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Activation of a 66-Kilodalton Human Endothelial Cell Matrix Metalloprotease by *Streptococcus pyogenes* Extracellular Cysteine Protease

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Human umbilical vein endothelial cells (HUVECs) were used to gain insight into the molecular mechanism whereby the major extracellular protease from group A streptococci damages host tissue. HUVECs exposed to streptococcal cysteine protease (SCP) for various times exhibited cytopathic effect and cell detachment from the culture vessel. Gelatin substrate zymography showed that a time- and concentration-dependent increase in the level of activity of an approximately 66-kDa gelatinase occurred in culture medium taken from cells exposed to enzymatically active SCP. This gelatinase comigrated in gelatin zymograms with the activated form of purified recombinant matrix metalloprotease 2 (MMP-2) and had type IV collagenase activity. In contrast, medium taken from cells exposed to inactivated (boiled) SCP and cells exposed to SCP inhibited by treatment with *N*-benzyloxycarbonyl-leucyl-valyl-glycine diazomethyl ketone lacked the 66-kDa gelatinase. Appearance of the 66-kDa gelatinase activity was also prevented by 1,10-phenanthroline, a zinc chelator and MMP inhibitor. Inasmuch as proteolytically active SCP is required for the emergence of this gelatinase and MMP activation occurs by proteolytic processing, the 66-kDa gelatinase may be a proteolytic cleavage product of a latent MMP expressed extracellularly by HUVECs. Direct SCP treatment of culture supernatant taken from HUVECs not exposed to SCP also produced the 66-kDa gelatinase. The data show that SCP activates an MMP produced by human endothelial cells, a process that may contribute to endothelial cell damage, tissue destruction, and hemodynamic derangement observed in some patients with severe, invasive group A streptococcal infection.

Group A streptococci (GAS) cause a wide variety of human pathologic conditions, including pharyngitis, glomerulonephritis, myositis, carditis, necrotizing fasciitis, a toxic-shock-like syndrome involving multiple organ failure, and acute rheumatic fever (1, 2, 11, 13). Invasive GAS infection can be characterized by severe tissue destruction and endothelial cell damage (22). The molecular mechanisms responsible for this damage are unknown. However, GAS strains causing severe, invasive disease are reported to produce higher levels of extracellular protease than strains associated with other infections (5, 21). In addition, a correlation has been observed among soft tissue necrosis, toxic-shock-like syndrome, including multiple organ involvement, and level of protease production (44). The major extracellular protease produced by GAS is a cysteine protease also known as streptococcal pyrogenic exotoxin B. Virtually all GAS strains express streptococcal pyrogenic exotoxin B (25, 39), and in vitro, some strains produce up to 150 mg of protease per liter of culture under appropriate conditions (16). Streptococcal pyrogenic exotoxin B is secreted as a 40-kDa precursor that is converted to a 28-kDa proteolytically active form under reducing conditions (20, 39). (Throughout this paper, the term streptococcal cysteine protease [SCP] will be used to refer to the 28-kDa mature form to differentiate it from the 40-kDa enzymatically inactive precursor.)

SCP degrades the extracellular matrix (ECM) proteins fibronectin and vitronectin (25). The ECM is involved in several critical cellular functions, such as maintenance of cell morphology and tissue integrity (9, 50). The tissue damage observed in some patients with invasive GAS infection may be partially due to direct ECM destruction caused by SCP. However, the fulminant progression of the tissue damage suggests that pathologic processes other than merely direct ECM degradation by SCP may be involved. For example, it is possible that SCP triggers host factors to participate in ECM breakdown.

Adherent cells, including endothelial cells, produce enzymes capable of degrading the ECM (3, 4, 7, 12). These enzymes are known as matrix metalloproteases (MMPs), and they are secreted during both normal and pathologic processes (4, 29). As a consequence of their considerable importance in homeostatic and pathologic processes, MMPs have been the subject of intensive research. MMPs participate in angiogenesis, tumor metastasis, uterine resorption, mammary involution, embryonic development, wound healing, growth and differentiation, bone resorption, periodontal tissue destruction, and other processes requiring ECM remodeling (4, 28, 29, 35, 42, 45). Thirteen MMPs have been described, and they are characterized by several distinct properties. First, all of these enzymes are secreted as inactive precursors containing a PRCGXPDV sequence in the propeptide region. The cysteine residue in this sequence interacts with a zinc atom present at the catalytically active site (46). Disruption of the interaction between this cysteine residue and zinc atom by proteolysis or chemical means results in activation of the protease. Activation is accompanied by autocatalytic cleavage on the carboxy-terminal side of the PRCGXPDV sequence (29, 41, 46). Second, all MMPs are inhibited by metal chelators and tissue inhibitors of metalloproteases (29). Third, all MMPs degrade ECM components and are subclassified as collagenases, gelatinases, and stromelysins on the basis of substrate specificity. Fourth, the MMPs have substantial levels of amino acid identity. Structural

