BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **231,** 222–226 (1997)
ARTICLE NO. RC965978

Superantigen-Induced Stromelysin Production from Rheumatoid Synovial Cells

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Received December 9, 1996

Superantigens activate a large number of T cells in a Vβ-restricted manner after binding to MHC class II molecules on the antigen presenting cells (APC). Superantigens also activate APC directly by interacting with their ligands, MHC class II molecules. In the present study, we examined the effects of superantigens on matrix metalloproteinases (MMPs) secretion from rheumatoid synovial fibroblasts. We demonstrated that stimulation of interferon (IFN)-γ-treated synovial fibroblasts with staphylococcal enterotoxin A (SEA) selectively induced the secretion of stromelysin, a neutral MMP, from synovial fibroblasts. Pretreatment of synovial fibroblasts with cycloheximide, an inhibitor of protein synthesis,prevented MMP-3 secretion from rheumatoid synovial cells suggesting that protein synthesis was required for SEA-induced MMP-3 secretion after SEA binding to MHC class II molecules. Our data suggest that in the synovium, bacterial superantigens are potent inducers of stromelysin which plays a critical role in articular destruction observed in inflammatory joint disease.

A common feature of inflammatory arthritis, including rheumatoid arthritis (RA), is the presence of synovial inflammation and subsequent cartilage destruction (1, 2). Although the precise mechanism(s) of cartilage destruction is not fully understood, results of several studies have demonstrated the presence of an excess amount of proteinases in the synovial fluid of inflammatory arthritis (3-5). Stromelysin, a neutral matrix metalloproteinase (MMP) capable of degrading several components of extracellular matrix (ECM), is believed to be a key enzyme during cartilage destruction (6-8). In addition to this activity, stromelysin (MMP-3) also activates other MMPs by proteolytic cleavage of the pro-peptide domain at the N-terminus (9, 10).

The bacterial superantigens, a group of proteins, including staphylococcal enterotoxins (SEs) and toxic shock syndrome toxin-1 (TSST-1), do not require antigen processing when they interact with their natural ligand MHC class II molecules on antigen presenting cells (11-14). After binding to MHC class II molecules, superantigens can activate a large proportion of T cells bearing particular T cell receptor (TCR) Vβ chains (11, 15). Recent studies have demonstrated a preferential expansion of T cells with limited TCR Vβ chains and suggested the existence of Vβ-specific superantigens in the rheumatoid synovium (16, 17). Engagement of MHC class II molecules by superantigens is quickly followed by activation of class II positive antigen presenting cells (18). SEs also activate human synovial fibroblasts and induce an up-regulation of inflammatory cytokines genes (19).

In the present study, we investigated the effects of SEs on MMPs secretion from rheumatoid synovial fibroblasts after engagement with MHC class II molecules. Our results demonstrated that stimulation of rheumatoid synovial cells with staphylococcal enterotoxin A (SEA) selectively induced MMP-3 secretion from these cells.

MATERIALS AND METHODS

Reagents. Staphylococcal enterotoxin A and B were purchased from Sigma Chemical Co. (St. Louis, MO). Anti-TNF-α neutralizing monoclonal antibody (Ab) was kindly provided by Dainihon Pharmaceutical Co. (Osaka, Japan). Rabbit anti-interleukin (IL)-1β neutralizing antibody was kindly provided by Otsuka Pharmaceutical

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Abbreviations: MHC, major histocompatibility complex; MMP, matrix metalloproteinase; IFN-γ, interferon-γ; SEA, staphylococcal enterotoxin A; TCR, T cell receptor; TNF-α, tumor necrosis factor-α; IL-1-β, interleukin-1β; TIMP-1, tissue inhibitor of metalloproteinse-1.

0006-291X/97 $25.00

Co. (Tokushima, Japan). Human recombinant interferon (IFN)-γ was a gift from Shionogi Pharmaceutical Co. (Osaka). Anti-MMP-3 monoclonal Ab was purchased from Oncogene Science (Cambridge, MA). Anti-tissue inhibitor of metalloproteinase-1(TIMP-1) antibody was purchased from Fuji Chemical Co.(Takaoka, Japan). Purified monoclonal antibodies to human HLA-DR and HLA-DQ were purchased from Immunotech S.A.(Marseille, France). All other reagents were obtained from Sigma Chemical Co.

