 33528 (Molecular Probes, Eugene, OR). During the 3-day incubation, the medium was changed daily. In some experiments, cells were incubated with 150 ng/ml of bFGF instead of Gas6.

BrdUrd Labeling Assay. Cells were pulsed for 2 h with 250 μM BrdUrd and then washed 3 \times 5 min with medium. Cells were fixed with ice-cold 80% ethanol for 30 min at -20°C, air dried, and the DNA denatured with 4 N HCl for 6 min at room temperature. After neutralization with 0.1 M sodium borate, the cells were washed 3 \times 5 min with PBS (0.14 M NaCl, 0.0027 M KCl, 0.1 M phosphate buffer, pH 7.4) containing 1% BSA and 1% normal goat serum, followed by incubation with anti-BrdUrd antibody (1:150 dilution; Becton Dickinson, Franklin Lakes, NJ) overnight at 4°C. Cells were then incubated with biotin-conjugated goat anti-rabbit IgG (1:500 dilution; Vector Laboratories, Burlingame, CA) for 1 h, followed by incubation with streptavidin-fluorescein (1:500 dilution; Amersham) for 1 h. Finally, the cells were incubated with 1 $\mu\text{g}/\text{ml}$ Hoechst 33528 in PBS for 30 min at room temperature, washed with PBS, and viewed with the aid of a fluorescence microscope (Zeiss AxioVert). The percentage of BrdUrd-labeled cells was calculated as the number of BrdUrd-labeled cells relative to the total number of cells as visualized by the Hoechst 33528 staining. Each experiment was repeated at least three times. All experiments were performed in triplicate, and counts were averaged from five different viewing fields.

Cell Death Assay. Mel 290 cells growing on 24-well plates were trypsinized and pooled. The cells were collected by centrifugation, washed with PBS, and resuspended in an equal volume of PBS and trypan blue (Sigma Chemical Co., St. Louis, MO). Cell suspensions were then pipetted onto a hemacytometer and counted under a microscope (Zeiss AxioPhot). The ratio of dead cells (blue) to the total amount of cells was determined by counting four different fields for each experimental condition.

Stable Transfection of an Antisense Axl Expression Construct. A 3,250-bp Axl cDNA fragment was excised from pLXSP-Axl1-4 [a gift from E. Liu (10)] with *Eco*RI and subcloned in reverse orientation into pcDNA3 (Invitrogen, Carlsbad, CA).

One day before transfection, 1 \times 10⁵ Mel 290 cells were plated on a 60-mm-diameter dish. Cells were transfected with 2.5 μg of pcDNA-AxlR using 7 μl of Lipofectamine (Life Technologies, Inc., Rockville, MD) in OptiMEM medium (Life Technologies) according to the manufacturer's protocol. The cells were incubated for 5 h at 37°C with the transfection DNA, after which maintenance medium containing 20% fetal bovine serum was added, and the incubation continued overnight. Fresh maintenance medium was added, and after 3 days cells were transferred to 150-mm-diameter dishes and grown in the presence of 6.55 ng/ml G418 (AgriBio, Miami, FL) until colonies could be seen. Individual colonies were harvested by trypsinization using cloning rings (BellCo Glass Inc., Vineland, NJ) and transferred to 24-well plates. Cells were expanded to 75-cm² flasks and then screened for the presence of the antisense construct by PCR analysis as well as for Axl expression by Western analysis.

Gas6-stimulated Gene Expression. RNA was isolated from Mel 290 cells and cells after Gas6 stimulation (as described previously) and processed to obtain double-stranded cDNA. The double-stranded cDNA was used in an *in vitro* transcription reaction to produce biotinylated cRNA, which was isolated with an RNeasy Mini kit (Qiagen Inc., Santa Clarita, CA). Biotinylated cRNA was then fragmented and hybridized to Affymetrix HG-U95A GeneChips (Santa Clara, CA) according to the manufacturer's recommendations. The results were evaluated using the Affymetrix Data Mining Tool.

