Expression of Receptor Tyrosine Kinase Axl and its Ligand Gas6 in Rheumatoid Arthritis

Evidence for a Novel Endothelial Cell Survival Pathway

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Angiogenesis and synovial cell hyperplasia are characteristic features of rheumatoid arthritis (RA). Many growth and survival factors use receptors belonging to the tyrosine kinase family that share conserved motifs within the intracellular catalytic domains. To understand further the molecular basis of cellular hyperplasia in RA, we have used degenerate primers based on these motifs and RNA obtained from the synovium of a patient with RA to perform reverse transcriptase-polymerase chain reaction. We report detection of the receptor tyrosine kinase (RTK) Axl in RA synovium and we document the expression pattern of Axl in capillary endothelium, in vascular smooth muscle cells of arterioles and veins, and in a subset of synovial cells in RA synovial tissue. Gas6 (for growth arrest-specific gene 6), which is a ligand for Axl and is related to the coagulation factor protein S, was found in synovial fluid and tissue from patients with RA and osteoarthritis. Axl expression and function was studied in human umbilical vein endothelial cells (HUVECs). Gas6 bound to HUVECs; soluble Axl inhibited this binding. Exogenous Gas6 protected HUVECs from apoptosis in response to growth factor withdrawal and from TNF α -mediated cytotoxicity. These findings may reveal a new aspect of vascular physiology, which may also be relevant to formation and maintenance of the abnormal vasculature in the rheumatoid synovium. (*Am J Pathol* 1999, 154:1171-1180)

Rheumatoid arthritis (RA) is characterized by hyperplasia of synovial cells, angiogenesis, and a chronic inflammatory cell infiltrate.¹ Although most attention has focused on the infiltration and activation of leukocytes in the synovial compartment in RA, increasing evidence suggests an important contribution from the resident cells, including endothelial cells and synovial cells.² Angiogenesis is

a characteristic feature of synovial inflammation in RA and adhesion of inflammatory cells to the endothelium is central to the maintenance of tissue inflammation.^{3,4}

A number of receptor tyrosine kinase (RTK)-ligand interactions have been identified that regulate vascular development and angiogenesis.⁵ Gene knockout mice have been particularly informative in understanding the role of RTKs in the developmental biology of the vascular system. Vascular endothelial cell growth factor (VEGF) is one of the key regulators of vascular development and has two RTK receptors, VEGFR-1 and -2. Mice lacking VEGFR-2 die early in embryonic development due to lack of endothelial and hemopoietic cells,⁶ whereas VEGFR-1-null mice generate both these cell types, but die because of failure to form early vascular structures.⁷ Another RTK involved in vascular development is Tie-2. Tie-2-null mice generate endothelial cells and early vascular patterning, but cannot organize an appropriate lattice of supporting cells to stabilize the developing vascular network.⁸ With the exception of physiological processes such as those in the female reproductive cycle and wound healing, angiogenesis is usually a pathological process in the adult.⁹ VEGF is probably the major regulator of angiogenesis in the adult, but basic fibroblast growth factor, platelet-derived growth factor, and hepatocyte growth factor signaling through cognate RTKs, as well as a variety of inflammatory cytokines and their receptors, can also cause angiogenesis.^{3,4} Endothelial cells are normally long-lived, but much less is known about survival signals in this cell type, particularly in the presence of tissue inflammation.

Cell growth and survival are active and interconnected processes that depend on the integration of signals from the external environment and the intrinsic differentiation programs of particular cells.¹⁰ In the absence of appropriate signals, cells die by apoptosis. Many growth factor receptors belong to the RTK family. RTKs have unique extracellular domains that specifically bind growth factor

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ligands but share homologous intracellular kinase domains that have intrinsic kinase activity and also bind signal transduction molecules. The presence of conserved motifs within the catalytic domain of RTKs has been exploited in the search for new members of the TK family.¹¹

As one approach to understanding the molecular basis of cellular hyperplasia in RA, we used reverse transcriptase-polymerase chain reaction (RT-PCR) to search for RTKs expressed in RA synovium. We report the identification of the RTK Axl in RA synovium and the expression of Axl in endothelial cells and vascular smooth muscle cells. We have also found Gas6, a recently discovered ligand for Axl, in synovial fluid. Exogenous Gas6 bound to human umbilical vein endothelial cells (HUVECs) and protected these cells from apoptosis in response to growth factor withdrawal and also from TNF α -mediated cytotoxicity. These findings may reveal a novel survival pathway for endothelial cells, which may be relevant to the pathology of RA.

