Apoptosis Induced by a Corneal-Endothelium–Derived Cytokine

Sammy H. Liu and John D. Gottsch

Purpose. The purpose of this study was to isolate and characterize cDNA clones encoding target proteins for autoantibodies in patients at high risk for transplant rejection.

Methods. A pool of 10 sera from patients at high risk for rejection who had undergone corneal transplantation was used for immunoscreening of an endothelial cDNA library, and the cDNA fragments were subcloned into prokaryotic expression vectors to generate recombinant fusion proteins. Cytotoxicity of recombinant protein was determined by a modified ^{51}Cr-release assay. Apoptosis induced by recombinant protein was determined by fluorescent dye–chromatin fragmentation assay and by TdT-dUTP terminal nick-end labeling (TUNEL) assay. An enzyme-linked immunosorbent assay was used to detect the presence of antibodies to recombinant protein in the sera of high-risk patients undergoing corneal transplantation and of control subjects.

Results. Screening of 500,000 plaques identified six positive clones, one of which demonstrated extensive homology with a novel tumor-derived cytokine termed endothelial monocyte–activating polypeptide (EMAP). EMAP was synthesized as a 39-kDa precursor that was proteolytically cleaved to generate an active 22-kDa cytokine. The mature peptide of EMAP alone was capable of inducing the death of cultured endothelial cells, whereas the propeptide was inactive. The protein synthesis inhibitor cycloheximide potentiated EMAP-induced apoptosis in endothelial cells. Cell death by apoptosis was evidenced by DNA fragmentation, extensive surface bleb formation, and chromatin condensation. A statistically significant difference was found in the level of antibodies specific to EMAP between patients at high risk for corneal transplant rejection and control subjects ($P < 0.001$). The antibody levels were elevated in patients with severe graft reaction when compared with patients with no graft reaction ($P < 0.001$).

Conclusions. These studies demonstrated that EMAP is a novel protein in corneal endothelial cells that is capable of inducing programmed cell death. Overexpression of this cytokine could initiate endothelial cell damage leading to stromal edema and corneal decompensation. (*Invest Ophthalmol Vis Sci.* 1999;40:3152–3159)

The corneal endothelium plays an important role in maintaining corneal transparency. Failure of the endothelium, as a result of intrinsic corneal diseases and disorders such as Fuchs' dystrophy and corneal allograft rejection, can lead to sight-reducing corneal edema. There is little regenerative capacity of the human corneal endothelium in these diseases, and the increased edema that is often noted is irreversible. There have been few reports regarding endothelial antigens.[1–3] The identification of endothelial antigens has been hampered by the difficulty of obtaining sufficient quantities of them to test for a possible biologic effect. This study has sought to circumvent this problem with an alternative strategy of isolating mRNA directly from the endothelium to construct an expression cDNA library. To identify cDNA clones that may express endothelial antigens, we used a pool of 10 sera from patients at high risk for corneal transplant rejection for immunoscreening the cDNA library. It was hoped this approach would identify and characterize novel antigens that may be of restricted cellular distribution as well as low cellular expression. With this method, we were able to clone a corneal endothelium-derived cytokine that displays extensive homology with a novel tumor-derived cytokine.[4,6] This cytokine has been designated as endothelial monocyte–activating polypeptide (EMAP).

Materials and Methods

Bovine Corneal Endothelial cDNA Library Construction

Bovine eyes were obtained from a local abattoir and kept on ice until dissection. The endothelium was denuded by gentle scraping of the cornea. Total RNA was isolated from corneal endothelial cells by reagent (TRIzol; Life Technologies, Gaithersburg, MD). Double-stranded cDNA was synthesized from 25 μg mRNA according to the manufacturer's protocol (Stratagene; La Jolla, CA). *Xho*I and *Eco*RI linker–primer adapters were incorporated into the cDNA to create the restriction sites at the 5′ and 3′ ends of the cDNA. The cDNA was size selected (>1 kb) by gel filtration, ligated into a vector (UniZAPXR; Stratagene), and packaged using extract (Gigapack II; Stratagene). The packaged DNA was titered and found to contain

From the Wilmer Eye Institute, Johns Hopkins University School of Medicine, Baltimore, Maryland.

Submitted for publication March 17, 1999; revised July 21, 1999; accepted August 2, 1999.

Commercial relationships policy: N.

Corresponding author: Sammy H. Liu, Wilmer Eye Institute, Johns Hopkins University School of Medicine, Baltimore, MD 21287-9142.

2.5×10^7 recombinants. After packaging, the library was amplified in XL-1 blue cells (Stratagene).

Serum Samples

A pool of sera was used from 10 patients who had had multiple corneal graft rejections. These sera were obtained from banked samples kept and administered by the Collaborative Corneal Transplantation Study (CCTS).[7,8] The pooled sera were absorbed with bovine corneal epithelium and stroma extracts coupled to cyanogen bromide-activated Sepharose (Sigma, St. Louis, MO). Bovine corneal epithelium and stroma extracts were prepared as described previously.[9] The epithelium and stroma extracts (10 mg protein/ml gel) were separately mixed with CNBr-activated Sepharose overnight at 4°C. After blocking the remaining active groups with 1 M ethanolamine, the gels were extensively washed with acetate buffer (0.1 M; pH 4.0) and bicarbonate buffer (0.1 M; pH 8.3) containing 0.5 M NaCl. The yield of coupling in the protein-Sepharose conjugate was approximately 40%. Two milliliters of pooled sera was mixed with 1 ml stromal protein–Sepharose conjugate in an end-over-end mixer for 2 hours at room temperature and then overnight at 4°C. After centrifugation, the supernatants were mixed with 1 ml epithelial protein–Sepharose conjugate in a similar manner. The sera were also absorbed with five strips of 2×5-cm nitrocellulose membrane previously saturated in 20% *Escherichia coli* extracts. A pool of sera from five normal subjects was preabsorbed and tested for reactivity with the positive clone. Absorbed sera were stored at −20°C in the presence of 0.05% sodium azide until used for immunoscreening.