Real-Time PCR. RNA was isolated from Mel 290 cells and reverse transcribed as described above. Real-time PCR was performed with SYBR mix (Applied Biosystems, Foster City, CA) using Cyr61-specific primers (sense, 5'-GGAAGCCTTGCTCATTCTTGA-3'; antisense, 5'-TCCAATCGTGGCTGCATTAGT-3') and GUS-specific primers (sense, 5'-GGAATTTTGC-CGATTTCATGA-3'; antisense, 5'-AGGAACGCTGCACTTTTTGG-3') as an internal standard using an Icyler (Bio-Rad) according to the manufacturer's instructions. All reactions were performed in triplicate. Relative expression levels and SDs were calculated using the comparative method as described (36).

RESULTS

Differential Expression of the Axl Receptor. To understand the molecular basis of uveal melanoma, suppression subtractive hybridization was used previously to identify genes with altered levels of expression in uveal melanoma cells as compared with normal uveal melanocytes (31). The comparison was made using mRNA obtained from an established uveal melanoma cell line, Mel 290, derived from a human biopsy and mRNA from uveal melanocytes isolated from donor eyes and passaged in culture for a limited time. Using suppression subtractive hybridization to screen for genes with elevated expression levels in melanoma cells, the receptor tyrosine kinase Axl was identified. Axl has been found to be up-regulated in a number of tumors and tumor cell lines (10, 37) but has not been studied in the eye.

Differential expression of Axl in normal uveal melanocytes and malignant melanoma cells was confirmed by RNase protection assay (Fig. 1A). The assay was performed with RNA of normal uveal melanocytes and Mel 290 cells using antisense Axl and GAPDH probes. Using GAPDH as an internal standard, steady-state Axl mRNA could be detected in melanoma cells but not in normal melanocytes.

Relative expression levels of the Axl protein were determined by immunoblotting using whole-cell extracts (Fig. 1B). Axl migrates as a doublet because of differential glycosylation (38). Axl was detected at much higher levels in Mel 290 cells than normal uveal melanocytes. A duplicate blot was used to stain for GAPDH as a control for loading artifact. This difference in Axl expression between Mel 290 and UM cells was not a consequence of the differences in growth media, because replacing the UM media with Mel 290 media for 24–48 h did not alter the levels of Axl in the uveal melanocyte culture (Fig. 1B). Axl expression in Mel 290 cells likewise was unaltered by changing to UM media (data not shown). The findings from RNase protection assays and immunoblotting confirm the results from the suppression subtractive hybridization assay that the expression of Axl is up-regulated in the malignant Mel 290 cells relative to normal uveal melanocytes.

FISH Analysis. In previous studies of Mel 290 cells, the amplification of certain genes corresponded to structural changes in a region of chromosome 1p observed by routine karyotyping (31). No similar duplications were observed for chromosome 19, the site of the Axl gene. To examine this issue more closely and to attempt to identify any structural alterations associated with the amplification of Axl expression, a BAC clone containing the entire coding region of Axl was used for FISH analysis of a Mel 290 chromosomal preparation. On inspection, two copies of the gene were indicated; no translocation or double minute chromosomes associated with the Axl gene were observed (Fig. 2). At this time, the sort of structural mutations

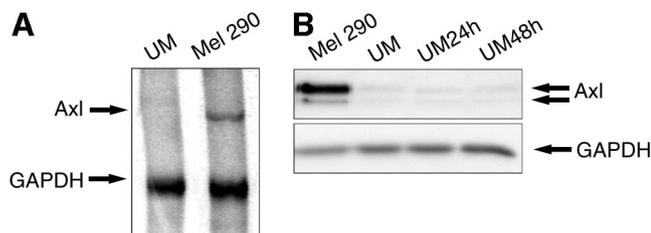


Fig. 1. Axl expression is up-regulated in the uveal melanoma cell line Mel 290. *A*, RNase protection analysis of total RNA isolated from normal uveal melanocytes (UM) and the uveal melanoma cell line Mel 290 using antisense Axl and GAPDH probes. *B*, Western analysis of total cellular extracts from the uveal melanoma cell line Mel 290 and uveal melanocytes, using antibodies specific for Axl and GAPDH. UM24 h and UM48 h, UM cells grown for 24 and 48 h, respectively, in Mel 290 medium.