Materials and Methods

Patients

Synovial fluid samples were obtained from three RA patients, one ballet dancer with a traumatic knee effusion, and one patient with psoriatic arthritis. Synovial tissue samples were obtained from eight patients with RA and six with osteoarthritis (OA) at the time of joint replacement surgery.

Cloning of Axl via RT-PCR from RA Synovial Tissue

A cloning technique based on RT-PCR using degenerate oligonucleotides was used to search for RTKs in RA synovium. RNA was extracted from the synovium of a patient with RA as described below and used to generate cDNA using a cDNA synthesis kit (Amersham, Buckinghamshire, UK). Primers corresponding to sequence motifs within the catalytic domains of protein tyrosine kinase (PTK) family members were used as described elsewhere.¹² The PCR products obtained were gel purified and digested with *Bam*HI and *Eco*RI before ligation to *Bam*HI/*Eco*RI-digested pBluescript II plasmid DNA (Stratagene, La Jolla, CA). Following transformation, plasmid DNA was isolated from individual bacterial colonies and the sequence of the insert was determined using the PCR oligonucleotide primers, a *Taq* Dye Deoxy Terminator Cycle Sequencing Kit (Perkin Elmer, Norwalk, CT) and an ABI automated sequencer (Perkin Elmer).

RNA Analysis

Total RNA was extracted from synovium using guanidinium thiocyanate and electrophoresed in agarose containing formaldehyde. Poly A-+ mRNA was extracted from SV40-transformed synovial cells (SV40.SYN)¹³ us-

ing oligo(dT)-cellulose. Northern blots were performed by capillary transfer to nylon membranes (Hybond N+, Amersham) and hybridized to ³²P-labeled, full length Axl cDNA (Mega Prime DNA labeling system, Amersham). Axl cDNA was provided by Dr. Johannes Janssen.

Immunohistochemistry

Synovial tissue specimens from patients with RA were fixed in paraformaldehyde and embedded in paraffin. Paraffin sections 5 μ m thick were dewaxed, hydrated, and incubated in methanol containing 3% peroxidase. Sections were digested with pepsin for 10 minutes at 37°C (Digest-All Kit, Zymed Laboratories, San Francisco, CA), then washed in phosphate-buffered saline (PBS). Immunohistochemistry was performed using a streptavidin-horseradish peroxidase system with 3-amino-9-ethylcarbazole as the chromogen, according to the manufacturer's instructions (Histostain-Plus Kit, Zymed Laboratories). The primary antibody (anti-Axl) was diluted 1:20 in PBS and incubated on the sections overnight at 4°C in a humidified chamber. As a negative control, normal rabbit IgG (Sigma Chemical Co., Steinheim, Germany) was substituted for anti-Axl at a corresponding protein concentration. The sections were counterstained with hematoxylin and mounted (DAKO, Glostrup, Denmark).