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studies have demonstrated that MMPs have a modular structure consisting of an activation domain, a zinc-binding catalytic domain, a proline-rich hinge region, and a C-terminal vitronectin- or hemopexin-like domain (6, 29). In addition, the two gelatinases (MMP-2 and MMP-9) possess a fibronectin-like gelatin-binding domain (29). MMP-1, -2, -3, and -9 are produced by endothelial cells, although MMP-3 is made only at a low level (12, 19, 34).

Despite their considerable importance in a variety of physiologic processes, relatively little research has been performed to investigate the role of MMPs in host-parasite interactions. It has been reported that MMPs can be activated by bacterial exoenzymes, including *Pseudomonas aeruginosa* elastase and a *Porphyromonas gingivalis* protease (17, 28, 36, 45, 48). Lipoarabinomannan, a *Mycobacterium tuberculosis* cell wall component, induces increased levels of MMP expression (10). Evidence indicates that MMP activation caused by these bacterial virulence factors contributes to host tissue damage. Because damage to endothelial cells is observed in severe GAS infections (22) and SCP causes cytopathic effect (CPE) in cultured human umbilical vein endothelial cells (HUVECs) (25), experiments were undertaken to explore the possibility that SCP activates MMPs. The data demonstrate that treatment of HUVECs with SCP activates a 66-kDa MMP that is a type IV collagenase.

MATERIALS AND METHODS

Endothelial cell culture. HUVECs were isolated from human umbilical cords by the method of Kubota et al. (32). The veins were rinsed with Hanks' balanced salt solution (HBSS; Gibco, Gaithersburg, Md.) and incubated with 140 mg of *Clostridium histolyticum* collagenase (Gibco) per liter in HBSS for 30 min. Endothelial cells released from several cords were pooled, pelleted by centrifugation at 1,200 × *g* for 5 min, and suspended in 20 ml per cord of complete M199, which is composed of M199 (Gibco), 20% fetal calf serum (Hyclone, Logan, Utah), 17 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Sigma, St. Louis, Mo.), 33 mM sodium bicarbonate (Sigma), 2% penicillin-streptomycin (5,000 U of penicillin per ml, 5 mg of streptomycin per ml; Gibco), 2 mM L-glutamine (Gibco), 90 mg of heparin (185.5 U/mg; Sigma) per liter, and 20 mg of endothelial cell growth factor (Sigma) per liter. Cells were grown in 100-mm-diameter tissue culture plates coated with 0.2% gelatin (Sigma) and incubated at 37°C with 5% CO_2 until confluent. The medium was changed twice weekly. When confluency was reached, cells were passaged by detaching with trypsin-EDTA (Gibco), pelleting by centrifugation at 1,200 × *g* for 5 min, and suspending and plating in a 3× volume of complete M199. Cells were used in passages 1 to 5.

MMP activation. SCP was purified from *Streptococcus pyogenes* MGAS 1719 and MGAS 289 grown in Chemically Defined Medium (JRH BioSciences, Lenexa, Kans.) (25). These strains express two common allelic variants of SCP (25). Protein concentration was measured by protein assay (Pierce, Rockford, Ill.). SCP was activated by the addition of β-mercaptoethanol to a final concentration of 10 mM. Proteolytic activity was assessed with a casein-impregnated agarose plate assay (Bio-Rad, La Jolla, Calif.). SCP purity was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (33).

Purified SCP was added to serum-free medium (complete M199 without serum, 0.1% bovine serum albumin) to final concentrations of 1, 5, and 10 μg/ml. For inhibition experiments, an SCP inhibitor (*N*-benzyloxycarbonyl-leucyl-valyl-glycine diazomethyl ketone [Z-LVG-CHN_2]; Enzyme Systems Products, Livermore, Calif.) was added to SCP at a final concentration of 2 μg/ml before being added to serum-free medium. Confluent HUVECs grown in gelatin-coated six-well tissue culture plates were washed twice in HBSS and incubated for 0, 1, 2, 6, and 24 h in 1.5 ml of serum-free medium containing SCP or SCP plus inhibitor per well. Culture supernatants were collected at each time point. Cells were washed twice in HBSS and removed from the plate with trypsin-EDTA. Detached cells were pelleted by centrifugation at 1,200 × *g* for 5 min and suspended in 0.5 ml of HBSS per well. Cells were lysed by addition of SDS to a final concentration of 1%, and cell debris was removed by centrifugation at 1,200 × *g* for 5 min. Plates from which cells had been collected were washed twice in HBSS. ECM material was scraped from the plates and collected in 0.5 ml of HBSS per well.