Synovial cells cultures. The experimental protocol was approved by the local ethics committees and a signed consent form was obtained. Synovial tissue samples were obtained from patients with RA during synovectomy. The synovial membranes were minced aseptically, then dissociated enzymatically with collagenase (4.0 mg/ml, Sigma) in RPMI 1640 for 4 hours at 37°C. The obtained cells were plated on culture dishes and allowed to adhere. To eliminate nonadherent cells from synovial cell preparations, the plated cells were cultured for 18 hours with RPMI 1640, supplemented with 10% FCS at 37°C in humidified 5% CO_2 in air. Cells were then washed extensively with phosphate buffered saline (PBS) solution. Adhering synovial cells were removed by adding trypsin-EDTA and washed with PBS containing 2% FCS. The collected synovial cells were used at the third or fourth passages for subsequent experiments. Synovial cell preparations were less than 1% reactive with monoclonal antibodies CD3, CD20(Coulter Immunology, FL), Leu M3(Becton Dickinson, CA) and anti-human von Willebrand factor (Immunotech, Marseille, France) which, respectively, define an antigen on mature T lymphocytes, monocytes/ macrophages, and vascular endothelial cells. Cells were plated in 6-well culture plate (Coster, Cambridge, MA) for 24 hours, then washed three times with PBS. In the next step, the synovial cells were treated with IFN-γ for 72 hours to induce MHC class II expression. The cells were washed and resuspended with serum free-RPMI medium in the presence or absence of staphylococcal enterotoxins. The conditioned medium was collected after 48 hours of incubation, centrifuged (3500 $\times$ g for 5 min) to remove debris, and analyzed by immunoblot or gelatin zymography.

Immunoblot analysis. Synovial fibroblast-conditioned media were subjected to polyacrylamide gels. Fractionated proteins were transferred to nitrocellulose membrane. The filters were blocked for 90 min using 5% non-fat powdered milk in TBS (50 mM Tris, 0.15 M NaCl, pH 7.5) containing 0.1% Tween 20, washed with TBS and incubated at room temperature for 2 hours with mouse anti-MMP-3 monoclonal antibody (1:100 dilution) or mouse anti-TIMP-1 monoclonal antibody (1:250 dilution). Filters were later washed with TBS and incubated with 1:1000 dilution of donkey anti-mouse IgG antibodies, coupled with horseradish peroxidase. The enhanced chemiluminescence (ECL) system (Amersham) was used for detection. Filters were subsequently exposed to the film for 15 sec and processed.

Gelatin zymography. Synovial fibroblast-conditioned media were incubated at 37°C for 20 min in SDS sample buffer without a reducing agent and then electrophoresed on 8% polyacrylamide gels containing 1% gelatin at 4°C. After electrophoresis, the gels were washed in 2.5% Triton-X 100 to remove SDS, and incubated with 50 mM Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl, 10 mM $CaCl_2$ and 0.02% NaN_3 for 16 hours at 37°C and stained with 0.1% Coomassie Brilliant Blue R250.

RESULTS

When human synovial cells were cultured with 100 u/ml of IFN-γ for 72 hours, the cells expressed MHC class II molecules on their surface as described previously (20) (data not shown). We examined whether