cDNA Library Screening

Portions of the cDNA library were plated at a density of 35,000 plaque-forming units per 135-mm petri dish and the plaques induced to produce fusion protein according to the protocol of Huynh et al.[10] After a 3-hour incubation at 42°C, a nitrocellular filter (Schleicher & Schuell, Keene, NH) saturated with 10 mM isopropyl thiogalactose (Life Technologies) was overlaid with the agar overnight at 37°C to induce the expression of β-galactosidase fusion proteins. After that, the filters were blocked with 1% bovine serum albumin (BSA) in Tris-buffered saline plus 0.05% Tween 20 (TBS-Tween) for 5 hours at room temperature and incubated with 1:100 diluted sera overnight at 4°C. After several washings with TBS-Tween, the bound antibodies were detached by incubation with anti-human IgG alkaline phosphatase (Sigma) for 2 hours at room temperature. The original positive plaque was preplated and rescreened sequentially until all progeny of plaques were recognized by the sera. Screening of 500,000 plaques identified six positive clones that were sequenced. DNA sequencing was performed on an automated DNA sequencer (Perkin Elmer, Foster City, CA). One clone was 87% homologous with a novel tumor-derived cytokine, EMAP,[4,6] and was chosen for detailed characterization in the present study.

Cloning and Expression of Propeptide and Mature Peptide of the EMAP Cytokine

Oligonucleotide primers were designed from the known nucleotide sequence of bovine EMAP cDNA and were used to amplify the cDNA for cloning of the pro region (peptides 1–146) and the mature region (peptides 147–310) of the EMAP cytokine. The sense primer contained a synthetic *Stu*I site, and the antisense primer contained a synthetic *Hind*III site to facilitate directional cloning. Synthesized DNA was amplified by polymerase chain reaction (PCR) using purified EMAP cDNA as a template. The PCR-based cloning strategy has been essentially described previously.[11] The PCR products after digestion with *Stu*I and *Hind*III were ligated into an expression vector, pPROEX-HTb (Life Technologies). The resultant plasmid, pPROEX (peptides 1–146) or pPROEX (peptides 147–310), was separately transformed into DH5-competent cells followed by selective growing of the recombinants on Luria-Bertani (LB) agar plates containing ampicillin (100 μg/ml). Plasmid DNA was obtained from positive colonies using a plasmid extraction kit (Qiagen, Chatsworth, CA). Correct cloning and the full-length sequence were verified by sequencing the insert DNA.

A single bacterial colony containing either the pPROEX (peptides 1–146) or the pPROEX (peptides 147–310) plasmid was inoculated into 10 ml LB broth plus ampicillin (100 μg/ml) and incubated overnight at 37°C with shaking. The following day, 500 ml LB broth containing ampicillin was inoculated with 5 ml of the overnight culture and incubated at 37°C to an absorbance (600 nm) of 0.5. Isopropyl thiogalactose was added to a final concentration of 1 mM. The bacteria were allowed to grow and express for 2 hours at 37°C with agitation. The bacterial cells were collected and lysed for protein purification. The pPROEXHT expression vector was constructed to incorporate a hexohistidine sequence at the amino terminus of the fusion protein. This hexohistidine tag allowed affinity purification of the fusion protein on an immobilized metal (Ni-NTA) column. The concentration of the fusion protein was determined using the Bradford protein reagent (Bio-Rad, Hercules, CA).

Endothelial Cell Cultures

Bovine eyes were washed three times with saline and were immersed for 6 minutes in commercial neomycin-gramacidin-polymyxin B solution to which 100 U/ml penicillin G and 50 μg/ml gentamicin were added. After further rinsing with saline, the corneas were excised from the eyes, and endothelial cells were isolated by trypsin treatment (0.25% trypsin for 5 minutes) of cornea endothelia. Endothelial cells were cultured in complete medium consisting of Medium 199 (Life Technologies) supplemented with 20% fetal calf serum (FCS), 100 μg/ml endothelial cell growth factor (Becton Dickinson, Bedford, MA), and 12 U/ml sodium heparin. All growing surfaces were pretreated with gelatin. Cells were passed at a ratio of 1:3 when they reached confluence. Cells from passages two and three were used for the experiments.

Cytotoxicity Assay

The cytotoxic effects of recombinant peptides of EMAP on corneal endothelial cells were determined by ^{51}Cr (sodium chromate, 1 mCi/ml; NEN, Boston, MA) retention, as described by Pohlman and Harlan.[12] Corneal endothelial cells were allowed to attach to 48-well, fibronectin-coated culture plates (Becton Dickinson) at a density of 5×10^4 cells in 0.1 ml Medium 199 plus 20% FCS per well for 4 hours. Cells were incubated overnight with 0.5 μCi per well of ^{51}Cr. Without washing the labeled cells or decanting the labeling medium, 0.1 ml Medium 199 alone or medium containing various concentrations of recombinant peptide of EMAP, with or without