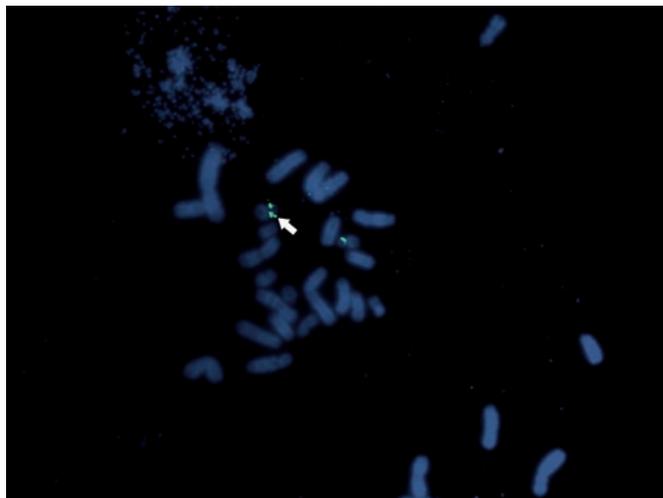


Fig. 2. Chromosome analysis of the Axl gene in Mel 290 cells. Mel 290 cells were analyzed by FISH using a BAC clone containing the Axl gene as described in "Materials and Methods." Arrow indicates two labeled copies of the Axl gene.

underlying the amplification of other genes in Mel 290 has not been observed for Axl.

Axl Expression in Primary Uveal Melanoma. The results thus far demonstrate the presence of Axl in uveal melanoma cells maintained in culture. To determine whether Axl is present in primary uveal melanomas and, therefore, may contribute to the phenotype of actual tumors, RT-PCR was performed to detect Axl mRNA in archival specimens and fresh tumor specimens of uveal melanoma (Fig. 3). Axl was detected in all melanoma samples tested, which included six archival specimens originating from both spindle cell and epithelioid tumor subtypes, and a fresh mixed cell tumor. We have used real-time PCR to further assess expression levels in tumor specimens. In addition to the samples shown in Fig. 3, eight of nine tumor specimens tested showed a significant increase in Axl expression compared with uveal melanocytes of 3–240-fold (data not shown).

To ensure that the expression of Axl did not originate from a minor population of non-tumor cells contained within the excised tumor mass (endothelial cells or fibroblasts), frozen sections were labeled with an antibody specific for Axl. The immunostaining was found to be uniformly associated with tumor cells throughout the specimen (Fig. 4).

Effect of Gas6 on the Mitogenesis of Mel 290 Cells. Binding of the ligand Gas6 to the Axl receptor has been described to induce cell cycle reentry in serum-starved NIH 3T3 cells and to enhance cell survival in the absence of growth factors (28). However, the role of Axl-mediated signaling has not been studied in cancer cells. To determine whether Gas6 can mediate survival and/or mitogenesis of ocular melanoma cells through their Axl receptors, Mel 290 cells were serum starved for 48 h in media with 0.5% serum, followed by complete serum withdrawal, at which time, defined as T_0 , Gas6 either was added in one condition or not added to the control condition. The effect of Gas6 on mitogenesis was measured by BrdUrd incorporation and on cell survival by trypan blue exclusion.

When serum was completely withdrawn from Mel 290 cells for three days, BrdUrd incorporation decreased >6-fold, dropping to 14% compared with controls (Fig. 5, *A* and *B*). Adding Gas6 to the medium of serum-starved Mel 290 cells for 3 days, however, resulted in BrdUrd incorporation levels that were virtually unchanged (79%) from cells growing in the presence of serum (Fig. 5, *A* and *B*). These data illustrate that Gas6 enables serum-starved Mel 290 cells to

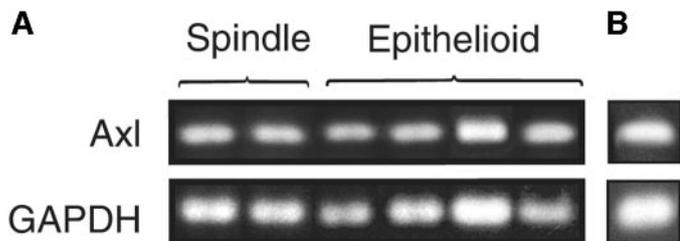


Fig. 3. Axl is expressed in both spindle-type and epithelioid primary uveal melanomas. A, RT-PCR analysis of RNA isolated from paraffin sections of uveal melanoma using gene-specific primers. B, RT-PCR analysis of RNA isolated from fresh uveal melanoma tissue of mixed cell type.