Substrate gel zymography. Substrate gel zymography was performed as previously described (7, 27, 30, 49) with 1 mg of bovine type I gelatin per ml, 1 mg of casein per ml, or 0.5 mg of human placenta type IV collagen (Sigma) per ml. In the standard zymography procedure, MMPs are electrophoresed in an enzymatically inactive form. After electrophoresis, the conditions are then altered to activate the enzymes. The standard zymography procedure involves a complex series of steps. First, samples of culture supernatant, cell lysate, and extracellular matrix material were concentrated 25-fold with Microcon 3 concentrators (Amicon, Beverly, Mass.). Second, samples were mixed with the same volume of zymogram sample treatment buffer (0.4 M Tris HCl [pH 6.8], 20% [vol/vol] glycerol [Sigma], 5% [wt/vol] SDS [Gibco], 0.03% [wt/vol] bromophenol blue [Bio-Rad]) and electrophoresed on 10% polyacrylamide gels containing gelatin, casein, or type IV collagen at 150 V for approximately 1 h. To activate MMPs, gels were incubated in 2.5% Triton X-100 (Sigma) for 1 h to remove SDS and then incubated for 18 h at 37°C in enzyme assay buffer (50 mM Tris HCl [pH 7.5], 200 mM NaCl, 5 mM $CaCl_2$ [Sigma]). Gels were stained with zymogram staining solution (0.5% [wt/vol] Coomassie brilliant blue G-250 [Bio-Rad], 30% [vol/vol] methanol [EM Science, Gibbstown, N.J.], 10% (vol/vol) glacial acetic acid [J. T. Baker, Phillipsburg, N.J.]) for 1 h, and destained in 30% (vol/vol) methanol–10% (vol/vol) acetic acid. Gelatin-, collagen-, or casein-degrading activity appeared as a clear zone in a dark blue background after Coomassie blue staining.

To differentiate whether the 66-kDa SCP-activated MMP is a precursor or mature form, some samples were treated with *p*-aminophenylmercuric acetate (APMA; Sigma) before zymography. Incubation of culture supernatant with 2 mM APMA for 2 h at 37°C causes a disruption of the cysteine-zinc interaction that normally maintains the MMPs in an inactive, precursor form (38). Disruption of this cysteine-zinc interaction causes autocatalyzed proteolytic conversion of the MMP precursor to the mature form MMP. If the 66-kDa SCP-activated MMP is an MMP precursor, then it should be converted to a lower molecular mass by APMA treatment.

Densitometric analysis was performed with a Howtek Scanmaster 3+ scanner and Whole Band Analyzer software (BioImage, Ann Arbor, Mich.) with a Sun Sparcstation 5 computer. For experiments examining inhibition of MMP activity, zymograms were incubated in enzyme assay buffer with 10 mM 1,10-phenanthroline (Sigma), 10 mM EDTA (Sigma), or 5 mM dithiothreitol (Sigma) (9, 26). Purified recombinant MMP-2 was a gift from W. G. Stetler-Stevenson (National Cancer Institute, Bethesda, Md.).

SCP digestion of proteins in culture supernatant. SCP (10 μg/ml) was incubated with 20 μl of culture supernatant (taken from HUVECs incubated in serum-free medium without SCP) for 0, 1, 2, 4, 6, and 24 h at 37°C and analyzed by zymography. To test for SCP proteolytic activity, human plasma fibronectin (Sigma) was digested by SCP and analyzed by Western immunoblotting (25).

Degradation of type IV collagen. Immulon 1 microtiter plates (Dynatech, Chantilly, Va.) were coated overnight at 37°C with 100 μg of human placenta type IV collagen (Sigma) per ml in phosphate-buffered saline (PBS). The plates were washed three times with enzyme assay buffer (50 mM Tris HCl [pH 7.5], 200 mM NaCl, 5 mM $CaCl_2$). HUVEC culture supernatants used for collagen degradation assays were concentrated fivefold with Microcon 30 concentrators to remove tissue inhibitors of metalloproteases, which have molecular masses below 30 kDa. Twofold serial dilutions of HUVEC culture supernatants were made in enzyme assay buffer across each row of the microtiter plates. Two micrograms of Z-LVG-CHN_2 (SCP inhibitor) per ml was added to culture supernatants to inhibit collagenase activity due to SCP. Plates were incubated 18 h at 37°C and then washed three times in enzyme-linked immunosorbent assay (ELISA) wash buffer (10 mM Tris HCl [pH 7.4], 0.9% NaCl, 0.05% Tween 20), and the second wash was left on for 30 min at 37°C for blocking. One hundred microliters of a 1:5,000 dilution of rabbit anti-type IV collagen (Rockland, Gilbertville, Pa.) was added to each well, and the plates were incubated for 1 h at 37°C. After the plates were washed three times, 100 μl of a 1:2,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad) in ELISA wash buffer was added to each well. After incubation at 37°C for 1 h, antibody reactivity was detected with a Bio-Rad peroxidase substrate kit and the A_{490} was measured. Absorbance values at each dilution for duplicate trials were averaged, and titers were defined as the reciprocal of the dilution at which the average absorbance value was 50% of maximum absorbance.