Protein Analysis

Protein was extracted from synovial tissue specimens using 25 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 0.1% TritonX-100, and protease inhibitors (10 μ mol/L E-64, 100 μ mol/L leupeptin, 10 mmol/L EDTA, 1 μ mol/L pepstatin, 1 mmol/L phenylmethylsulfonyl fluoride, 10 mmol/L 1,10-phenanthroline, and 10 μ mol/L Z-Phe-Ala-CHN₂, all from Sigma). Synovial tissue lysates were ultracentrifuged at 50,000 rpm for 1 hour at 4°C and frozen at -80°C. Synovial fluids were centrifuged at 2000 rpm for 10 minutes at 4°C to remove cellular components and treated with hyaluronidase at 37°C for 1 hour. For primary cells and cell lines, protein was extracted using 25 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, and 1% 3-3-cholamidopropyl-dimethylammonio-1-propanesulfonate containing protease inhibitors as above. Lysates were incubated on ice for 30 minutes and cell debris was removed by centrifugation in a microfuge at 13,000 rpm for 10 minutes at 4°C. The amount of protein was estimated using the Bio-Rad Protein Assay (Bio-Rad, Richmond, CA). Samples (75 μ g of synovial fluid or 25 μ g of synovial tissue lysate) were run on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and transferred to nitrocellulose (Hybond-C, Amersham). Membranes were blocked with 10% skim milk powder in Tris-buffered saline, 0.1% Tween 20 (TBS-T) and incubated with primary antibody diluted 1:1000 in 1% skim milk powder in TBS-T. In some experiments, Axl and Gas6 anti-sera were pre-incubated for 30 minutes at room temperature with 5 μ g of the extracellular domain of Axl fused to human immunoglobulin (Axl-Ig) or recombinant human Gas6 (rhGas6)

as specificity controls. After washing in TBS-T, blots were incubated with sheep anti-rabbit horseradish peroxidase (Silenus, Hawthorn, Australia) diluted in 1% skim milk powder in TBS-T and proteins were visualized using the ECL detection system (Amersham). Gas6, anti-Gas6 rabbit polyclonal antibody, and Axl-Ig peptide were provided by Dr. Brian Varnum and anti-Axl rabbit polyclonal antibody was provided by Dr. Edison Liu.

Cell Culture Conditions

For *in vitro* culture of synovial lining cells, synovial tissue from RA patients was dissected and minced into 2–3 mm pieces. The tissue was washed in RPMI and dissociated for 1.5 hours at 37°C with gentle agitation in RPMI containing 2.4 mg/ml dispase II (Boehringer Mannheim, Mannheim, Germany), 1 mg/ml collagenase Type II (Sigma), and 100 µg/ml DNase I (Boehringer Mannheim) in RPMI. The tissue was then ground gently through a sieve and washed several times in RPMI containing 10% fetal bovine serum (FBS, Life Technologies, Auckland, New Zealand). Cells were cultured in RPMI containing 10% FBS at 37°C, 5% CO₂, with medium changes at 24 hours to remove nonadhering cells and debris. Early passage HUVECs were cultured at 37°C in 5% CO₂ in complete medium consisting of M199 (Earle's salts) medium for endothelial cells supplemented with 25% (v/v) conditioned medium, 20% (v/v) FBS, 50 ng/ml transferrin (Boehringer Mannheim), 10 ng/ml endothelial cell growth supplement (Sigma), 10 µg/ml insulin (Sigma), and 2 mmol/L glutamine (Life Technologies, Grand Island, NY). The cells were seeded in 24-well plates precoated in 2.5% (w/v) gelatin (BDH Chemicals, Poole, UK) in PBS at approximately 5 × 10⁵ cells per well.

Gas6 Binding to HUVECs

HUVECs were detached using trypsin/EDTA and incubated in complete M199 medium either with or without 500 ng/ml Gas6 for 1 hour at 37°C. In some experiments, 10 µg/ml soluble Axl-Ig was added in addition to Gas6. After washing in PBS containing 2% FBS, the cells were stained with anti-Gas6 polyclonal antibody at 10 µg/ml or an irrelevant rabbit antibody, washed, and stained with fluorescein isothiocyanate-labeled sheep anti-rabbit Ig (Silenus). Stained cells were analyzed on a Becton Dickinson (San Jose, CA) FACScan.

Cell Survival Assay

Cells were grown in complete medium (containing serum and growth factors), M199 medium (to induce cell death by apoptosis), or M199 medium supplemented with 100 ng/ml Gas6. The medium was changed every 48 hours and cell death was monitored at days 1, 2, 5, and 8 after detachment of the cells using trypsin/EDTA. Viable cells were counted using trypan blue exclusion. There were three replicates for each time point and the experiment was performed three times.