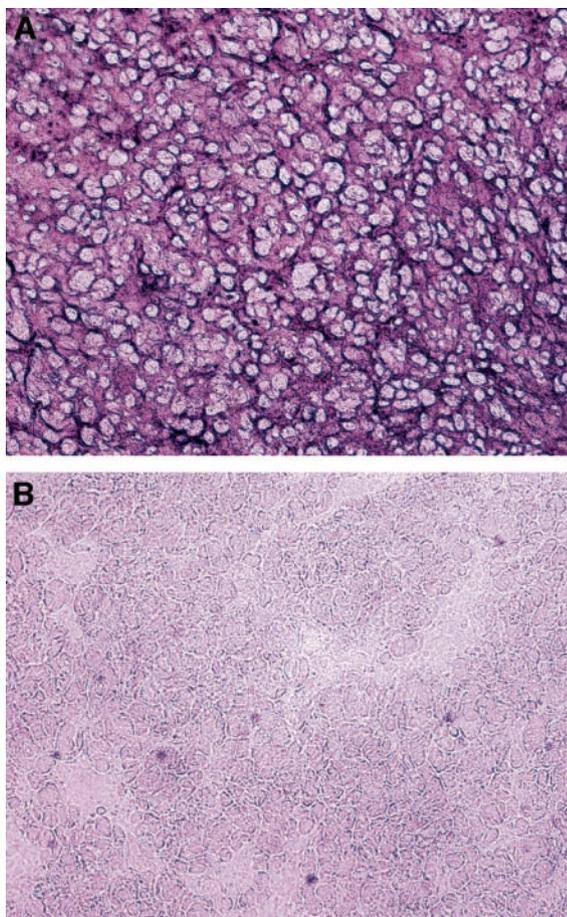


Fig. 4. Axl is homogeneously expressed throughout uveal melanoma tumor tissue. Immunohistochemistry on frozen sections of a uveal melanoma incubated with the anti-Axl antibody 17-4 (A) or normal rabbit IgG (B).

maintain a mitogenic rate that is very similar to cells growing in 0.5% serum-containing media (which in turn is comparable with cells growing in complete medium).

Mitogenesis also can be stimulated by bFGF when added to serum-starved NIH 3T3 cells, although it is known to act via a different receptor than Gas6. Therefore, serum-starved Mel 290 cells were incubated with bFGF for 3 days instead of Gas6. Addition of bFGF to serum-starved cells did not increase BrdUrd incorporation (Fig. 5B).

Specificity of the Axl and Gas6 Effects. Other members of the Axl tyrosine kinase receptor family, c-Mer and Sky/Rse, have been shown to bind Gas6, albeit at a lower K_d (19). Therefore, to determine whether the effect of Gas6 on Mel 290 cells is mediated by the Axl receptor, a stably transfected Mel 290 cell line carrying an antisense Axl expression construct was used in the same Gas6 assays as de-

scribed above. This stable cell line expresses reduced levels of Axl receptor (Fig. 5C). Fig. 5D illustrates that BrdUrd incorporation in cells containing the antisense Axl construct was reduced similarly in the presence or absence of Gas6, indicating that Gas6 mediates its effects on Mel 290 mitogenesis through the Axl receptor. The difference in mitogenesis levels in the absence of Gas6 between the Mel290 cells (14%; Fig. 5B) and the antisense Axl cells (29%; Fig. 5D) may result from the fact that the antisense Axl cell line was selected as a single colony during the stable transfection procedure, perhaps leading to a slightly modified response to serum starvation as compared with parental Mel 290 cells.

Effect of Gas6 on the Survival of Mel 290 Cells. Next, cell death was measured by trypan blue exclusion (see "Materials and Methods" and Fig. 6). If serum was completely removed from Mel 290 cells, approximately half of the cells were dead on the third day. However, if the cells were placed in Gas6-containing medium for 3 days, only 18% of the cells died, as judged by their ability to exclude the vital dye. If 10% serum was added to the cells for 3 days instead of Gas6, cell death on the third day was 4–5%. These data suggest that withdrawing serum from Mel 290 cultures leads to an incremental increase in cell death, but Gas6 is able to partially prevent this detrimental effect to the cells. However, it cannot completely protect the cells to the degree obtained with complete medium.