RESULTS

SCP activates gelatinase in HUVEC culture supernatants. Kapur et al. (25) observed that cultured HUVECs treated with SCP detach from the tissue culture plate and display CPE. Experiments were undertaken to determine if ECM degradation and HUVEC CPE are due solely to a direct proteolytic activity of SCP or if activation of MMPs is associated with the process. Confluent HUVECs were washed twice in HBSS to remove inhibitors, including MMP inhibitors, present in the medium. Cells were then incubated in serum-free medium containing 0, 1, 5, or 10 μg of SCP per ml for times ranging from 0 to 24 h. Consistent with previous observations (25), some detachment and CPE occurred in HUVECs incubated with 10 μg of SCP per ml for 6 h. At 24 h, detachment of most cells had occurred at all concentrations of SCP but, in contrast, control HUVECs incubated in serum-free medium alone remained attached to the culture vessel surface (data not shown).

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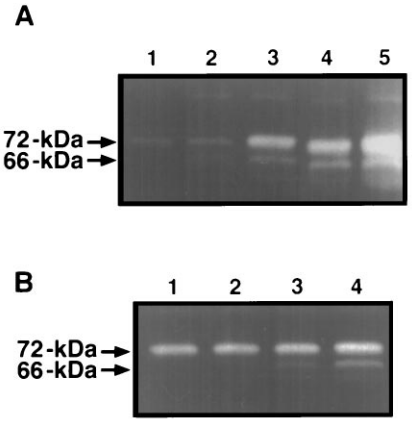

FIG. 1. Time- and SCP-concentration-dependent activation of 66-kDa gelatinase. Confluent HUVECs were washed to remove serum components and incubated in serum-free medium containing 0, 1, 5, or 10 μg of SCP per ml for times ranging from 0 to 24 h. Culture supernatants were collected and analyzed by gelatin substrate zymography. Degradation of gelatin by proteins present in these culture supernatants results in clear areas on the gel. (A) Zymogram of culture supernatant from HUVECs incubated in serum-free medium containing 10 μg of SCP per ml for 0 h (lane 1), 1 h (lane 2), 2 h (lane 3), 6 h (lane 4), or 24 h (lane 5). (B) Zymogram of culture supernatants from HUVECs incubated with serum-free medium alone (lane 1) or serum-free medium containing 1 μg of SCP per ml (lane 2), 5 μg of SCP per ml (lane 3), or 10 μg of SCP per ml (lane 4) for 6 h. Molecular masses are given and are based on a comparison with molecular mass standards and migration of known MMPs.

Culture supernatants collected at each time point and at each SCP concentration were analyzed by gelatin and casein zymography to measure gelatinase and caseinolytic activities, respectively. All samples obtained from cells incubated in serum-free medium without SCP had one major band of gelatinolytic activity with an apparent molecular mass of 72 kDa. In contrast, culture supernatant obtained from cells incubated with SCP had a second gelatinolytic band with an apparent molecular mass of approximately 66 kDa. The amount of this 66-kDa gelatinase activity increased in a time- and concentration-dependent manner (Fig. 1). The 66-kDa band also appeared in gelatin zymograms of culture supernatants taken from HUVECs incubated with a naturally occurring variant of SCP isolated from strain MGAS 289. No protease activity was detected in samples studied by casein zymography (data not shown).

It is possible that SCP also activated gelatinases that were not present at a sufficient concentration in the culture supernatant to be detected by zymography. To test this possibility, culture supernatants were concentrated 25-fold by ultrafiltration and analyzed by zymography. No additional SCP-activated gelatinase activity was observed.

To rule out the possibility that activation of the 66-kDa gelatinase was due to some other substance present in the purified SCP preparation, HUVECs were incubated with serum-free medium containing 1% β-mercaptoethanol, 10% Tris-ethanol buffer, 10% Matrex Red Dye A, or PBS. These reagents were used in SCP purification and should be present only in trace amounts, if at all, in the solution containing purified protease. No 66-kDa gelatinase activity was observed in HUVECs incubated with these materials for up to 24 h (Fig. 2).

