Patient Submitted for publication June 29, 2004; revised September 1, 2004.

Primary open-angle glaucoma (POAG) is a common ocular disease characterized by optic nerve damage and visual field loss and is often associated with elevated intraocular pressure (IOP). POAG is more prevalent and more likely to result in irreversible blindness in African Americans than in whites. POAG is probably caused by a variety of cellular insults, leading individually or collectively to cell death in retinal ganglion cells (RGCs) and in the trabecular meshwork (TM). In the past few years, genetic factors have been identified in POAG, although the underlying pathophysiology remains to be elucidated.

A potential cell marker of POAG is CD44H. CD44H is a type I transmembrane glycoprotein that is expressed on many cell types of neuroectodermal and mesenchymal origin. CD44H is a receptor for hyaluronic acid (HA), which is decreased in the TM of patients with POAG, and for several other ligands, including osteopontin, TGF-β receptor, and matrix metalloproteinases. CD44H undergoes a variety of activation-dependent, cell-type-specific, post-translational modifications that can affect its ligand specificity and affinity.

CD44H plays a critical role in the survival of many cell types through its interaction with multiple signaling pathways, including Ras, PKC, and NF-kB, focal adhesion kinase (FAK) and mitogen-activated protein kinase (MAPK), and the PI3K–PKB/Akt pathways. In several cell types, CD44H, HA, and androgen receptor stimulation enhance cell survival.

The 32-kDa ectodomain fragment of CD44 is released from membrane CD44H by proteolytic cleavage as a soluble form, sCD44. Emerging evidence suggests that sCD44 inhibits cell growth. We have shown that the aqueous humor of patients with POAG has an increased concentration of 32-kDa sCD44. The purpose of this study was to determine whether exogenous sCD44 is cytotoxic to TM cells and the RGC-like cell line (RGC-5). sCD44 cytotoxicity was blocked by heat inactivation, pretreatment with a pan-caspase inhibitor, and coadministration of anti-CD44 antibody or HA. In addition, coadministration of anti-CD44 antibody or HA, androgen receptor stimulation enhance cell survival.

The 32-kDa ectodomain fragment of CD44 is released from membrane CD44H by proteolytic cleavage as a soluble form, sCD44. Emerging evidence suggests that sCD44 inhibits cell growth. We have shown that the aqueous humor of patients with POAG has an increased concentration of 32-kDa sCD44. The purpose of this study was to determine whether exogenous sCD44 is cytotoxic to TM cells and the RGC-like cell line (RGC-5). sCD44 cytotoxicity was blocked by heat inactivation, pretreatment with a pan-caspase inhibitor, and coadministration of anti-CD44 antibody or HA. In addition, coadministration of a potential neuroprotective agent, the androgen 17α-methyl testosterone (17α-MT), also prevented sCD44 cytotoxicity.