TNF α -Mediated Cytotoxicity

Cells were grown in M199 base medium supplemented with 0, 10, or 100 ng/ml Gas6. At day 1, 10⁻⁸ mol/L TNF α (Boehringer Ingelheim, Frankfurt, Germany) was added to half the wells without changing the medium to induce cell death by apoptosis. Fresh Gas6 was added at day 2 and cells were harvested by trypsinization at day 5. Viable cells were counted using trypan blue exclusion. There were four replicates for each condition and the experiment was performed three times.

Cell Cycle Analysis by Flow Cytometry

HUVECs collected from a TNF α -mediated cytotoxicity experiment were washed in PBS, fixed in 70% ethanol, and stained with propidium iodide as described previously.¹⁴ Measurement of propidium iodide fluorescence and analysis of the cell cycle was performed on a Becton Dickinson FACScan using CellFit SOBR computer software. The combination of dead and apoptotic cells was measured by counting the percentage of events to the immediate left of the G1 histogram peak. Ten thousand events were collected for each sample.

Statistical Analysis

Student's *t*-test was used to measure the difference between group means.

Results

Cloning of Axl from Rheumatoid Synovial Tissue Using RT-PCR for Tyrosine Kinases

To detect members of the PTK family we performed RT-PCR using degenerate oligonucleotide primers that correspond to sequences within the catalytic domain of PTK family members. RNA was extracted from the synovium of a patient with RA and, after synthesis of cDNA, tyrosine kinase sequences were amplified using PTK-I and PTK-II oligonucleotides as primers.¹² A PCR product of approximately 200 bp was obtained, purified, and subcloned into pBluescript II. After transformation of competent *E. coli*, individual bacterial colonies were picked and plasmid DNA isolated for sequence determination. Members of the jak family of tyrosine kinases were the clones most frequently obtained, but DNA sequences from multiple distinct colonies revealed 100% homology with the RTK Axl,¹⁵ also known as UFO.¹⁶ We chose to study the expression and possible function of this RTK in RA in more detail.

Axl Expression in RA Synovium

Northern Blotting

Northern blot analysis was performed to assess the level of Axl expression in synovial tissues. Human Axl mRNA occurs as two transcripts of 4.9 and 3.4 kb. These

Figure 1. Northern blot analysis of Axl mRNA expression in synovial tissues. PolyA⁺ mRNA was extracted from an SV40-transformed synovial cell line, SV40.SYN (lane 1), and total mRNA from synovium of patients with RA (lanes 2–5) and OA (lane 6). cDNA containing the entire Axl coding sequence was used as the probe (upper panel). The same Northern blot was subsequently probed with glyceraldehyde-3-phosphate dehydrogenase cDNA to control for RNA loading (lower panel).

gene products are generated by alternative splicing of exon 10 and differential usage of two imperfect polyadenylation sites.¹⁷ Figure 1 shows Axl mRNA in synovial tissues from several patients with RA and one patient with OA. OA was used as a control for rheumatoid joint disease. Both mRNAs are present in all samples, as well as in the SV40.SYN cell line, showing that Axl expression is not unique to RA.

Immunohistochemistry

We next performed immunohistochemistry to determine which cell types express Axl within RA synovial tissue (Figure 2). The most striking finding was of Axl expression associated with blood vessels, in particular endothelial cells. In subsynovial capillaries, Axl was expressed in endothelial cells (Figure 2, D and E). However, in larger blood vessels, both arterioles and veins, Axl expression was confined to smooth muscle cells (Figure 2, B and C). Expression was also seen in some but not all synovial lining cells in RA synovium (Figure 2F). Normal rabbit IgG used at the same protein concentration as the Axl polyclonal antibody gave widespread nonspecific staining (Figure 2A).