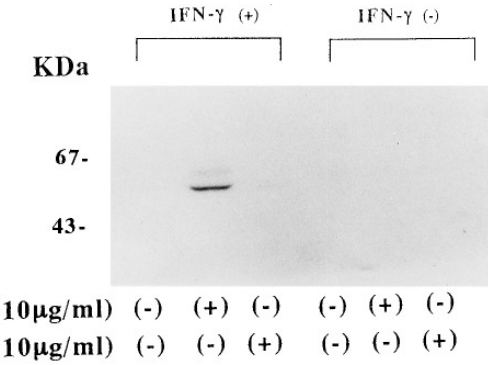

FIG. 1. Superantigen-induced stromelysin secretion from rheumatoid synovial fibroblasts. Synovial fibroblasts were untreated or pretreated with 100 u/ml of IFN-γ. The cells were then stimulated with SEA or SEB in serum-free RPMI medium for 48 hours. The conditioned medium was analyzed by immunoblot using anti-stromelysin monoclonal antibody. Prestained molecular weight markers are shown on the far left line. A representative example of four similar results.

the engagement of MHC class II molecules by SEs induces secretion of MMPs from synovial cells. IFN-γ treated or untreated synovial cells were cultured with serum-free RPMI medium in the presence or absence of SEA or SEB (10 μg/ml) for 48 hours. The conditioned medium was collected and electrophoresed on 8% SDS-polyacrylamide gel and immunoblotted by anti-stromelysin (MMP-3) Ab. Untreated synovial cells did not secrete MMP-3 following culture with SEA or SEB. However, MMP-3, which migrates as a 57 KDa band, was detected in the conditioned media when IFN-γ-pretreated synovial cells were stimulated with SEA (Fig. 1).

The secretion of gelatin-degrading metalloproteinases (gelatinases) was also examined by gelatin zymography. As illustrated in Fig. 2, synovial cells constitutively secreted gelatinases with apparent *Mr* of 72000 and 92000. The secretion of these gelatinases was not influenced by SEA stimulation in IFN-γ-pre-treated or untreated synovial cells. Although tissue inhibitor of metalloproteinase (TIMP-1) secretion from synovial cells was analyzed by immunoblot, there was no difference in TIMP-1 secretion between untreated and SEA-stimulated synovial cells (data not shown).

We also examined the dose-response effect of SEA on MMP using various concentrations of SEA and IFN-γ. For this purpose, synovial cells were pretreated with 100 u/ml of IFN-γ for 72 hours, then stimulated by various concentrations of SEA (0.1-10 μg/ml). After 48 hours of incubation, the conditioned media were analyzed by anti-MMP-3 immunoblots. SEA stimulation induced MMP-3 secretion from synovial cells in a dose-dependent manner (Fig. 3A). In addition, when synovial cells were pretreated with increased concentra-

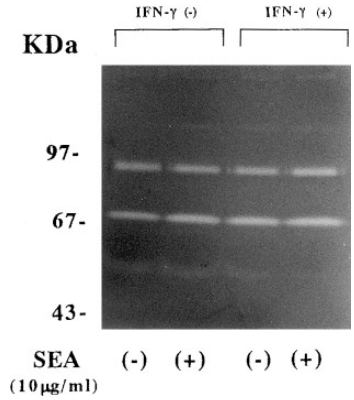

FIG. 2. Superantigen stimulation did not increase gelatinases secretion from rheumatoid synovial fibroblasts. Untreated or IFN-γ-pretreated synovial cells were stimulated with SEA in serum-free RPMI medium for 48 hours. The conditioned medium was electrophoresed on a gelatine substrate gel and stained with Coomassie Brilliant blue R-250. Clear zones represent gelatinolytic activity. A representative example of three similar results.

tions of IFN-γ before the addition of SEA (10μg/ml), MMP-3 secretion was also enhanced by IFN-γ in a dose-dependent manner (Fig. 3B).

In the next step, we used cycloheximide, an inhibitor of protein synthesis, to determine whether de novo protein synthesis was required for SEA-mediated MMP-3 secretion from synovial cells. IFN-γ-pretreated synovial cells were incubated with SEA (10 μg/ml) in the presence of various concentrations of cycloheximide.