Methods

Purification of 32-kDa sCD44

sCD44 was purified from 200 mL fetal calf serum (FCS), which contained 5 ng/mL sCD44, or human serum (Sigma-Aldrich, St. Louis, MO), which contained 450 ng/mL sCD44, by solubilizing in 20 mL of urea buffer (8 M urea, 50 mM MES [pH 6.5, 2-(N-morpholino)ethane sulfonic acid] and several protease inhibitors, to obtain a final concentration of 0.75 M urea) overnight at 4°C while mixing. Urea-treated serum was diluted 1:2 with phosphate-buffered saline (PBS) and filtered with a 100-kDa molecular weight cutoff (MWCO) concentrator at 5400 g for 50 minutes at room temperature. The 100-kDa filtrate was reconstituted in refolding buffer (400 mM l-arginine, 100 mM Tris-HCl, 2 mM proapoptotic pathways. (Invest Ophthalmol Vis Sci. 2005; 46:214–222.) DOI:10.1167/iovs.04-0765
EDTA, 5 mM reduced glutathione, 0.5 mM oxidized glutathione, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 0.5 μg/mL leupeptin [pH 8.0]) on a magnetic stirring plate for 48 hours at 4°C and concentrated with 10-kDa MWCO concentrators at 5400 g for 20 minutes at room temperature. The purified serum, which contained the 32-kDa sCD44, was incubated with anti-CD44 antibody (BU52; 1.5 μg/mL; Ancell, Bayport, MN) for 16 hours at 4°C, incubated with rabbit anti-mouse IgG secondary antibody conjugated to agarose (5 μL/μg of primary antibody; Sigma-Aldrich) for 90 minutes at room temperature, and centrifuged at 5000 g for 7 minutes at room temperature. The supernatant was discarded, and the resultant pellet was rinsed with PBS, eluted with 0.5 M NaCl and PBS and 0.2 M glycine [pH 2.5]38,39 and then neutralized with 1 M Tris-HCl [pH 7.5], to obtain the purified 32-kDa sCD44. Purified sCD44 was further concentrated by the use of a 10-kDa MWCO concentrator at 5400 g for 4 minutes. The molecular weight and purity were verified by two-dimensional (2-D) polyacrylamide gel electrophoresis (PAGE) and silver staining, and also by immunoblot analysis. 2-D PAGE was performed with 1 ng equivalent of sCD44, as determined by CD44-specific ELISA.8 The first and second dimensions were performed according to the manufacturer's instructions (Invitrogen, Carlsbad, CA; and Bio-Rad, Hercules, CA, respectively). The isolated sCD44 was revealed to be a 32-kDa protein by silver staining (Silver Stain Plus; Bio-Rad). For the immunoblot analysis of the isolated sCD44 protein, the sCD44 equivalent of 100 pg was loaded on a 4% to 15% linear-gradient gel and subjected to PAGE under reducing and nonreducing conditions. The proteins were transferred to nitrocellulose membranes and incubated with anti-CD44 antibody (BU52; 1:500 dilution; Ancell) for 16 hours at 4°C. The membranes were incubated with goat anti-mouse IgG-horseradish peroxidase (HRP)–conjugated secondary antibody (1:3000 dilution; Bio-Rad) for 1 hour at room temperature. The membranes were then incubated with chemiluminescent substrate (ECL Plus; Amersham Pharmacia Biotech, Piscataway, NJ) and developed according to the manufacturer's instructions.

**TM Cells, sCD44 Specificity and Controls, Dose-Response Study, and FCS Medium Concentration**

Bovine31 and human TM32 cells were plated at a cell density of 40,000 cells per well in 24-well plates and grown until confluent in 0.5 mL Dulbecco's modified Eagles' medium (DMEM) containing 10% FCS, as previously described. Cells were washed twice with PBS, and the medium was changed to DMEM containing 0.1% FCS for treatment with sCD44. To verify sCD44 specificity, the sCD44 preparation was boiled for 5 minutes to heat inactivate the protein. In addition, cells were incubated with graded amounts (8–200 μM) of pan-caspase inhibitor33(Z-VAD-FMK; Sigma-Aldrich) diluted in dimethyl sulfoxide (DMSO) for 2 hours before sCD44 treatment. sCD44 was also premixed for 2 hours at room temperature with graded amounts (0.01–100 μg) of anti-CD44 monoclonal antibody (Ancell) or with graded amounts (0.1–100 μg) of purified HA. Human umbilical cord HA (Sigma-Aldrich) was purified by size exclusion chromatography on a Sepharose CL-4B column (Pharmacia, Uppsala, Sweden), as previously described.34 In addition, human TM cells were grown in 10% FCS and changed to media containing 0.1%, 1%, or 10% FCS, to determine whether higher concentrations of FCS would affect cell viability. Purified sCD44 isolated from FCS and human serum was diluted in PBS and filtered with 0.2-μm sterile filters (Fisher Scientific, Pittsburgh, PA) to treat bovine and human TM cells, respectively.