Western Blotting

To confirm our immunohistochemistry findings, protein lysates from a number of relevant cell types were analyzed for Axl expression by Western blotting (Figure 3A). Axl is a 140-kd glycosylated protein.¹⁴ Soluble Axl-Ig was used a positive control (molecular weight, 110 kd). Primary cultured RA synovial cells showed low level, but detectable Axl expression and this was greater in the

synovial cell line SV40.SYN. However, in accord with the immunohistochemistry results, HUVEC protein lysates were strongly positive for Axl. A number of lower molecular weight immunoreactive bands were also detected in HUVECs, consistent with multiple glycosylation sites in the extracellular domain of Axl.¹⁸ The specificity of the anti-Axl antibody was shown by pre-incubation with the soluble Axl-Ig peptide (Figure 3B) before addition to a Western blot. The 110-kd band of Axl-Ig was almost completely abolished, indicating antibody specificity for the extracellular domain of Axl. Rabbit IgG showed no reactivity against HUVEC lysates or Axl-Ig (data not shown).

Gas6 in Synovial Tissue and Fluid

Gas6 has been identified as a ligand for Axl.^{19,20} Protein lysates from synovial tissue of patients with either RA or OA were used in a Western blot to detect expression of Gas6 (Figure 4A). Using a polyclonal anti-Gas6 antibody, a major immunoreactive protein of approximately 75 kd corresponding to recombinant human Gas6 could be identified in all synovial tissue specimens, although the intensity was generally greater in the RA synovial tissues. Equivalent amounts of protein, as estimated by the Bio-Rad protein assay, were loaded in each lane. Specificity of the anti-Gas6 antibody was confirmed by competition with recombinant human Gas6 (Figure 4B). Figure 4C shows Western blot analysis of synovial fluids from patients with RA, psoriatic arthritis, and a noninflammatory joint effusion probed with the anti-Gas6 antibody. A band of approximately 75 kd was identified in all synovial fluid specimens, corresponding to the expected size of Gas6. A second band of approximately 90 kd was also seen, possibly corresponding to a previously described Gas6 splice variant.²¹ The proteolytic products of this variant are thought to be approximately 36 and 50 kd,²² and immunoreactive bands of this size were identified in the Western blot of synovial fluid (Figure 4C) but not synovial tissue.

Gas6 Binds to HUVECs

Gas6 has been shown to be a ligand for Axl and the related RTKs Sky and Mer.^{19,23–25} To confirm that Gas6 is a physiological ligand for Axl expressed by HUVECs, Gas6 was added to HUVECs and the cells were stained with an anti-Gas6 antibody. As shown in Figure 5, HUVECs bound added Gas6 and this was competed out in the presence of soluble Axl-Ig.

Gas6 Prolongs Survival of Endothelial Cells Following Growth Factor Withdrawal

Gas6 is able to protect a variety of cells from apoptosis induced by complete growth factor depletion.^{26–28} To determine whether Gas6 has similar activity in HUVECs, we induced apoptosis by complete growth factor depletion (Figure 6). In the growth factor-deprived cultures, cell

Figure 2. Immunohistochemistry of Axl expression in RA synovium. **A:** Immunoreactivity with an equivalent concentration of normal rabbit IgG. **B-F:** Immunoreactivity with rabbit anti-Axl polyclonal antibody (staining for Axl appears as a red precipitate). Axl expression in vascular structures (**B**), in arteriole and capillary (**C**), in subintimal veins (**D**); in capillary (**E**), in synovial lining cells and capillaries (**F**). Final magnification, $\times 100$ (**A** and **B**); $\times 400$ (**C**, **D**, and **F**); $\times 1000$ (**E**).

viability had decreased at 24 hours and by day 8 all cells were dead. In contrast, when Gas6 was added to growth factor-deprived HUVECs, there was an initial drop in viability at 24 hours, but thereafter cell viability was retained. Rescue from apoptosis by Gas6 was statistically significant at days 5 ($P < 0.001$) or 8 ($P < 0.002$). HUVECs produced some endogenous Gas6 under normal culture conditions (data not shown), but cell-associated Gas6 was clearly unable to rescue HUVECs to the same extent.