Analogous to the mitogenesis experiments described above, the stable cell line containing the antisense Axl construct was used to determine whether the effect of Gas6 on survival of Mel 290 cells is

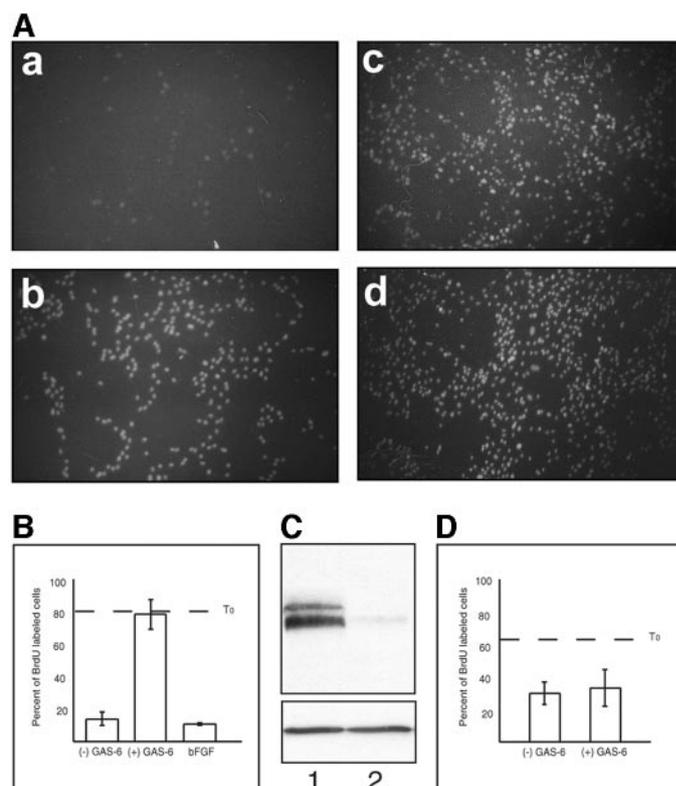


Fig. 5. Gas6 induces mitogenesis in Mel 290 cells. A, serum-starved Mel 290 cells were grown in the absence (a and c) or presence (b and d) of Gas6 and then labeled with BrdUrd (a and b) or Hoechst 33528 (c and d). B, percentage of BrdUrd-labeled cells grown in the absence or presence of Gas6 or the presence of bFGF. Bars, SD. C, Western analysis of total cellular extracts of Mel 290 cells (Lane 1) and a Mel 290 cell line transfected with an antisense Axl construct (Lane 2) using antibodies specific for Axl (top panel) and GAPDH (bottom panel). D, mitogenesis in Mel 290 cells stably transfected with an antisense Axl expression construct in the absence or presence of Gas6. T_0 , start of complete serum withdrawal and addition of growth factors. (The same results were obtained using either Mel 290 cells or Mel 290 cells stably transfected with an empty vector as the control for comparison with the antisense Axl construct). Bars, SD.