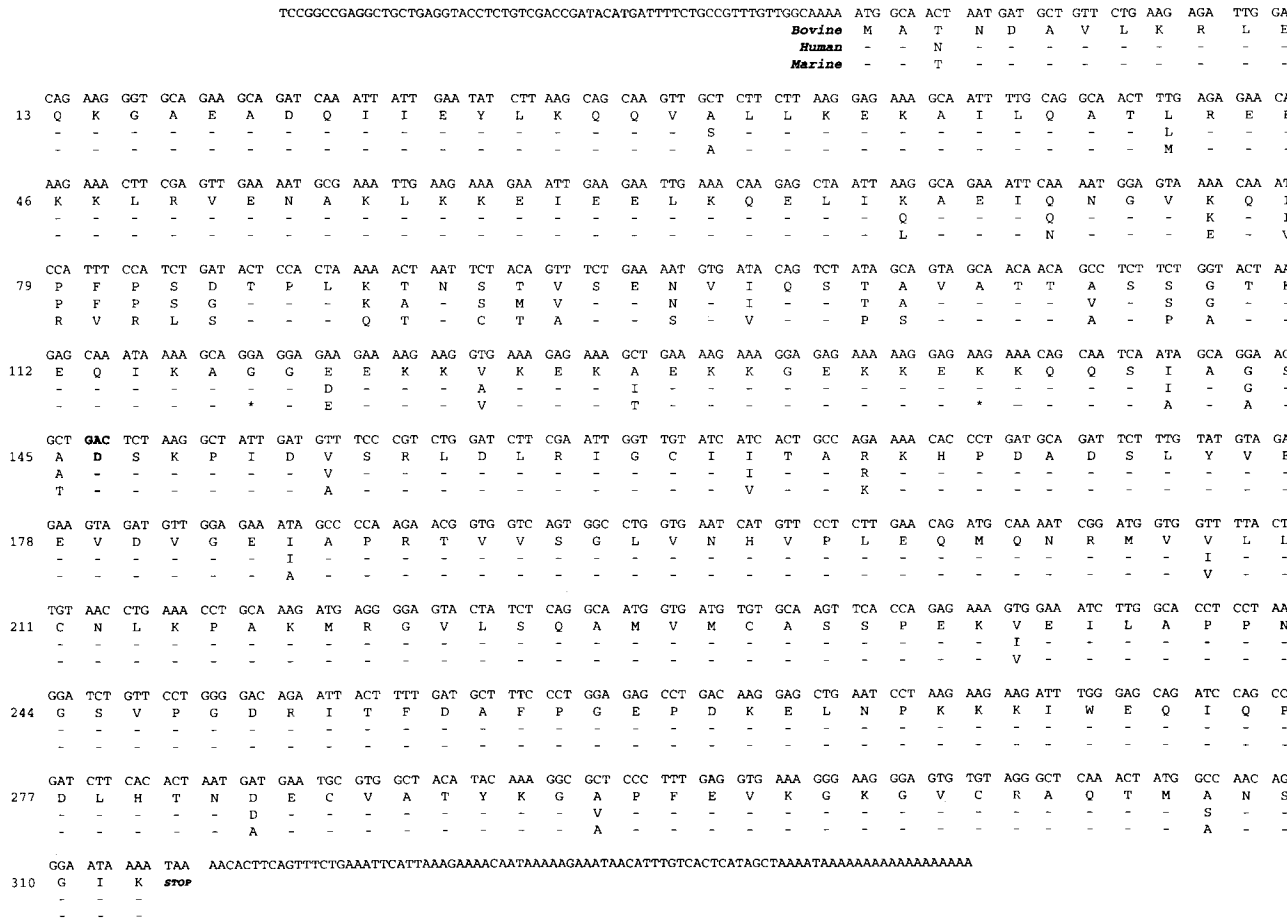

FIGURE 1. Complete nucleotide and deduced amino acid sequences of bovine corneal endothelium-derived EMAP cytokine. Human and murine sequences were from Kao et al.[5] *Dashes* indicate positions of the same amino acid residues shared by all sequences. The *bold letter* indicates the aspartic acid residue (Asp-146) believed to be involved in generating the mature EMAP cytokine.

fixed amounts of cycloheximide (CHX, Sigma) was added in triplicate. After 24 hours' incubation at 37°C, each well was washed five times with phosphate-buffered saline (PBS) plus 5% FCS to remove detached cells and unbound ^{51}Cr. The residual adherent cells were lysed by adding 0.2 ml aqueous 1% solution of Triton X-100 (Sigma) and incubating for 30 minutes at room temperature. Aliquots were counted (gamma counter; Packard Instruments, Meriden, CT), and the percentage of cytotoxicity was then calculated as counts per million (cpm) control − cpm test/cpm control × 100, where cpm control was obtained from the cells incubated in medium alone, and cpm test was obtained from the cells incubated with the recombinant peptide of EMAP with or without CHX.

Fluorescent Dye–Chromatin Fragmentation Assay

Endothelial cells were allowed to attach to an eight-chamber fibronectin-coated slide dish (Becton Dickinson) for 5 hours at a density of 5×10^4 cells/chamber in Medium 199 plus 10% FCS at a final volume of 0.3 ml. The propeptide and mature peptide of EMAP were added at a final concentration of 30 μg/ml, with or without the constant amounts of CHX (20 μg/ml). At 24 hours, cultured cells were rinsed twice in PBS, and 0.3 ml 70% ethanol containing 100 μg/ml DNA dye (Hoechst 33258; Molecular Probes, Eugene, OR) was added to each well and incubated for 30 minutes at room temperature. Two hundred cells were counted and scored for the incidence of apoptotic chromatin changes under a fluorescence microscope.

TdT-dUTP Terminal Nick-End Labeling (TUNEL) Assay

Endothelial cells were treated with 30 μg/ml of either the propeptide or mature peptide with or without 20 μg/ml CHX. The TUNEL assay was performed according to the protocol of the manufacturer (Boehringer–Mannheim, Indianapolis, IN). After 24 hours, the cultures were fixed with 4% paraformaldehyde in PBS. The slides were rinsed three times with PBS and incubated with 0.3% H_2O_2 in methanol for 30 minutes at room temperature to block endogenous peroxidase. The slides were rinsed with PBS and then incubated in 0.1% Triton X-100 in 0.1% sodium citrate for 2 minutes at 4°C. They were rinsed twice with PBS, and 50 μl TUNEL reaction mixture was applied to the sample. The slides were incubated at 37°C for 60 minutes and rinsed three times with PBS. Fifty microliters of converter peroxidase was added, and the slides were incubated for 30 minutes at 37°C. After three rinses in PBS, the slides were incubated for 10 minutes in 50 μl diaminobenzidine substrate solution at room temperature. Two hundred cells were counted under light microscope.

Enzyme-Linked Immunosorbent Assay

Serum samples were obtained from the CCTS from patients known to have had no graft reactions and from patients who had had multiple corneal graft rejections.[7,8] A severe graft reaction was defined by observed clinical signs of immunologic rejection with the presence of one or more of the following signs: more than five keratic precipitates, cells in the stroma, an endothelial rejection line, or both increased thickness and increased aqueous cells.[7,8] Twenty-three control samples were identified from a group of patients who had undergone cataract surgery and had no active corneal disease or collagen vascular disorder.