Gas6 Protects HUVECs from $TNF\alpha$ -Induced Cell Death

$TNF\alpha$ is known to induce apoptosis of some cell types, especially upon withdrawal of growth factors.²⁹ Gas6 has

been found to rescue $TNF\alpha$ -treated NIH3T3 cells from apoptosis.²⁷ We therefore examined the ability of Gas6 to protect HUVECs against $TNF\alpha$ -mediated apoptosis. As shown in Figure 7, $TNF\alpha$ efficiently induced cell death in growth factor-starved HUVECs ($P < 0.001$) and 100 ng/ml (but not 10 ng/ml) of Gas6 partially protected HUVECs from $TNF\alpha$ -induced cytotoxicity ($P < 0.001$).

Gas6 Retains HUVECs in the Cell Cycle and Reduces Cell Death

Flow cytometric analysis was used to demonstrate the survival effects of Gas6. Figure 8 shows representative cell cycle profiles of HUVECs under conditions of growth factor deprivation (Figure 8A) and after treatment with

Figure 3. Western blot analysis of Axl in protein extracts from isolated cell types. **A:** Axl-Ig (lane 1), protein lysates from cultured rheumatoid synovial lining cells (lane 2), an SV40-transformed synovial cell line (SV40.SYN) (lane 3), and HUVECs (lane 4). Equal amounts of protein were loaded in each lane. The Western blot was probed with an anti-Axl rabbit polyclonal antibody. **B:** Axl-Ig was loaded in lanes 1 and 2. The Western blot was probed with anti-Axl (lane 1) or anti-Axl that had been pre-incubated with Axl-Ig (lane 2).

Gas6 (Figure 8B), $\text{TNF}\alpha$ (Figure 8C), or both (Figure 8D). Dead or apoptotic cells accumulate in the hypodiploid region to the left of the vertical marker. Cells retained in the various phases of the cell cycle at the time of sampling appear to the right of the vertical marker. The percentage of apoptotic or dead cells was significantly higher in the growth factor-starved cells compared to starved cells supplemented with Gas6 ($65 \pm 4\%$ compared with $49 \pm 3\%$, $P < 0.001$). Rescue from $\text{TNF}\alpha$ -induced apoptosis by Gas6 was incomplete but statistically significant. This experiment was performed three times with a total of seven replicates for each condition. Because most $\text{TNF}\alpha$ -treated cells were killed it was difficult to determine the true hypodiploid region, but there was at least $85 \pm 4\%$ cell death with $\text{TNF}\alpha$, compared with $76 \pm 6\%$ in the presence of Gas6 ($P < 0.01$). The G1

peak was obliterated in cells treated with $\text{TNF}\alpha$ (Figure 8C) but remained obvious when Gas6 was added (Figure 8D), indicating retention of cells in the cell cycle. The percentage of cells in the G1 phase of the cell cycle was significantly higher in the serum-starved, Gas6-treated group compared to cells without Gas6 ($20 \pm 3\%$ compared with $13 \pm 3\%$, $P < 0.001$).

Discussion

Resident synoviocytes, endothelial cells, and chondrocytes undergo dramatic changes in response to chronic joint inflammation in diseases such as RA. Understanding the activation, proliferation, survival, and apoptotic pathways of these cells is crucial to understanding this disease. Tyrosine kinases are closely associated with the regulation of growth, survival, and signaling in a wide variety of cells. To study the molecular basis of cellular hyperplasia in RA, we have used RT-PCR to search for RTKs expressed in rheumatoid synovial tissue. This approach has been used by a number of investigators to characterize TKs that are overexpressed by tumors³⁰ but has not been previously used to study the pathology of RA. We identified Axl, an RTK originally discovered through its association with chronic myeloid leukemia.^{15,16} Using immunohistochemistry, we found Axl expressed by some synovial cells, but the most striking expression was associated with vascular structures, in particular with smooth muscle cells and endothelial cells. Endothelial cell expression of Axl has not been previously noted and so we decided to investigate potential functional effects of Axl and its ligand Gas6 in this cell type, using HUVECs as a model.