**RGC-5, TM Cells, Cell Specificity of sCD44, and 17-α-MT Rescue**

The cell specificity of sCD44 was tested in six cell lines: the transformed rat RGC-like cell line (RGC-5),35,36 human TM cells,32 bovine TM cells,31 NIH 3T3 fibroblasts, aortic smooth muscle cells (CRL-1999; American Type Culture Collection [ATCC], Manassas, VA), and human cortical neuronal-like cells (CRL-10,442; ATCC). The RGC-5 was transformed with psi2 E1A virus and expressed Thy-1, Brn-3C, N-methyl-D-aspartate (NMDA) receptor, GAGA-B2 receptor and synaptophysin.36 Bovine TM and RGC-5 cells were treated with purified sCD44 and 17-α-MT (Sigma-Aldrich), to test whether the cytotoxic effects of sCD44 could be blocked by a neuroprotective factor.

**Cell Morphology and Counting**

Cells were briefly washed twice with 0.5 mL 0.02% EDTA solution and briefly treated with 0.5 mL of 1× trypsin solution (Sigma-Aldrich) for 30 seconds. The trypsin solution was removed, and the plates were lightly tapped and/or briefly heated to facilitate removal of the cells from the wells. When all the cells were released, 0.2 mL complete medium was added to stop trypsinization. With a pipette, a single-cell suspension was obtained and transferred to a separate tube. The tubes were vortexed, and 0.1 mL of the cell suspension was added to 8.9 mL electrolyte solution (Isoton II; Coulter, Miami, FL). Each well was counted in a cell counter (Coulter) in triplicate. Cell viability was determined by adding 20 μL of filtered 0.4% trypan blue + PBS solution to 20 μL of the cell suspension, and 10 μL was injected into a hemocytometer. Under a phase-contrast microscope, viable and nonviable cells were photographed. The cell viability was expressed as the absolute number of viable cells in the experimental group (experimental percentage of cell viability times the total number of experimental cells) versus the absolute number of viable cells in the control group (control percentage cell viability times the total number of control

**Figure 1.** (A) SDS-PAGE and immunoblot analysis of purified sCD44. sCD44 was purified from FCS or human serum by differential centrifugation and immunoprecipitation. Lane 1: native FCS; lane 2: isolated sCD44 from FCS; lane 3: isolated sCD44 from human serum; lane 4: isolated sCD44 from human serum under nonreducing conditions. Note sCD44 migrated as a single 32-kDa band under reducing conditions and as a larger aggregate under nonreducing conditions. (B) Isolated sCD44 from human serum separated by 2-D electrophoresis and stained with silver stain plus revealed a 32-kDa protein with an isoelectric point of 5.4 to 7.0.
cells). Data are expressed as the mean ± SD and were analyzed by Student’s t-test. P < 0.05 was considered statistically significant.

**RESULTS**

**Purification of 32-kDa sCD44**

sCD44 was isolated by a multistep purification process consisting of urea solubilization and immunoprecipitation. The yield of sCD44 from FCS and human sera was ~20% and ~5%, respectively. Under reducing conditions, the 32kDa sCD44 migrated as a single band; however, under nonreducing conditions, sCD44 migrated predominantly as larger complexes with apparent molecular masses greater than 250-, 190-, and 120-kDa (Fig. 1A). 2-D gel electrophoresis of isolated sCD44 revealed, by silver staining, a 32-kDa protein with an isoelectric point ranging from 5.4 to 7.0 (Fig. 1B).

**Dose- and Time-Dependent Effect and Specificity of sCD44 in Bovine and Human TM Cell Viability**

A dose- and time-dependent response to sCD44 was observed in bovine TM cell viability (Fig. 2). After 24 hours, cell viability was statistically significantly decreased with incubation of 1.0 ng/mL sCD44 (P < 0.02) in comparison with control cell viability. As the dose of sCD44 was increased, cell viability significantly decreased (P < 0.005). After 48 hours, a dose of 0.1 ng/mL sCD44 resulted in a statistically significant decrease in cell viability (P < 0.02) in comparison with control cell viability. From 24 to 48 hours, cell viability significantly decreased (P < 0.005) with each dose of sCD44.