SCP-activated gelatinase is a metalloprotease. To confirm that the 66-kDa gelatinase activated by SCP is a metalloprotease, 1,10-phenanthroline, a zinc chelator and metalloprotease inhibitor, was included in the enzyme assay buffer used in the zymography procedure. This compound inhibited all gelatinase activity, a result consistent with the idea that the SCP-activated gelatinase is an MMP. Moreover, inclusion of either of two other metalloprotease inhibitors (EDTA and dithiothreitol) also prevented the appearance of the 66-kDa band (data not shown).

SCP activates an inactive MMP. MMPs are secreted as inactive precursors which are activated by autocatalyzed proteolysis of a propeptide sequence (31, 38). This proteolysis is triggered by disruption of the normal interaction between a cysteine residue in the propeptide and a zinc atom located at the enzyme active site (29, 46), a regulatory mechanism known as a cysteine switch. Cleavage of enzymatically inactive MMP precursors by other proteases (for example, plasmin) or chemical treatments, such as incubation with APMA, disrupt this cysteine-zinc interaction and thereby activate MMPs (38). MMP precursors are also activated in the final stage of the zymography procedure, a process that allows visualization of both the precursor and the mature form. Therefore, the 66-kDa gelatinase could be either a precursor or a mature MMP. To differentiate between these two possibilities, a known activator of MMPs (APMA) was incubated with the SCP-treated culture supernatants before zymography. If the 66-kDa gelatinase is an inactive MMP precursor, then APMA treatment should disrupt the cysteine-zinc interaction and cause an autocatalyzed conversion to the mature active form. In APMA-treated samples, this proteolytic conversion should occur before, rather than during, zymography. Therefore, compared with that of samples not treated with APMA, a lower molecular mass form should be observed on zymograms. If the 66-kDa gelatinase is the activated form of an MMP, then there should be no change in size after APMA treatment. When the experiment was conducted, no decrease in size of the 66-kDa band occurred after APMA treatment of the culture supernatants (Fig. 3), indicating that this 66-kDa gelatinase is not an inactive MMP precursor. As a control to verify that APMA treatment can activate MMPs, culture supernatant from HUVECs not exposed to SCP was treated with APMA and subjected to zymography. The resulting zymogram had a band of approximately 68 kDa. Taken together, these results suggest that the SCP-activated gelatinase is a product of a larger MMP that has undergone proteolytic cleavage to form a product of 66 kDa.

The APMA data do not reveal whether SCP directly or indirectly causes the proteolysis, or up-regulates the expression, of the precursor, thereby resulting in the appearance of more of the active form. A first step to determine if the 66-kDa gelatinase arises through direct proteolysis by SCP is to verify that SCP is active in tissue culture medium. Therefore, 0.5 μg

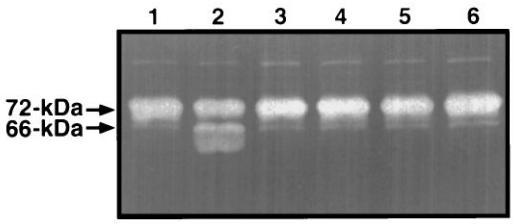

FIG. 2. Lack of effect of materials present in SCP preparations on 66-kDa gelatinase activation. HUVECs were incubated for 24 h with serum-free medium (lane 1) or serum-free medium containing 10 μg of SCP per ml (lane 2), 1% β-mercaptoethanol, a reducing agent used to activate SCP (lane 3), 10% Tris-ethanol buffer used in the SCP purification protocol (lane 4), 10% Matrex Red Dye A used for purification of SCP (lane 5), or PBS (lane 6). Culture supernatants were collected and analyzed by gelatin substrate zymography.

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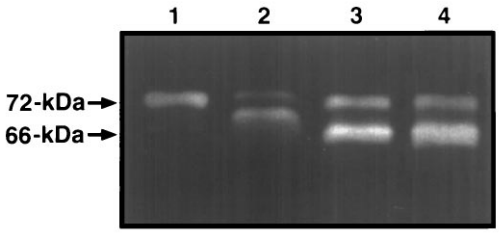

FIG. 3. Activation of HUVEC MMPs by APMA. HUVECs were incubated in serum-free medium alone (lane 1) or in medium containing 10 μg of SCP per ml (lane 3) for 24 h, and culture supernatants were subjected to gelatin substrate zymography. Culture supernatants were also treated with 2 mM APMA at 37°C for 2 h to activate MMP precursors before zymography. Lane 2 shows APMA treatment of culture supernatant from HUVECs not exposed to SCP. Lane 4 shows APMA treatment of culture supernatant from HUVECs exposed to 10 μg of SCP per ml for 24 h.