Serum antibody levels against EMAP were determined by enzyme-linked immunosorbent assay (ELISA), as described previously.[9] Immulon-2 plates (Dynatech, Chantilly, VA) were coated overnight at 4°C with 100 μl recombinant EMAP (1 μg/ml) per well in carbonate buffer (pH 9.6). The remainder of the test was performed at room temperature. Unbound sites were blocked with 1% BSA in PBS. Sera were diluted 1:500 with PBS containing 0.05% Tween 20 and 0.5% BSA and then added (100 μl/well) to each well. The plates were incubated for 2 hours, and an alkaline phosphatase–conjugated goat anti-human IgG (Sigma) was added followed by the substrate *p*-nitrophenyl phosphate (Sigma). The plates were thoroughly washed with PBS containing 0.05% Tween 20 between the additions of reagents. The results were read with a spectrophotometer (Titertek Multiskan; Flow Laboratories, Rockville, MD) at 405 nm. Statistical analyses of anti-EMAP levels were performed by χ^2 test and one-way analysis of variance to compare findings in different groups.

Results

Cloning of a Corneal Endothelium-Derived Cytokine cDNA

Immunoscreening of a bovine corneal endothelial cDNA library was accomplished with a pool of sera from the CCTS of 10 patients at high risk for corneal transplant rejection.[7,8] Screening of 500,000 plaques identified six positive clones that were sequenced. One clone was 87% homologous with a novel tumor-derived cytokine EMAP.[4,5] Figure 1 shows the nucleotide sequence of corneal endothelium-derived EMAP cDNA and the deduced amino acid sequence. The coding region of EMAP is 936 nucleotides long. There are 69 nucleotides in the 5′ untranslated region, which includes the ATG initiator codon. The 3′ untranslated region of 75 nucleotides contains a TAA terminator codon. The putative polyadenylation signal sequence AATAAA is located 30 nucleotides upstream from the beginning of the poly(A) sequence at the 3′ end of the message. Therefore, a full-length cDNA encoding bovine EMAP cytokine was obtained.

The protein encoded by the sequence from nucleotide positions 70 to 1006 is composed of 312 amino acids and has a calculated molecular mass of 39,466 Da (39 kDa). The full sequence of this 39-kDa protein does not contain a hydrophobic stretch of amino acids of sufficient length to characterize it as a secretory signal sequence. This has led to speculation that EMAP may be synthesized as a 39-kDa inactive precursor that may be proteolytically cleaved at the C-terminus of aspartate residue 146 to generate an active 22-kDa cytokine.[4–6] However, the actual means by which the EMAP precursor is processed to the mature form remain unknown. Our approach to this problem was to express the putative pro region (peptides 1–146) and the mature region (peptide 147–310) of EMAP in *E. coli*, and then investigate the ability of both peptides of EMAP to induce cytotoxicity and apoptosis in cultured corneal endothelial cells, as presented in the following experiments. It is important to note that the deduced amino acid sequence of the mature region of bovine EMAP showed 96% homology to its murine and human counterparts, whereas the putative pro region of EMAP showed greater sequence variation (74%) among these three species (Fig. 1).

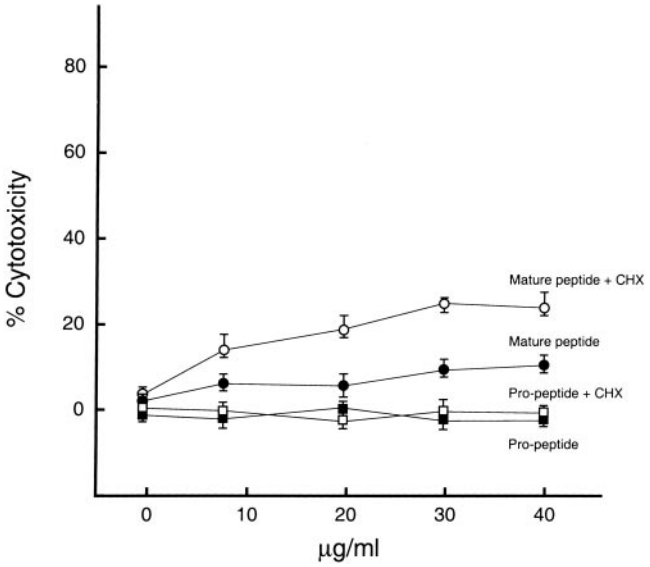

Figure 2. The cytotoxic effects of propeptide and mature peptide of EMAP on corneal endothelial cells were determined by a modified ^{51}Cr-release assay. Endothelial cells were cultured in the presence of various concentrations of EMAP peptides with or without 20 μg/ml CHX, and the percentage of cytotoxicity was measured after a 24-hour incubation. Experiments were repeated three times, and means ± SD are shown.

Cytotoxic Effect of EMAP on Corneal Endothelial Cells

The cytotoxic effects of recombinant propeptide and mature peptide of EMAP on cultured corneal endothelial cells were determined by a modified ^{51}Cr-release assay. The propeptide of EMAP was not toxic to endothelial cells over a 24-hour incubation, whereas mature peptide of EMAP alone had a low but significant cytolytic activity (12% ± 2%; $P < 0.01$) against endothelial cells (Fig. 2). Cytoxicity through the mature peptide can be stimulated by the protein synthesis inhibitor, CHX (20 μg/ml), which, alone, was not toxic to endothelial cells. Coincubation of corneal endothelial cells with CHX and mature peptide significantly increased cytotoxicity at 24 hours (Fig. 2). Heating recombinant mature peptide to 80°C for 30 minutes completely abolished its cytotoxic effect (data not shown). Coincubation of endothelial cells with CHX and the propeptide of EMAP, however, did not provoke cytotoxicity.