The decrease in human TM cell viability in sCD44-treated cells in comparison with control TM cells indicated a dose- and a time-dependent response. After 24 hours, the cell viability of human TM cells treated with a 0.1 ng/mL dose of sCD44 was not significantly different from the control cell viability; however, larger doses of sCD44—1 ng/mL (P < 0.02; data not shown) and 10 ng/mL (P < 0.0002)—resulted in statistically significant decreases in cell viability in comparison with control TM cells. After 48 hours, all doses of sCD44 (0.1, 1.0, and 10.0 ng/mL) significantly decreased human TM cell viability in comparison with control cell viability. The influence of FCS concentration on cell viability was evaluated by changing the FCS concentration from 0.1% to 1% or 10%. With the use of higher concentrations of FCS, treatment with the lower doses of sCD44 (0.1 and 1.0 ng/mL) resulted in cell viability similar to that of the control. With the 10 ng/mL dose of sCD44, the cell viability of sCD44-treated human TM cells in comparison with control human TM cells was significantly decreased after 24 and 48 hours (P < 0.01), even in the presence of higher concentrations of FCS (Table 1).

To verify the cytotoxicity of sCD44, four controls were used to determine that sCD44 was cytotoxic to bovine TM cells: (1) An isolated sCD44 preparation was boiled for 5 minutes to heat-inactivate the protein, which resulted in loss of toxicity in cell viability (P < 0.02) in comparison with control cell viability. From 24 to 48 hours, cell viability significantly decreased (P < 0.005) with each dose of sCD44.

**TABLE 1. sCD44 and Human TM Cell Viability**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose sCD44* (ng/mL)</th>
<th>n</th>
<th>24 hours†</th>
<th>P</th>
<th>48 hours</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% FCS</td>
<td>0.0 (control)</td>
<td>4</td>
<td>81.3 ± 4.6</td>
<td>—</td>
<td>80.5 ± 2.4</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>4</td>
<td>76.8 ± 5.5</td>
<td>ins§</td>
<td>70.0 ± 5.8</td>
<td>0.02</td>
</tr>
<tr>
<td>1.0% FCS</td>
<td>0.0 (control)</td>
<td>4</td>
<td>48.0 ± 6.9</td>
<td>0.0002</td>
<td>47.8 ± 5.0</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>4</td>
<td>86.8 ± 8.7</td>
<td>—</td>
<td>85.3 ± 4.6</td>
<td>—</td>
</tr>
<tr>
<td>10.0% FCS</td>
<td>0.0 (control)</td>
<td>4</td>
<td>79.8 ± 3.4</td>
<td>0.02</td>
<td>78.8 ± 3.5</td>
<td>ins</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>4</td>
<td>68.8 ± 3.6</td>
<td>0.01</td>
<td>65.8 ± 8.0</td>
<td>0.01</td>
</tr>
<tr>
<td>10.0% FCS plus†</td>
<td>0.0 (control)</td>
<td>8</td>
<td>84.8 ± 3.2</td>
<td>—</td>
<td>81.3 ± 2.8</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>5</td>
<td>84.0 ± 4.0</td>
<td>ins</td>
<td>80.2 ± 2.3</td>
<td>ins</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>5</td>
<td>73.4 ± 4.8</td>
<td>0.01</td>
<td>73.4 ± 4.0</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td>10.0% FCS plus†</td>
<td>0.1</td>
<td>—</td>
<td>—</td>
<td>75.7 ± 6.7</td>
<td>ins</td>
</tr>
<tr>
<td></td>
<td>10.0% FCS plus†</td>
<td>10.0</td>
<td>—</td>
<td>—</td>
<td>49.3 ± 6.4</td>
<td>0.000001</td>
</tr>
</tbody>
</table>

* The dose of purified sCD44 isolated from human serum was determined by ELISA.
† Cell count was determined by a cell counter and viability by trypan blue staining.
‡ Additional sCD44 was added after 24 hours.
§ Significance was determined by Student’s t-test comparing control with treatment.