of SCP was incubated with 5 μg of fibronectin in serum-free medium for 2 h and analyzed by Western blot analysis. As expected, digestion of fibronectin was observed (data not shown). If, as we suspect, the 66-kDa gelatinase activity arises through proteolysis of a larger MMP by SCP, then culture supernatants from HUVECs incubated with inactivated or inhibited SCP should not exhibit this 66-kDa protein. HUVECs were incubated in serum-free medium with SCP inhibited by Z-LVG-CHN_2 or with boiled (inactivated) SCP. A 66-kDa gelatinase activity was not observed in culture supernatants from cells incubated with boiled SCP or SCP plus inhibitor at any concentration or time point (Fig. 4), a result indicating that SCP must be proteolytically active to give rise to the 66-kDa gelatinase.

To provide additional evidence that the 66-kDa gelatinase arises from a latent MMP expressed extracellularly by HUVECs and not solely synthesized de novo by the cells, 10 μg of active SCP per ml was incubated with cell-free HUVEC culture supernatant for times ranging from 0 to 24 h and analyzed by zymography. As shown in Fig. 5, incubation of HUVEC culture supernatants with SCP resulted in the appearance of the 66-kDa gelatinase. Therefore, SCP can lead to proteolytic conversion of a latent MMP secreted by HUVECs to an active form through either a direct or an indirect mechanism.

SCP-activated gelatinase is a type IV collagenase. As shown in Fig. 6, the two major gelatinases observed in culture supernatant from HUVECs exposed to SCP migrated at the same

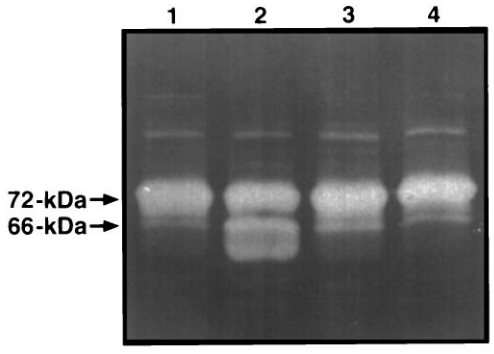

FIG. 4. Inactivated SCP fails to activate 66-kDa gelatinase. Gelatin substrate zymography was performed on culture supernatants from HUVECs incubated in serum-free medium alone (lane 1) or serum-free medium containing 10 μg of SCP per ml (lane 2), 10 μg of SCP plus 2 μg of the SCP inhibitor Z-LVG-CHN_2 per ml (lane 3), or 10 μg of SCP which had been inactivated by boiling per ml (lane 4).

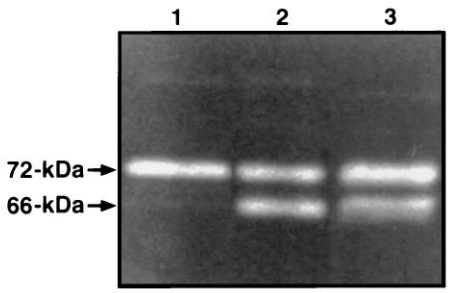

FIG. 5. SCP activation of an MMP precursor secreted by HUVECs. Culture supernatant was collected from HUVECs incubated in serum-free medium without SCP. To determine if SCP can activate an MMP secreted into this supernatant by HUVECs, the culture supernatant was incubated with 10 μg of SCP per ml for 6 h and subjected to gelatin substrate zymography. Lane 1 shows the culture supernatant before SCP treatment. Lane 3 shows the supernatant after SCP treatment. Lane 2 shows the result of treatment of HUVECs (instead of culture supernatant) with 10 μg of SCP per ml for 6 h. The approximately 66-kDa band is produced both by treatment of culture supernatant with SCP (lane 3) and by treatment of HUVECs with SCP (lane 2), suggesting that SCP activates an MMP secreted by the HUVECs rather than that it solely induces increased levels of expression of the 66-kDa gelatinase.

molecular masses as the latent and active forms of recombinant MMP-2 on gelatin zymograms, a result suggesting—but not proving—that SCP may activate MMP-2. Because MMP-2 is a type IV collagenase, the ability of these gelatinases to degrade type IV collagen was examined by zymography. Both the 72- and 66-kDa gelatinases had type IV collagenase activity (Fig. 7). Densitometric analysis of type IV collagen zymograms revealed a 78.5% increase in the level of total collagenase activity in lanes containing culture supernatants from cells exposed to SCP compared with that of cells exposed to serum-free medium alone.