Axl expression has been found in myeloid, erythroid, and megakaryocytic leukemic cell lines,³¹ in myeloid leukemias,³² and in colonic³³ and hepatocellular carcinomas.³⁰ ARK (the murine counterpart of Axl) is expressed within mesenchymal elements by day 12.5 of murine embryonic development³⁴ and is broadly expressed in adult mouse tissues.¹⁶ Less is known about the cellular distribution of Axl, but rat vascular smooth muscle cells,^{35,36} human chondrocytes,³⁷ human CD34+ hemopoietic stem cells, and mature myeloid hemopoietic cells³² have been shown to express Axl. Our study is the first to show clear expression of Axl by human endothelial cells and vascular smooth muscle cells *in situ* and provides further support for the possibility that Axl may be involved in vascular structure or function. We found an intriguing pattern of Axl expression: in small capillaries, Axl was expressed by endothelial cells, whereas in larger arterioles and veins, surrounding smooth muscle cells were Axl-positive. The extracellular domain of Axl contains adjacent fibronectin type III and immunoglobulin-like repeats^{16,38} and homophilic binding between the extracellular domains of Axl has been demonstrated.³⁹ This suggests a role in cell adhesion which could be relevant to tube formation in angiogenesis. Vascular smooth muscle cell expression has been previously noted in the rat and may suggest involvement of Axl in some other aspect of vascular function.^{35,36} Clearly, the

Figure 4. Western blot analysis of Gas6 in synovial tissue and fluid. Equal amounts of protein were loaded in each lane. **A:** synovial tissue from four separate patients with RA (lanes 2–5) or OA (lanes 6–9). Recombinant human Gas6 (lane 1) was used as a positive control. **B:** Gas6 probed with anti-Gas6 (lane 1) or with anti-Gas6 antibody pre-incubated with Gas6 (lane 2). **C:** synovial fluids from three separate patients with RA (lanes 2–4), a traumatic joint effusion (lane 5), and one patient with psoriatic arthritis (lane 6). Gas6 was used as a positive control (lane 1).

Figure 6. Effect of Gas6 on growth factor-deprived HUVECs. HUVECs were grown in complete medium (diamonds) or induced to undergo apoptosis by withdrawal of growth factors in the presence (triangles) or absence (circles) of 100ng/ml Gas6. Cell viability was assessed at indicated time points by trypan blue exclusion. Results are presented as the mean $\pm$ SD of data collected from three replicates in a representative experiment. The experiment was performed three times.

phenotype of mice with a targeted deletion of Axl will be of great interest in this regard, both in the basal state and in response to inflammatory and angiogenic stimuli.

One ligand for Axl has been identified as Gas6.^{19,20} Gas6 was originally discovered and named due to its production by cells in the quiescent phase of the cell cycle.⁴⁰ Gas6 is a multimodular protein with an N-terminal γ -carboxyglutamic acid (Gla) domain, epidermal growth factor-like repeats, and a sex hormone-binding globulin-like domain.⁴¹ The last feature may be sufficient for receptor binding and activation.^{42,43} Gas6 requires vitamin K-dependent γ -carboxylation and has homology to Protein S, a key protease regulator of coagulation.⁴¹ The full spectrum of Gas6 biological activity is currently under investigation, but it is of interest that Protein S and several other serum proteases including thrombin,⁴⁴ urokinase-type plasminogen activator,⁴⁵ and factor Xa⁴⁶ have also been found to contribute to inflammatory pathways.

Gas6 has a number of properties that may be relevant to vascular biology. Gas6 expression has been documented in unstimulated endothelial cells^{41,47} and conditioned media from a bovine endothelial cell line was used to stimulate Axl phosphorylation and subsequently to purify Gas6 as an Axl ligand.²⁰ Gas6 was also found in conditioned media of rat vascular smooth muscle cells that had been treated with thrombin and endothelin.⁴⁸ Gas6 can promote adhesion between Axl-expressing cells⁴⁹ and can elicit chemotaxis of vascular smooth muscle cells.⁵⁰ Both of these properties are reminiscent of the Tie-2 ligand angiopoietin-1 and Gas6 could be similarly involved in formation or modeling of the vasculature. Avanzi et al⁴⁷ reported that Gas6 inhibited adhe-

Figure 5. Detection of Gas6 binding to HUVECs by flow cytometry. **A:** HUVECs stained with an irrelevant rabbit polyclonal antibody (at an equivalent concentration to the test antibody). **B:** HUVECs stained with an anti-Gas6 polyclonal antibody either with (filled line) or without (dotted line) the addition of Gas6 to the cells for 1 hour. **C:** addition of Gas6 and soluble Axl to HUVECs to compete out Gas6 binding.