Mediation of Apoptosis by EMAP

To examine whether the EMAP-mediated cell death of cultured endothelial cells is an apoptotic process, the cells were incu-

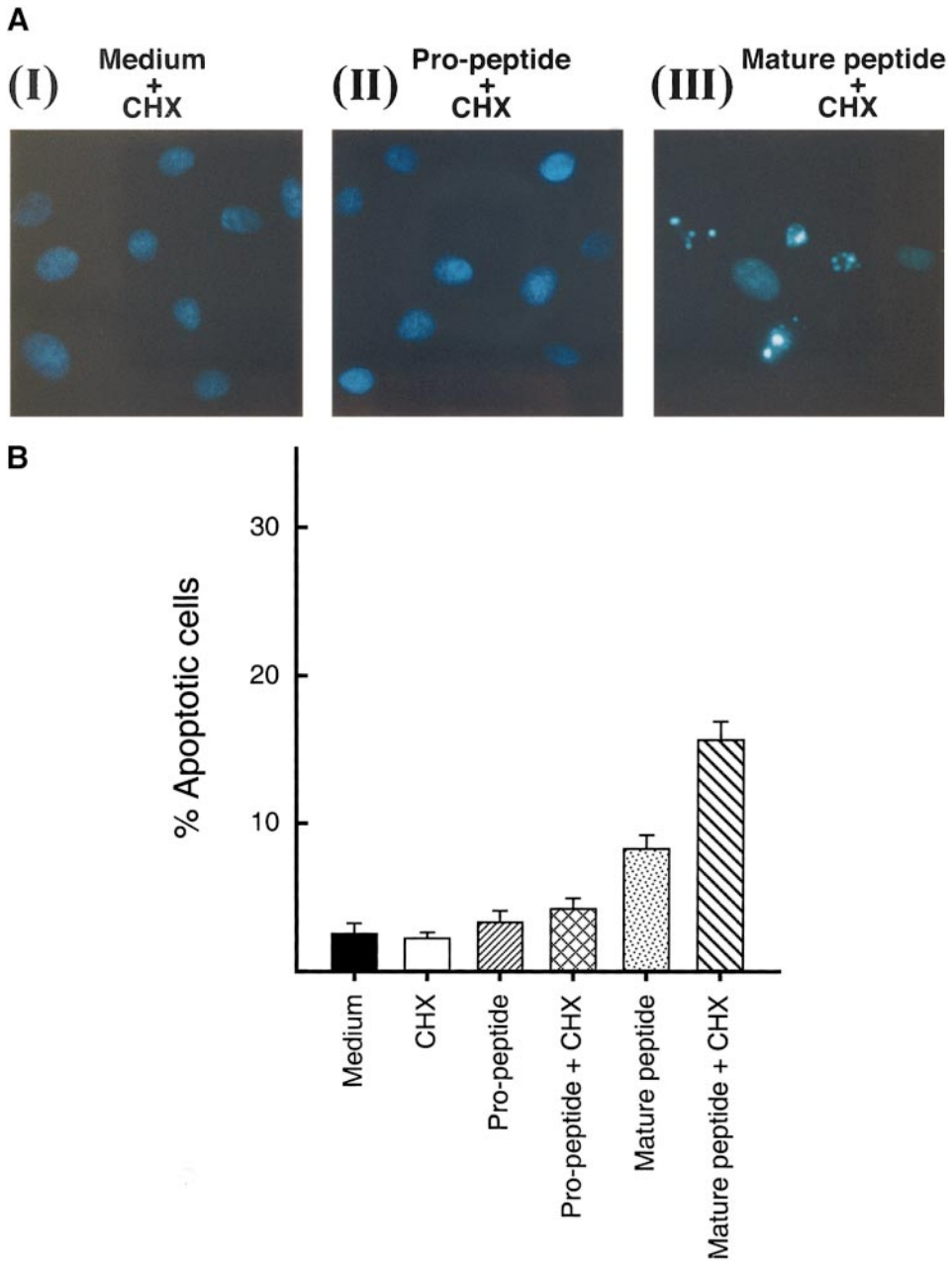

FIGURE 3. (**A**) Nuclear chromatin condensation in endothelial cells grown for 24 hours and examined with DNA dye. Cells treated with (I) medium plus CHX and (II) the propeptide plus CHX showed a normal nuclear morphology, whereas treatment with (III) the mature peptide plus CHX induced apoptosis as shown by multiple, brightly stained condensed nuclei. (**B**) The percentage of endothelial cells exhibiting morphologic changes of apoptosis. Two hundred cells were counted and scored for the incidence of apoptotic chromatin changes. Values represent the mean ± SD of results from three independent experiments.

bated for 24 hours with 20 μg/ml propeptide or mature peptide of EMAP in the presence or absence of 30 μg/ml CHX. The mature peptide alone was capable of inducing apoptosis in cultured endothelial cells. Addition of CHX to the cells potentiated EMAP-mediated apoptosis. The cells in the presence of mature peptide and CHX exhibited dramatic morphologic changes. As revealed by the DNA-specific stain (Hoechst; Molecular Probes), the chromatin of these cells condensed and segregated into masses at the nuclear membrane followed by fragmentation of the nucleus (Figs. 3A, 3B). Cells retained a normal morphology with uniform nuclei and intact plasma membranes when cultured with the same amount of the propeptide and CHX. To confirm that DNA fragmentation occurred in endothelial cells undergoing chromatin condensation by treatment with the mature peptide and CHX, an in situ TUNEL assay was used to detect DNA breakage (Figs. 4A, 4B). On average, approximately 12% to 15% of cells treated with the mature peptide and CHX showed fragmentation. The percentage of apoptotic cells in the culture was underestimated, because growing the cells in the presence of mature peptide and CHX caused these cells to detach from the tissue culture dish, and the lost cells could not be scored. Two percent to 3% of the endothelial cells treated with the propeptide and CHX were apoptotic.