FIGURE 2. Dose and time course of sCD44 and bovine TM cell viability treated with sCD44: (A) after 24 hours; (B) after 48 hours. The data represent the mean of results (n = 6) in a representative experiment. Error bars, SD. *P < 0.02; **P < 0.001, sCD44 treatment versus control.
(Fig. 3A); (2) pretreatment with 40 μM pan-caspase inhibitor prevented (P < 0.01) cell cytotoxicity (Fig. 3A); (3) premixing the isolated sCD44 preparation with anti-CD44 antibody neutralized the cytotoxic effects of sCD44 in a dose-dependent manner (Fig. 3B); (4) the isolated sCD44 preparation was premixed with HA for 2 hours to bind the sCD44 to graded amounts of HA. HA blocked the cytotoxic effects of sCD44 in a dose-dependent manner (Fig. 3C).

**Morphologic Changes in sCD44-Treated TM Cells**

Bovine TM cells revealed morphologic changes that included cell rounding, cell detachment, and cell aggregation within 24 hours of incubation with sCD44 (Fig. 4). Control bovine TM cells were uniform and squamous in appearance (Fig. 4A). The morphology of bovine TM cells treated with a 0.1 ng/mL dose of sCD44 was indistinguishable from that of control cells at 24 hours (Fig. 4B). As the concentration of sCD44 was increased to 10 and 40 ng/mL, cell rounding, detachment, and aggregation became more evident. These results are consistent with the cell viability data shown in Figure 2.

**Dose- and Time-Dependent Effect of sCD44 in RGC-5 Cell Line Viability**

sCD44 also exhibited a dose- and time-dependent neurotoxicity in the RGC-5 cells (Table 2). The administration of 1 ng/mL dose of sCD44 resulted in a significant decrease in cell viability after 6 (P < 0.01), 12 (P < 0.0001), and 24 (P < 0.02) hours. A sCD44 dose of 40 ng/mL was more toxic than a lower dose of 1 ng/mL, and the decrease in RGC-5 cell viability with 40 ng/mL sCD44 was highly significant after 6, 12, and 24 hours (P < 0.0001).
Table 2. sCD44 and RGC-5 Viability

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose sCD44* (ng/mL)</th>
<th>6 Hours</th>
<th>P</th>
<th>12 Hours†</th>
<th>P</th>
<th>24 Hours</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0</td>
<td>82.9 ± 2.4</td>
<td></td>
<td>83.5 ± 4.7</td>
<td></td>
<td>81.5 ± 2.9</td>
<td></td>
</tr>
<tr>
<td>sCD44</td>
<td>1.0</td>
<td>79.2 ± 2.6</td>
<td>0.01‡</td>
<td>73.8 ± 2.3</td>
<td>0.00004</td>
<td>78.2 ± 2.8</td>
<td>0.02</td>
</tr>
<tr>
<td>sCD44</td>
<td>4.0</td>
<td>76.5 ± 2.3</td>
<td>0.00002</td>
<td>68.6 ± 2.2</td>
<td>0.000002</td>
<td>63.6 ± 5.5</td>
<td>0.000002</td>
</tr>
</tbody>
</table>

n = 9 in all groups.

* The dose of purified sCD44 isolated from human serum was determined by ELISA.
† Cell count was determined by a cell counter and viability by trypan blue staining.
‡ Significance was determined by Student’s t-test comparing control with treatment.