Because SDS present in the zymograms may denature collagen, an ELISA-based assay was used to confirm that the SCP-activated MMP can degrade nondenatured type IV collagen. Type IV collagen-coated microtiter plates were incubated with HUVEC culture supernatants containing SCP inhibitor to ensure that collagen degradation was not due to SCP activity. Collagen remaining on the plate after digestion was detected with rabbit serum specific for type IV collagen. Absorbance values at each dilution for duplicate trials were averaged, and titers were defined as the reciprocal of the dilution at which the average absorbance value was 50% of the maximum absorbance value. Therefore, lower titers indicated that

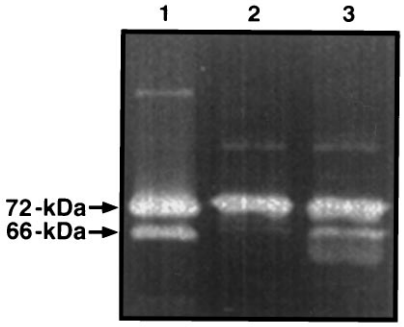

FIG. 6. Comigration of SCP-activated gelatinase with purified recombinant MMP-2. Gelatin substrate zymography was performed with purified recombinant MMP-2 (lane 1), culture supernatant from HUVECs incubated with serum-free medium (lane 2), and culture supernatant from HUVECs incubated with 10 μg of SCP per ml (lane 3) for 6 h.

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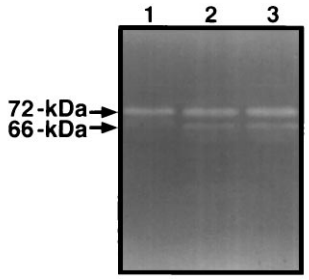

FIG. 7. Degradation of type IV collagen by HUVEC culture supernatants. Culture supernatants from HUVECs exposed to serum-free medium alone (lane 1) or to serum-free medium containing 10 μg of SCP per ml from strain MGAS 1719 (lane 2) or MGAS 289 (lane 3) for 24 h were analyzed by type IV collagen zymography.

less immunoreactive collagen remained on the plate and greater collagen degradation occurred. Culture supernatant from HUVECs exposed to 10 μg of SCP per ml for 24 h had a titer (3,252) 15-fold less than that of culture supernatant from HUVECs incubated in serum-free medium alone for the same period of time (48,696). As a positive control, 0.03 μg of purified recombinant MMP-2 was used in the same assay and had a titer of 551. These results document increased degradation of type IV collagen by culture supernatant from HUVECs exposed to SCP.

DISCUSSION

SCP-activated gelatinase. Our data show that incubation of HUVECs with SCP results in the appearance of a new 66-kDa MMP activity. Although our data do not definitively identify the SCP-activated MMP, several lines of evidence support the hypothesis that the 66-kDa gelatinase is an active form of MMP-2 or MMP-9. First, this gelatinase activity is inhibited by zinc and calcium chelators, indicating that metal ions are required for activity. Therefore, the 66-kDa gelatinase is a metalloprotease. Second, the enzyme degrades gelatin and type IV collagen, proteins that are substrates for several MMPs, including MMP-2 and MMP-9 (4). Third, the two major gelatinases observed in HUVEC culture supernatants exhibited apparent molecular masses of 72 and 66 kDa and comigrated with purified recombinant MMP-2 on zymograms. Fourth, HUVECs have been reported to produce MMP-1, MMP-2, MMP-3, and MMP-9 (12, 14, 19, 34). MMP-1 (52 kDa) and MMP-3 (57 kDa) have molecular masses lower than the 66-kDa MMP observed in this study. Therefore, it is unlikely that the SCP-activated MMP is one of these enzymes. It is more likely that the 66-kDa MMP is MMP-2, which has a 66-kDa active form, or MMP-9 which has been processed by SCP to form a 66-kDa protein. It is also a formal possibility that the SCP-activated 66-kDa gelatinase is a previously uncharacterized MMP. Studies are under way to definitively identify the activated MMP.

SCP activation of MMPs and GAS pathogenesis. Relatively few pathogenic bacteria have been reported to express virulence factors that activate host MMPs. *Pseudomonas aeruginosa* elastase has been found to activate MMP-2 made by corneal fibroblasts, a process thought to participate in ocular infection pathogenesis (28, 36, 37). Similarly, poorly characterized proteases expressed by the oral organisms *Porphyromonas gingivalis* and *Treponema denticola* have been reported to activate MMP-1 and MMP-8 (45). Activation of these MMPs is thought to initiate a pathogenetic pathway that is responsible for much of the tissue destruction observed in periodontal disease. In addition, a cell wall component of *Mycobacterium tuberculosis* has been reported to induce expression of MMP-9 (10).