Determination of Human Antibody Levels to EMAP

Serum samples obtained from patients at high risk for corneal transplant rejection and from control subjects were tested for antibodies to EMAP by ELISA. Antibody levels were expressed as the absorbance values at 1:500 dilution of each test serum. Statistically significant differences in the levels of specific an-

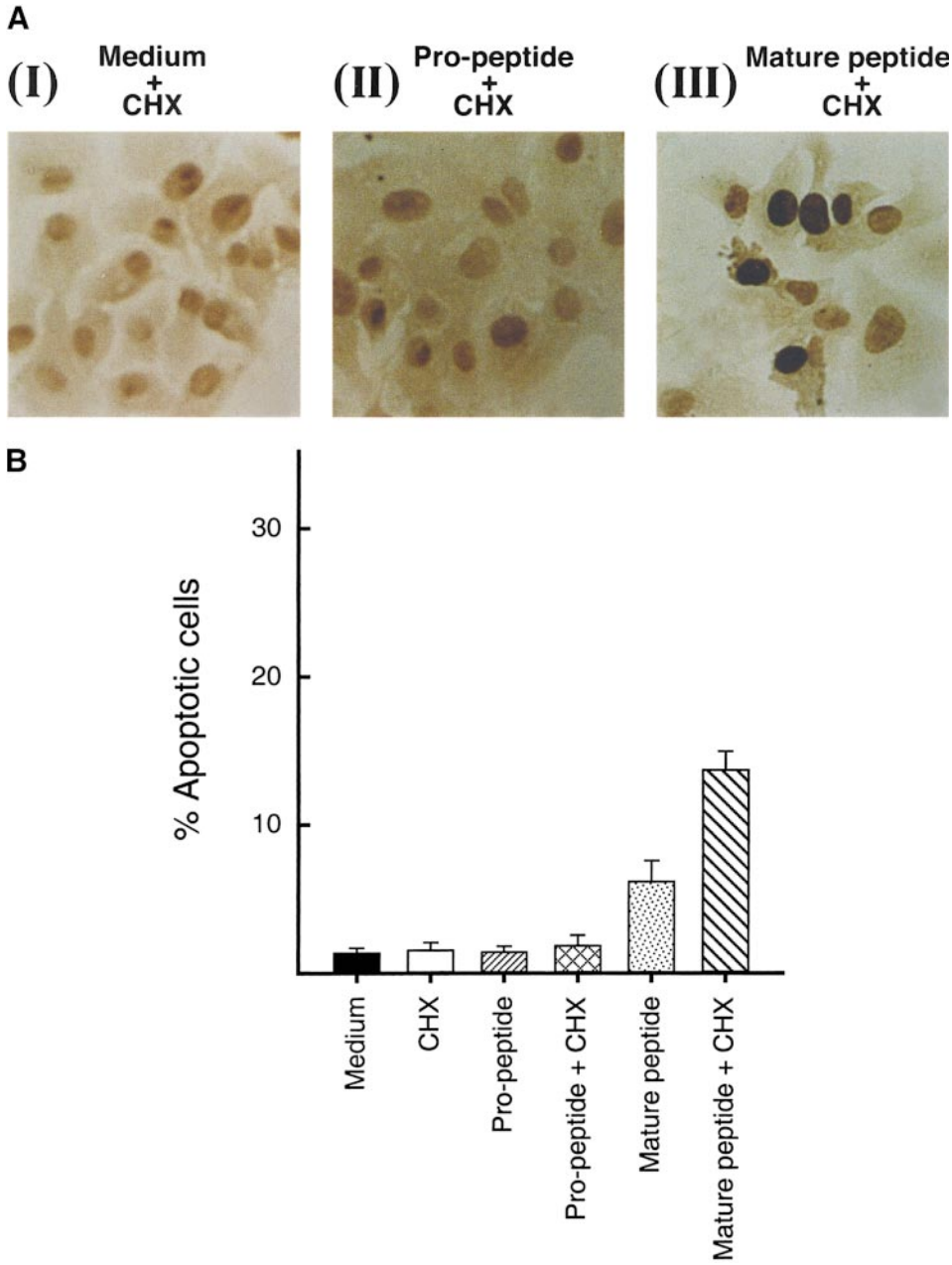

FIGURE 4. (**A**) Apoptosis induced by EMAP was shown by TUNEL assay. Endothelial cells treated with (I) medium plus CHX and (II) the propeptide plus CHX showed only rare cells staining with TUNEL, whereas the treatment with (III) the mature peptide plus CHX induced apoptosis, shown by intense staining with TUNEL. (**B**) The percentage of apoptotic cells was determined by dividing the number of TUNEL-positive cells by the total number of cells counted. Two hundred cells were counted for each group. Values represent the mean ± SD of results from three independent experiments.

tibodies to EMAP were found between patients with severe graft rejection and control patients, between patients with severe graft rejection and those with no reaction, and between those with no reaction and control subjects ($P < 0.001$, $P < 0.001$, and $P < 0.001$ respectively). Only the control subjects against EMAP had tightly clustered results, whereas the levels of anti-EMAP antibodies in patients who had had corneal transplant rejection showed wide variability (Fig. 5).

DISCUSSION

In this study, we used autoantibodies from patients at high risk for corneal transplant rejection to probe and isolate cDNA clones from a bovine corneal endothelial library. One clone encodes a 39-kDa precursor of a novel cytokine. HLAs have been considered to be the major barrier to successful transplantation, although there is apparently no correlation between an HLA match and subsequent graft survival.[7,8] However, rejection occurs in the absence of detectable lymphocytotoxic antibodies, suggesting that antigenic systems other than HLA could be involved in graft rejection. We have provided evidence of a possible non-HLA antigen that may play a role in the immune response during graft rejection.