Morphologic Changes in the sCD44-Treated RGC-5 Cell Line

RGC-5 treated with sCD44 also displayed morphologic alterations. The transformed RGC-5 cells were interconnected neuronal-like cells with extended axonal processes. After 6 hours of incubation with sCD44, minimal changes were observed. After 12 hours, cell processes and cell bodies of the RGC-5 cells were swollen, and a marked decrease in the number of cells was observed. After 24 hours of incubation with sCD44, the swelling of the cell bodies increased in cells that received the higher dose of sCD44 (Fig. 5).

sCD44, Cell Viability, and Cell Specificity

The cell specificity of sCD44 was tested after 24 and 48 hours in six different cell lines, by using a dose of 10 ng/mL sCD44 (Fig. 6). The most susceptible cell lines were the human and bovine TM cell lines. The cell viability of bovine TM cells was 55% and of human TM cells was 59% after 48 hours. Aortic smooth muscle cells showed resistance to sCD44, as the cell viability, expressed as a fraction of the control cells, stayed near 100%. 3T3 fibroblasts were also somewhat resistant to sCD44; the cell viability was 87%. Human cortical neuronal-like cells showed an initial decrease in cell viability that was accompanied by cell swelling after 24 hours, but was followed by recovery, and the cell viability was ~90% after 48 hours. RGC-5 cell viability was 68% after 48 hours, the decrease was highly significant (P < 0.000001) in comparison with control RGC-5 viability.

The Effects of 17-α-MT on sCD44 Toxicity

To test whether the toxic effects of sCD44 could be reversed, the androgen 17-α-MT was coadministered with sCD44, and the cell viability of bovine TM cells was assessed at 12, 24, and 48 hours (Fig. 7). After 12 hours, 10 ng/mL sCD44-treated bovine TM cells showed significantly decreased cell viability in comparison with control cells (P < 0.05). The coadministration with 1 or 10 ng/mL 17-α-MT and sCD44 initially did not significantly affect the viability after 12 hours; however, after 48 hours, coadministered cells were rescued and cell viability increased significantly (P < 0.005), compared with cells treated with 10 ng/mL sCD44 only.

The protective effects of 17-α-MT against sCD44 cytotoxicity were also observed in RGC-5 (Fig. 8). In RGC-5 cells treated with 10 ng/mL sCD44, the cell viability was 56% compared with control and was highly significant (P < 0.0001). Coadministration of 1 or 10 ng/mL 17-α-MT and 10 ng/mL sCD44 resulted in an increase in cell viability (75% with 1 ng/mL 17-α-MT and 68% with 10 ng/mL; P < 0.01) after 48 hours. Incubation with 1 or 10 ng/mL of 17-α-MT alone slightly increased RGC-5 cell viability (statistically insignificant difference from the control).

DISCUSSION

Exogenous 32-kDa sCD44 purified from FCS or human serum was isolated by urea solubilization and immunoprecipitation to homogeneity, as verified by 2-D PAGE and silver staining. The cytotoxicity of sCD44 was evaluated in cell culture. sCD44 was cytotoxic to TM cells and RGC-5 and caused a significant decrease in cell survival in a time- and dose-dependent manner. After 48 hours, treatment with 0.1 ng/mL sCD44 resulted in significant TM cell death. The cytotoxic effects of sCD44 on human TM cells were verified by four control experiments. Heat-inactivation eliminated sCD44 cytotoxicity; coadministration of sCD44 and anti-CD44 monoclonal antibody neutralized the effect of sCD44; coadministration of sCD44 and its ligand, HA, blocked sCD44 cytotoxicity; and pretreatment of TM cells with a pan-caspase inhibitor prevented sCD44 cytotoxicity. The optimum concentration of coadministration of an anti-CD44 monoclonal antibody with sCD44 was 10 μg/mL. Anti-CD44 monoclonal antibody alone was cytotoxic to human TM cells at a lower concentration (e.g., 0.1 μg/mL). Anti-CD44 antibody alone is reported to induce apoptosis in fibroblasts and leukemia cells in vitro. The optimum concentration of HA was 1 μg/mL when coadministered with CD44. These results suggest, therefore, that the isolated protein in this study was in fact sCD44. sCD44 was cytotoxic to human TM cells, and sCD44 activity was blocked by heat inactivation or coadministration of anti-CD44 neutralizing antibody or HA. Moreover, sCD44 cytotoxicity was prevented by pretreatment with a pan-caspase inhibitor, indicating that sCD44 cytotoxicity occurred as a result of apoptosis.