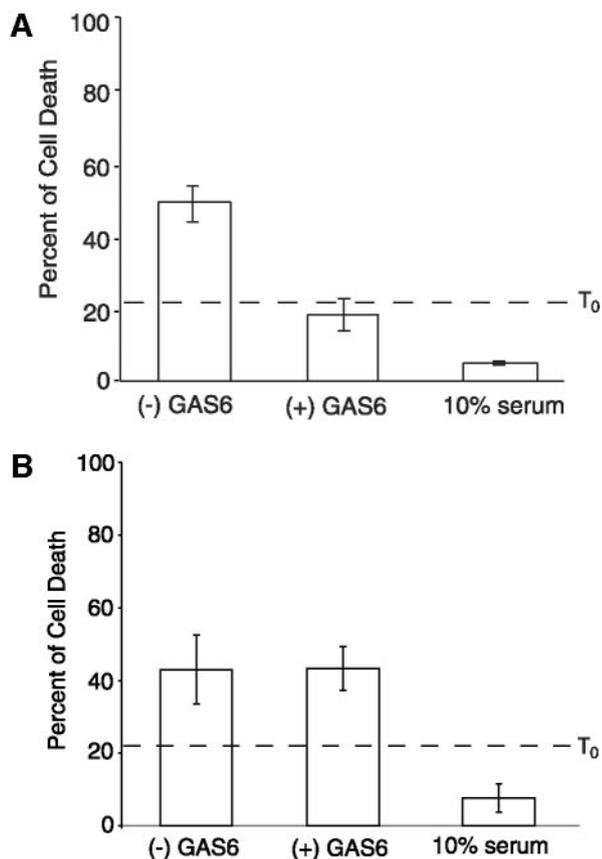


Fig. 6. Gas6 mediates survival in Mel 290 cells. *A*, percentage of cell death in Mel 290 cultures grown in the absence or presence of Gas6 or 10% serum. *B*, survival in Mel 290 cells stably transfected with an antisense Axl expression construct in the absence or presence of Gas6. T_0 , start of complete serum withdrawal and addition of Gas6. Bars, SD.

mediated by the Axl receptor. Fig. 6*B* illustrates that cell death in cells containing the antisense construct increased similarly between serum-starved cells growing in the presence or absence of Gas6, indicating that Gas6 also mediates its effects on Mel 290 survival through the Axl receptor.

The cumulative effect of Gas6 on cell number in the Mel 290 cultures is that it remains relatively unchanged. Although Gas6 has a positive mitogenic effect on serum-deprived Mel 290 cells, its action is counterbalanced by a higher percentage of cell death compared with cells maintained in medium with serum. If 10% serum is added to the medium instead of Gas6, the cell number increases ~5-fold after 3 days in comparison with cultures grown only in the presence of Gas6. Because the BrdUrd incorporation is similar in both cultures, these data suggest that the increase in cell number in cultures to which 10% serum has been added is mainly the result of a decreased number of cells dying.

Gas6-stimulated Gene Expression. To begin characterizing the cellular pathways by which ligand-stimulated Axl might influence tumor cell phenotype, RNA was isolated from Mel 290 cells after stimulation with Gas6 and processed for hybridization with Affymetrix HG-U95A GeneChips. Upon examination of the data, the most notable change compared with unstimulated cells was the 11-fold down-regulation of Cyr61. To verify this finding, real-time PCR was performed after the addition of Gas6 for 0, 30, 60, and 90 min. Transcript levels were normalized to GUS and then compared with T_0 (Fig. 7). As a control, a similar time course was performed without the addition of Gas6. Independent of Gas6, Cyr61 levels decrease somewhat over 60 min after complete serum withdrawal. In the absence of Gas6, Cyr61 levels then remained stable. However, in the presence of

Gas6, Cyr61 levels decreased further to a total of 17-fold, thus confirming results from the initial GeneChip analysis. Results were further confirmed by RNase protection assay (data not shown). As another control, a Gas6 time course was performed on the Mel 290 cell line stably transfected with an antisense Axl construct. Cyr61 levels decreased over 60 min, similarly to the Mel 290 time course, and then remained stable in the presence of Gas6 rather than decline further, indicating that Gas6 mediates the decrease in Cyr61 levels through the Axl receptor.

DISCUSSION

We demonstrate the expression of Axl in both an established cell line of uveal melanomas and in primary tumors. Stimulation of Axl by its ligand, Gas6, invokes mitogenesis, and a comparable effect was not obtained by the addition of other growth factors such as bFGF. Gas6 stimulation of Axl also reduces but does not completely eliminate cell death during serum starvation. The mitogenesis and survival observed in the presence of Gas6 require Axl, because down-regulation of the gene through the use of an antisense construct eliminates the effects. The net effect of Axl expression and subsequent stimulation by Gas6 is to maintain the number of uveal melanoma cells under conditions that otherwise would result in the loss of cells. It is therefore likely that the expression of Axl provides these tumor cells with some selective advantage.