Autoantibodies to various cytokines—tumor necrosis factor (TNF)-α, interleukin (IL)-2, IL-8, and interferon (IFN)— have been detected in sera from healthy donors.[13,14] These antibody levels, although highly variable, have been found to be increased in the circulation of patients with a variety of inflammatory diseases or in those subjected to cytokine therapy.[13,14] The role played by anticytokine autoantibodies in vivo is unclear. They may block the binding of a cytokine to its specific cell surface receptor, thereby neutralizing its biologic activity

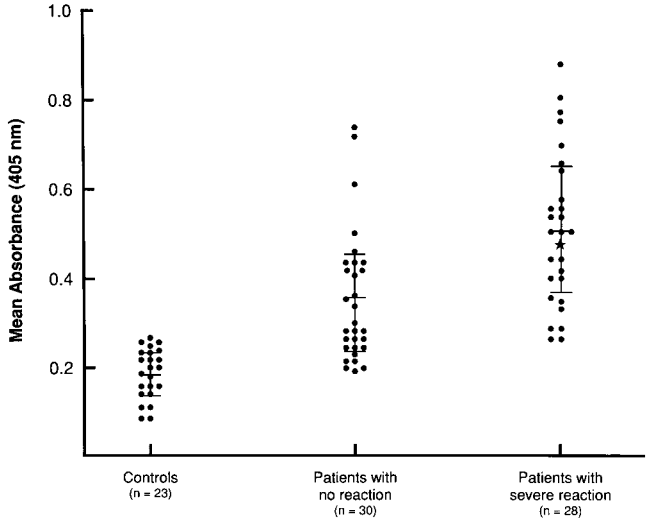

FIGURE 5. Serum samples from patients at high risk for corneal transplant rejection and control patients were tested by ELISA to determine their relative antibody levels to EMAP. Bar, mean ± SD. Statistically significant differences in the levels of specific antibodies to EMAP were found between patients with severe graft rejection and control patients, between patients with severe graft rejection and those with no reaction, and between those with no reaction and control patients ($P < 0.001$, $P < 0.001$, and $P < 0.001$ respectively). In the patients with severe graft rejection ⋆ represents the pooled sera used to screen the endothelial cDNA library.

in vivo. They may trigger complement-mediated cytotoxicity toward cells carrying membrane-bound cytokine. The recognition of EMAP in the serum of patients who experience transplant rejection is consistent with a possible disease association. However, recognition of EMAP by the immune system may be a secondary phenomenon related to cell destruction in the graft-rejection process. The increased levels of anti-EMAP antibodies in patients at high-risk for corneal transplant rejection with no graft reaction may suggest that these antibodies are not pathogenic, because no clinical signs of immunologic rejection are observed in those patients.[7,8] Meanwhile, the marked elevation of anti-EMAP antibodies in patients with severe graft reaction may reflect a state of hyperimmunization and is probably a consequence of the continuous synthesis and release of EMAP from damaged endothelial cells. Because corneal allograft rejection is primarily a T-cell–mediated disease,[15,16] future studies should be directed to determining whether cellular immune reactions against EMAP are involved in endothelial rejection. The observation that the human, bovine, and mouse EMAP genes are highly conserved may allow valuable animal models to be developed to study how this molecule plays a pathologic role in corneal graft rejection.

Human endothelial cells are insensitive to TNF-induced cytotoxicity but are killed when treated with TNF-α together with the inhibitors of RNA or protein synthesis.[12,17] These observations suggest that TNF-α can induce human endothelial cell death but that protective proteins are also synthesized. Several TNF-inducible genes have now been shown to protect cells from the cytotoxic effect of TNF. The induction of mitochondrial manganese superoxide dismutase,[18] heat shock proteins,[19] antiprotease plasminogen activator inhibitor type 2,[20] and the A20 zinc finger protein[21] are among the protective effects evoked by TNF. Furthermore, a TNF-inducible *bcl-2*–related A1 protein has been shown to protect endothelial cells against TNF-mediated apoptosis.[22] Consequently, blocking the synthesis of such protective proteins by the inhibition of translation or transcription sensitizes many cell types to TNF toxicity. We observed a similar tendency of CHX to potentiate EMAP-induced cytotoxicity and apoptosis in corneal endothelial cells. The identification and characterization of the protective proteins are relevant to the action of EMAP, because the molecular basis of EMAP-mediated cytotoxicity and apoptosis is not understood.

An active EMAP cytokine, which has a molecular mass of 22 kDa, was originally purified from the culture supernatants of murine methylcholanthrene A–induced fibrosarcomas.[4,5] However, cDNA sequence data from a number of species (human, mouse, and bovine) indicate that EMAP mRNA encodes a larger protein than that identified as the mature secreted cytokine. Analysis of the deduced amino acid sequence from EMAP cDNAs has not revealed a region that has sufficient hydrophobicity and length to qualify as a signal sequence. Therefore, some form of proteolytic processing is required to release active EMAP. A similar case had been demonstrated for another inflammatory cytokine, IL-1β, which has no distinguishable hydrophobic signal sequence. A specific cysteine protease, the IL-1β–converting enzyme (ICE), cleaves an inactive IL-1β precursor at the C-terminal side of Asp-116 to generate biologically active, mature IL-1β.[23,24] Eleven ICE-like proteases have been identified.[25,26] All these proteases cleave substrates that must have an aspartic acid in the P1 position. EMAP precursor has an aspartate residue (Asp-146) in the P1 position. It is possible that an ICE-like protease is involved in the processing of pro-EMAP to produce the active 22-kDa cytokine. In our study the mature peptides 147–310 of EMAP induced apoptosis in corneal endothelial cells, whereas the propeptide 1–146 was inactive. Therefore, in cases of both IL-1β and EMAP, removal of the amino terminal half appears to be essential for biologic activity. The ICE gene, a mammalian homologue of the *Caenorbabditis elegans* cell death gene *ced-3*, has been identified as an inducer of apoptosis in several cells.[27,28] TNF and Fas antigen are the best-characterized gateways to apoptosis. Recently, ICE-like protease has been demonstrated to be involved in TNF-induced and Fas-mediated apoptosis.[17,29,30] We plan to determine whether apoptosis induced in corneal endothelial cells by EMAP is accompanied by the activation of an ICE-like protease. Understanding the basic mechanisms mediating endothelial cell death may lead to the development of novel approaches for the treatment of corneal diseases featuring apoptosis.