The minimum dose of sCD44 required to decrease bovine TM cell viability was 0.1 ng/mL, which is extremely low in comparison with the sCD44 concentration present in normal aqueous humor. One possibility is that TM cells in vitro are more vulnerable to the apparent toxic effects of sCD44 because they lack survival factors that are present in normal aqueous humor. We tested the effects of higher concentrations of FCS. The TM cell viability of sCD44 treated cells was higher in 10% FCS medium than in 0.1% FCS medium; however, a 10 ng/mL dose of sCD44 in the presence of 10% FCS was cytotoxic to TM cells. This result indicates that the decrease in cell viability was from the cytotoxic effect of sCD44 and not the depletion of FCS. In addition, coadministration of HA blocked sCD44 cytotoxicity, which is particularly noteworthy because the HA concentration in aqueous humor of patients with POAG is decreased from that of normal patients (Navajas JR, et al. IOVS 2004;45:ARVO E-Abstract 3665).

sCD44-induced cell toxicity was cell-specific. RGC-5 and TM cells were the most susceptible cells to exogenous sCD44, whereas smooth muscle cells were resistant to sCD44. sCD44 treatment resulted in cell swelling of human cortical neuronal-like cells and RGC-5 cells. Fast and massive soma swelling is...
CD44 is known to occur in RGC cell death. CD44 is upregulated in the rat brain after ischemia; however, CD44−/− mice are more resistant to ischemia. These results illustrate an important adaptive phenomenon involving CD44 upregulation, which ultimately may be detrimental to neural tissue.

One possible mechanism for sCD44 toxicity is its interaction with membrane bound CD44 and/or with the epidermal growth factor receptors erbB2 and erbB3. Membrane-bound CD44 mediates heterodimer formation between erbB2 and erbB3. Because the erbB2-erbB3 complex activates the PI3K/PKB pathway, interference by sCD44 may interfere with the downstream survival pathway signaling and allow an extrinsic pathway of apoptosis.

A second mechanism by which sCD44 may kill cells is its internalization into mitochondria. CD44 is a normally occurring mitochondrial protein necessary for mitochondrial function. CD44−/− knockout mice have disrupted mitochondrial cristae. In the mice, the ratio of antiapoptotic Bcl-xl to proapoptotic Bak was shifted toward apoptosis. Pretreatment of cells with the pan-caspase inhibitor Z-VAD-FMK prevented sCD44 cytotoxicity. Recent evidence by Xu et al. also indicates that sCD44 may have the ability to induce apoptosis by itself. sCD44 contains three HA-binding motifs. A synthetic peptide having three HA-binding motifs inhibits cell growth in vitro; activates caspase-8, caspase-3, and poly(ADP-ribose) polymerase; and triggers apoptosis. An FITC-HA-binding protein analogue, P4, is internalized in MDA-435 breast cancer cells into mitochondria; the P4 analogue is cytotoxic and also inhibits vascular endothelial growth factor. Immunoprecipitation of P4 in cell lysates revealed that P4 binds Bcl-2 and Bcl-xl, both of which are regulators of the intrinsic pathway of apoptosis. Further support of the notion that HA-binding proteins localize to the mitochondria comes from metastatin, an HA-binding protein fragment of a link protein that has significant homology to sCD44. Metastatin has been isolated from bovine cartilage and inhibits tumor cell growth in vitro. Notably, the cytotoxicity of metastatin is blocked by premixing with HA in a fashion similar to our control experiment with sCD44. Moreover, the effect of metastatin on cytotoxicity is cell dependent, in that cells with low HA concentration are susceptible to metastatin, whereas cells with higher HA concentrations are more resistant. Thus, the results of our experiments with various cell lines suggest that TM and RGC-5 cells have low HA concentration and consequently are vulnerable to sCD44. In contrast, 3T3 fibroblasts and smooth muscle cells have high concentrations of HA and are more resistant to sCD44.