One mechanism that has been proposed for dormancy of micrometastases is through balanced proliferation and apoptosis while in a state of angiogenic suppression (39, 40). Axl and similar genes may have a role during periods of dormancy characteristic of some micrometastases. In patients with metastatic uveal melanoma, metastases likely arise by the time the primary tumor is detected. These metastases can remain relatively asymptomatic for periods up to 40 years but once activated, are usually fatal within a few (6–8) months. Axl expression may aid the survival of such long-lived micrometastases by helping to maintain a balance between cell division and cell death. In addition, angiogenic suppression may result from down-regulation of the angiogenic factor Cyr61, supporting the mechanism described for dormancy of micrometastases (39).

Here we demonstrate that the angiogenic factor Cyr61 is a principal gene affected by Gas6 stimulation of Axl in uveal melanoma cells. Cyr61 is a secreted, matrix-associated protein implicated in complex cellular events, including cell adhesion, migration, and proliferation

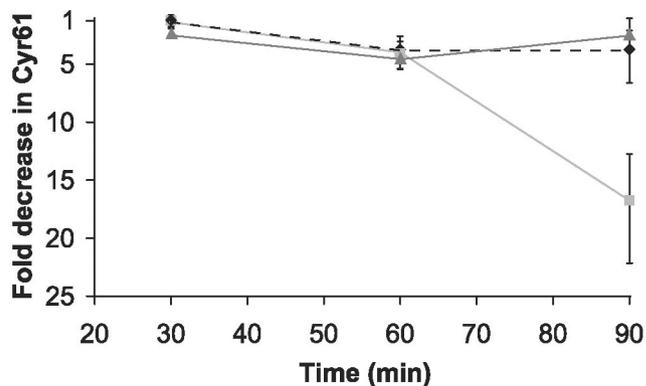


Fig. 7. Gas6 down-regulates Cyr61 mRNA in Mel 290 cells. Cyr61 mRNA levels in Mel 290 cells in the presence (■) or absence (◆) of Gas6 and Mel 290 cells stably transfected with an antisense Axl expression construct in the presence of Gas6 (▲) were measured by real-time PCR. Cyr61 levels were first normalized to GUS and then plotted as fold decreases relative to their values at T_0 (start of complete serum withdrawal and addition of Gas6). Relative transcript levels were calculated from the C_T values, and the range of each data point was calculated from the SDs (bars) as described (36). Data points were measured in triplicate.

(41–43). Cyr61 contributes to tumor formation and progression (44–47), and this array of activities appears to be mediated by different integrin receptors (41, 43, 48). Therefore, Cyr61 activity can be modulated by the level of Gas6 as shown here and by the availability of different receptor binding sites. By extension, the microenvironment at the site of metastasis similarly may regulate Cyr61 activity. It may be that decreased Cyr61 levels, because of Gas6, fail to stimulate growth of a tumor blood supply, thereby preventing outgrowth of micrometastases. Stimulation of other signaling pathways may then serve as a switch to end dormancy by: (a) shifting the balance between apoptosis and mitogenesis to enhance proliferation; and (b) increasing the expression of angiogenic factors that might include Cyr61, overriding Gas6. Some of these growth factor signaling pathways have been characterized in uveal melanoma cells and involve epidermal growth factor receptor and insulin-like growth factor receptor and c-Met (49–51). The ligands of these receptors, which include transforming growth factor- α , hepatocyte growth factor/scatter factor, and insulin-like growth factor, are produced in the liver, the primary site of uveal melanoma metastasis, stimulating tumor cell proliferation (52, 53). In addition, hepatocyte growth factor has been described as a promoter of angiogenesis (54).

Because Axl is a cell surface receptor, it may be an opportune target for immunotherapy, as devised recently for similar markers (55). Alternatively, more appropriate targets may be realized as the pathways involving Axl are elucidated.

Cancer is a multistage process involving the accumulation of different genetic changes, in the context of epigenetic events, leading to altered cellular pathways and phenotypes subject to selective pressures. Despite the heterogeneity of cancers, uncontrolled proliferation and diminished cell death are two hallmarks of neoplastic growth. Axl exerts its influence in both spheres; tumor cells are stimulated to progress through the cell cycle and divide, while these cells also avoid entering an apoptotic pathway. The expression of Axl, therefore, enhances tumor cell survival and reinforces malignant transformation, akin to other members of the family of receptor tyrosine kinases (56), and its characterization in uveal melanoma may be important to understanding the progression and treatment of the disease.

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