References

1. Panjwani N, Baum J. Rabbit corneal endothelial cell surface glycoproteins. *Invest Ophthalmol Vis Sci.* 1985;26:450–456.
2. Srivastava OP, Srivastava K, Shukla SD. Characterization of a 66-kilodaltons surface glycoprotein of the human corneal endothelium. *Invest Ophthalmol Vis Sci.* 1990;31:1982–1993.
3. Howell DN, Burchette JL, Paolini JF, Geier SS, Fuller JA, Sanfilippo F. Characterization of a novel human corneal endothelial antigen. *Invest Ophthalmol Vis Sci.* 1991;32:2373–2482.
4. Kao J, Ryan J, Brett G, et al. Endothelial-monocyte-activating polypeptide: a novel tumor-derived polypeptide that activates host-response mechanisms. *J Biol Chem.* 1992;267:20239–20247.

5. Kao J, Houck K, Fan Y, et al. Characterization of a novel tumor-derived cytokine. Endothelial-Monocyte-Activating Polypeptide II. *J Biol Chem* 1994;269:25106–25119.
6. Tas MPR, Houghton J, Jakobsen AM, et al. Cloning and expression of human endothelial-monocyte-activating polypeptide 2 (EMAP-2) and identification of its putative precursor. *Cytokine.* 1997;9:535–539.
7. The Collaborative Corneal Transplantation Studies Research Group. The Collaborative Corneal Transplantation Studies (CCTS). Effectiveness of histocompatibility matching in high-risk corneal transplantation. *Arch Ophthalmol.* 1992;110:1392–1403.
8. Maguire MG, Stark WJ, Gottsch JD, et al. Risk factors for corneal graft failure and rejection in the Collaborative Corneal Transplantation Studies. *Ophthalmology.* 1994;101:1536–1547.
9. Gottsch JD, Liu SH, Minkovitz JB, Goodman DF, Srinivasan M, Stark WJ. Autoimmunity to a cornea-associated stromal antigen in patients with Mooren's ulcer. *Invest Ophthalmol Vis Sci.* 1995;36:1541–1547.
10. Huynh TV, Young RA, Davis RW. Constructing and screening cDNA libraries in lambda gt10 and gt11. In: Glover D, ed. *Cloning techniques: A Practical Approach*. Oxford, UK: IRL Press; 1984.
11. Gottsch JD, Liu SH. Cloning and expression of human corneal calgranulin C (CO-Ag). *Curr Eye Res.* 1998;17:870–874.
12. Pohlman TH, Harlan JM. Human endothelial cell response to lipopolysaccharide, interleukin-1 and tumor necrosis factor is regulated by protein synthesis. *Cell Immunol.* 1989;119:41–52.
13. Bendtzen K, Svenson M, Jossen V, Hippe E. Autoantibodies to cytokines - friends or foes? *Immunol Today.* 1990;11:167–169.
14. Bendtzen K. Cytokines and natural regulators of cytokines. *Immunol Lett.* 1994;43:111–123.
15. He YG, Ross J, Niederkorn JY. Promotion of murine orthotopic corneal allograft survival by systemic administration of antiCD4 monoclonal antibody. *Invest Ophthalmol Vis Sci.* 1991;32:2723–2728.
16. Holland EJ, Chan CC, Wetzig RP, Palestine AG, Nussenblatt RB. Clinical and immunohistologic studies of corneal rejection in the rat penetrating keratoplasty model. *Cornea.* 1991;10:374–380.
17. Slowik MR, Min W, Ardito T, Karsan A, Kashgarian M, Pober JS. Evidence that tumor necrosis factor triggers apoptosis in human endothelial cells by interleukin-1-converting enzyme-like protease-dependent and -independent pathways. *Lab Invest.* 1997;77:257–267.
18. Wong GH, Alexander K, Elwell JH, Oberley LW, Goeddel DV. MnSOD induction by TNF and its protective role. In: Beutler B, ed. *Tumor Necrosis Factors: The Molecules and Their Emerging Role in Medicine.* New York: Raven Press; 1988;473–484.
19. Jaattela M, Wissing D, Bauer PA, Li GC. Major heat shock protein hsp 70 protects tumor cells from tumor necrosis factor cytotoxicity. *EMBO J.* 1992;11:3507–3572.
20. Medcalf RL, Kruithof EKO, Schleunig WD. Plasminogen activator inhibitor 1 and 2 are tumor necrosis factor/cachectin-responsive genes. *J Exp Med.* 1988;168:751–759.
21. Opipari AW, Hu HM, Yabkowitz R, Dixit VM. The A20 zinc finger protein protects cells from tumor necrosis factor cytotoxicity. *J Biol Chem.* 1992;267:12424–12427.
22. Karsan A, Yee E, Harlan JM. Endothelial cell death induced by tumor necrosis factor-α is inhibited by the *bcl-2* family member, A1. *J Biol Chem.* 1996;271:27201–27204.
23. Black RA, Kronheim SR, Sleath PR. Activation of interleukin-1β by a co-induced protease. *FEBS Lett.* 1989;247:386–390.
24. Kostura MJ, Tocci MJ, Limjuco G, et al. Identification of a monocyte specific pre-interleukin 1β convertase activity. *Proc Natl Acad Sci USA.* 1989;86:5227–5231.
25. Cohen GM. Caspases: the executioners of apoptosis. *Biochem J.* 1997;326:1–16.
26. Stroh C, Schulze–Osthoff K. Death by a thousand cuts: an ever increasing list of caspase substrates. *Cell Death Differ.* 1998;5:997–1000.
27. Yuan J, Shaham S, Ledoux S, Ellis HM, Horvitz HR. The *C. elegans* cell death gene *ced-3* encodes a protein similar to mammalian interleukin-1β-converting enzyme. *Cell.* 1993;75:641–652.
28. Whyte M. ICE/CED-3 proteases in apoptosis. *Trends Cell Biol.* 1996;6:245–248.
29. Enari M, Hug H, Nagata S. Involvement of an ICE-like protease in Fas-mediated apoptosis. *Nature.* 1995;375:78–81.
30. Los M, Vande Craen M, Penning LC, et al. Requirement of an ICE/CED-3 protease for Fas/Apo-1-mediated apoptosis. *Nature.* 1995;375:81–83.