A third mechanism for sCD44 cytotoxicity is to disrupt the essential interactions between membrane CD44 and HA. Under normal circumstances, HA in the extracellular matrix binds to the membrane CD44 receptor, influencing PKB activation. PKB signaling pathways include members of the Bcl-2 family, such as BAD; caspases-7, -8, and -9; and the forkhead family transcription factors, specifically FHKL1. Thus, when sCD44 disrupts the CD44-HA interaction, the downstream effect is that apoptotic proteins such as FHKL1 and BAD are released. In addition, sCD44 could bind CD44, and thereby prevent normal CD44 cell signaling. If sCD44 interacts with membrane-bound CD44 in a low-affinity manner similar to the HA oligomers studied by Ghatak et al., sCD44 may further suppress the PKB cell survival pathway by activating phosphatase and tensin homologue (PTEN), the phosphatase that inactivates PIP3, a key component in PKB activation. Once sCD44 disrupts PKB activation, several apoptotic proteins can be freed to induce cell death.

In this study, the androgen 17α-MT prevented and rescued cells from sCD44-induced death. Androgens are important mediators of neuroprotection and prevent cell death in neurode-
generative diseases and serum-deprived primary neuron cultures. In prostate carcinoma cells, it has been shown that downstream erbB2 signaling involves androgen receptor activation. In the absence of erbB2 signaling, 17α-MT may supplement this survival pathway in the presence of sCD44. In addition, application of testosterone has been shown to protect hippocampal neuron cultures from 17α/β-amyloid toxicity. There is remarkable similarity between the neuropathologies of Alzheimer’s and POAG. Both CD44 and the 17α/β-amyloid precursor are cleaved by presenilin-dependent gamma secretase in their transmembrane domain. The shed ectodomain of both of these proteins exhibit neurotoxicity and self-aggregation.

A common concept in neurodegenerative diseases is a toxic protein associated with the cause of a disease process (e.g., β-amyloid in Alzheimer’s disease, α-synuclein in Parkinson’s disease, and amylin in type II diabetes). Accumulation and misfolding of the toxic protein is linked to anomalies of mitochondrial function. It is now recognized that mitochondria are porous, allowing proteins, such as HA-binding proteins, to enter and leave. HA-binding proteins have in common a similar motif, (B-X7-B) in which B is a basic amino acid, usually arginine or lysine, and X is any amino acid except an acidic amino acid. Exactly why certain HA-binding proteins are trafficked into the mitochondria and become toxic is unclear. Multiple apoptotic pathways emanate from mitochondria, resulting in membrane depolarization and release of cytochrome c. Normally, many mitochondrial proteins (e.g., monoamine oxidase B and cytochrome b5), are held on mitochondrial membranes by mitochondrial tethering domains. The tethering domain consists of the arginine/lysine-enriched portion of the binding protein. Interestingly, sCD44 has a putative mitochondrial tethering domain; thus, sCD44 may compete for other mitochondrial tethering proteins, displace them, and interfere with normal mitochondrial function.

In conclusion, this study demonstrated the cell toxicity of sCD44 in a cell-specific manner, in that RGC-5 and TM cells were particularly vulnerable to exogenous sCD44 in vitro by...
activation of proapoptotic pathways. We have also demonstrated that the effects of sCD44 can be blocked by pretreatment with pan-caspase inhibitor or by coadministration of HA, anti-CD44 neutralizing antibody or the androgen 17α-MT.

Acknowledgments
The authors thank Jane Danley and Dolores Henning for editorial assistance.

References