Figure 7. HUVECs in growth factor-free conditions were treated with TNF α , both with and without Gas6. Results are presented as mean $\pm$ SD of four replicates from a representative experiment. The experiment was performed three times.

sion of neutrophils to stimulated, but not resting, HUVECs and speculated that Gas6 exerts a protective anti-inflammatory effect. Nakano et al⁵¹ showed that the Gla domain of Gas6 can specifically bind phosphatidylserine, a phospholipid normally positioned on the inner leaflet of the plasma membrane but thought to be exposed on dying cells, leading those investigators to propose a role for Gas6 in the clearance of apoptotic cells.

We found Gas6 in synovial tissue and fluid from patients with OA and RA. Endothelial cells,^{41,47} rat vascular smooth muscle cells,⁴⁸ and cultured human chondrocytes³⁷ have been found to produce Gas6 and these cell types are therefore potential sources of Gas6 in synovial fluid. However, to our knowledge Gas6 has not been detected in the serum, suggesting local production or an alteration of half-life within the joint. It is of interest that the levels of Gas6 were generally higher in RA synovial tissue, suggesting that Gas6 may be up-regulated or over-produced in the setting of joint inflammation.

Gas6 is now well characterized as a promiscuous ligand for the Axl subfamily but, in contrast to most RTK ligands, Axl-Gas6 interaction alone induces only modest mitogenic effects in some cells.^{26,35,37,48,52-54} However, Gas6 has been shown to protect a number of Axl-positive cells from stimuli that induce apoptosis.^{26-28,37} Other nonmitogenic properties of Gas6 include chemotactic effects on vascular smooth muscle cells⁵⁰ and up-regulation of osteoclast function.⁵⁵ A number of effects of Gas6 on vascular smooth muscle cells have been documented;^{28,35,48,50} however, much less is known about Axl-Gas6 interaction in endothelial cells. We chose HUVECs as a model system and have shown that these cells express Axl and bind Gas6. Upon growth factor withdrawal, exogenous Gas6 acted as a survival factor for HUVECs and protected them from TNF α -induced cytotoxicity. Little is known about regulation of endothelial cell survival and how it changes in inflammation.⁵⁶ The synovial cavity is a relatively hypoxic and acidotic envi-

Figure 8. Representative profiles from cell cycle analysis by propidium iodide staining. Growth factor-starved HUVECs (A) were treated with either 100 ng/mL Gas6 (B), 10⁻⁸ mol/L TNF α (C), or both (D) under identical conditions to the TNF α -induced cytotoxicity experiment. Cells were harvested for flow cytometric analysis of DNA content. Signal to the left of the vertical marker represents dead or apoptotic cells. The major peak to the right of the vertical marker represents cells in G1 of the cell cycle. The experiment was performed three times.

ronment and synovial effusions can result in ischemia of the synovium.⁵⁷ Inflammatory cytokines such as TNF are produced in high local concentrations in RA and attract leukocytes from the bloodstream. The major role of Axl-Gas6 interaction may therefore be in survival of the vasculature under conditions of cellular stress or injury.³⁶

Within the normal synovial joint, Axl and Gas6 could function as a survival pathway for endothelial cells and perhaps for vascular smooth muscle cells, synovial cells, and chondrocytes. Our results raise the possibility that Gas6 may also promote survival of activated endothelial cells, and perhaps other Axl-positive cells, within the hostile environment of the inflamed rheumatoid joint. In this way, a survival mechanism normally involved in tissue homeostasis could also contribute to maintenance of a pathological vasculature in RA.

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