Two of the hallmark features of many patients with severe invasive streptococcal infections, such as necrotizing fasciitis, are fulminant tissue destruction and endothelial cell damage (22). In many patients, this destruction occurs within hours and is so extensive that virtually no normal tissue architecture remains. Moreover, histologic examination reveals that tissue destruction occurs in areas where bacteria are not present (22). These observations imply that one or more extracellular bacterial products participate directly or indirectly in tissue damage. Importantly, a recent study of acute- and convalescent-phase sera obtained from 17 patients with invasive GAS infections has documented seroconversion to purified recombinant SCP, a result indicating that SCP is produced in vivo during episodes of human severe invasive disease (18). Our demonstration that SCP activates a 66-kDa gelatinase with type IV collagenase activity suggests a plausible pathway whereby an extracellular streptococcal product may participate directly and indirectly in some of the tissue destruction recorded for patients with severe invasive infections. Under this hypothesis, SCP assists in ECM destruction directly by proteolytic cleavage of the critical ECM proteins fibronectin and vitronectin (25, 39) and indirectly by activation of host MMPs.

Our study did not address the important issue of the mechanism of MMP activation. In principle, activation of host MMPs by SCP may occur by direct proteolysis of the inactive MMP precursor or, alternatively, by an indirect pathway whereby SCP activates an MMP activator, such as plasmin, or degrades one of the physiologic inhibitors of MMPs known as tissue inhibitors of metalloproteases. The results of preliminary experiments suggest that SCP does not directly cleave purified recombinant MMP-2 (8) under the conditions assayed, which suggests that the SCP-activated MMP is not MMP-2 or that activation occurs by an indirect route. Clearly it will be important to elucidate the exact pathway of MMP activation, and studies are under way to address this issue.

We discovered that SCP activation of an MMP made by HUVECs leads to greatly increased type IV collagen degradation. Type IV collagen degradation by HUVEC culture supernatants increased approximately 78.5%. Although substrate specificities of MMPs differ, all the MMPs degrade several ECM proteins. Therefore, it is likely that several ECM proteins, including other collagen types, are also detrimentally affected by SCP activation of an MMP. Degradation of collagen and other ECM proteins would have severe adverse effects on tissue and, hence, to the patient.

Many patients with invasive streptococcal disease die with hypotension, shock, and vascular collapse. Increased vascular permeability occurring as a result of increased collagen degradation and ECM breakdown due to MMP activation (41) may contribute to these pathologic processes. Increased vascular permeability would also contribute to enhanced dissemination of GAS to the extravascular compartment, a process that would ultimately add to the extensive soft tissue damage observed in some patients. Increased levels of collagenase activity due to SCP activation of MMPs may also contribute to this soft tissue destruction. Because MMPs are also made by other types of adherent cells, such as epithelial and smooth muscle cells, it is possible that SCP activates additional MMPs, depending on the tissue involved. Preliminary experiments indicate that this is the case.

One of the functions of several MMPs, including MMP-2, is to stimulate tumor necrosis factor alpha (TNF-α) production by cleaving pre-TNF-α to form the mature, biologically active

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form of the cytokine (15). In this context, three observations are noteworthy. First, patients with severe invasive GAS infections have been reported to have high levels of circulating TNF-α (40). Second, with a baboon model of invasive GAS disease, Stevens et al. (47) showed that TNF-α levels are increased in diseased animals. Third, we have recently discovered that in a rat lung model, SCP acts synergistically with streptolysin O to result in an extensive inflammatory response characterized by very high levels of TNF-α in bronchoalveolar lavage fluid (43). SCP also cleaves pre-interleukin-1β to generate biologically active interleukin-1β (24), an inducer of MMPs in fibroblasts (34). Taken together, these observations suggest that cascades involving SCP, interleukin-1β, MMPs, and TNF-α may be activated in the course of streptococcal disease. Studies are under way to test this notion.

In conclusion, we have documented that SCP activates a 66-kDa MMP produced by human endothelial cells. Activation of this host enzyme increases type IV collagen degradation, a process that may contribute significantly to the pathology of severe invasive GAS infections by increasing histologic damage, vascular permeability, dissemination of bacteria, soft tissue necrosis, and inflammation. We note that one of the implications of this research is that inhibitors of SCP, such as host antibody, would diminish MMP activation and the subsequent detrimental host effects caused by this activation. In this regard, the observations that humans with low acute-phase anti-SCP antibody levels are more likely to die during an invasive GAS episode (21) and that immunization of mice with purified SCP is protective (23) are germane.

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