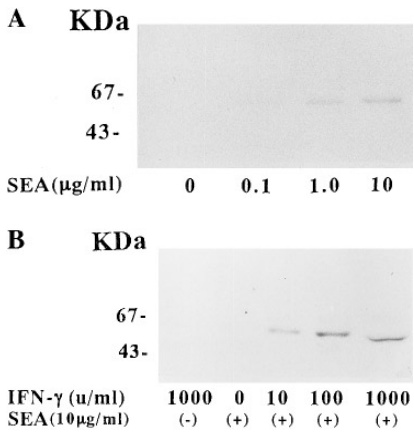

FIG. 3. Secretion of stromelysin from SEA-stimulated synovial fibroblasts. (A) Synovial fibroblasts were pretreated with 100 u/ml of IFN-γ for 72 hours. IFN-γ-pretreated synovial fibroblasts were stimulated with increasing concentrations of SEA for 48 hours. The conditioned medium was collected and analyzed by immunoblot using anti-stromelysin antibody. (B) Synovial fibroblasts were pretreated with increasing concentrations of IFN-γ for 72 hours, then stimulated with 10 μg/ml of SEA for 48 hours. The conditioned medium was collected and analyzed by immunoblot using anti-stromelysin antibody. A representative example of three similar results.

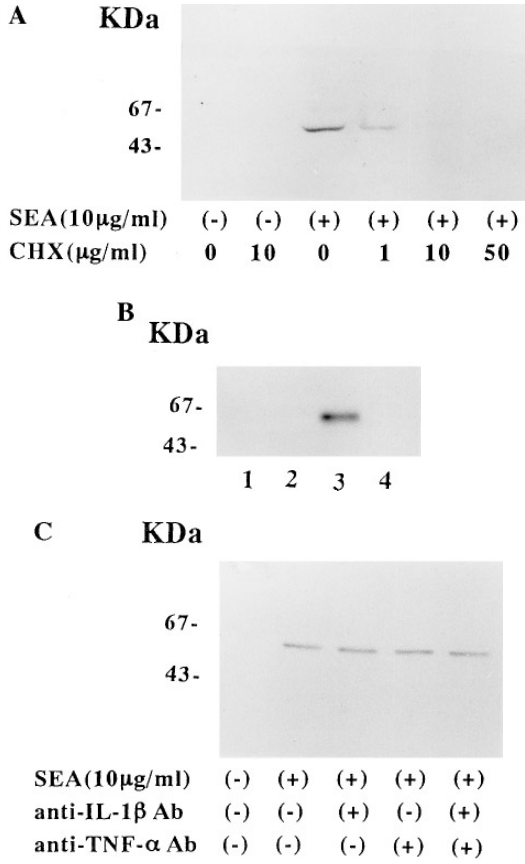

FIG. 4. The effects of cycloheximide, anti-MHC class II antibodies and anti-cytokine antibodies on SEA-induced stromelysin secretion from rheumatoid synovial fibroblasts. (A) Synovial fibroblasts were pretreated with IFN-γ (100 u/ml) for 72 hours, followed by one hour treatment with increasing dose of cycloheximide 48 hours prior to SEA stimulation. The conditioned medium was collected and analyzed by immunoblot using anti-stromelysin antibody. (B) IFN-γ-pretreated synovial fibroblasts were stimulated with SEA in the presence or absence of anti-HLA-DR and anti-HLA-DQ monoclonal antibodies for 48 hours.The conditioned medium was collected and analyzed by immunoblot using anti-stromelysin antibody. lane 1; IFN-γ-untreated, without stimulation, lane 2; IFN-γ-untreated, stimulated with SEA (10μg/ml), lane 3; IFN-γ-pretreated, stimulated with SEA, lane 4; IFN-γ-pretreated, stimulated with SEA in the presence of anti-HLA-DR and DQ monoclonal antibodies (10μg/ml). (C) IFN-γ-pretreated synovial fibroblasts were stimulated with SEA in the presence or absence of anti-IL-1β or anti-TNF-α antibodies for 48 hours. Rabbit polyclonal anti-human IL-1β antibody (1:1000 dilution) and mouse anti-human TNF-α monoclonal antibody (14E3, 1:500 dilution) were mixed in SEA-stimulated synovial cells culture. In these conditions, these antobodies were sufficient to neutralize 10 Iu/ml of human IL-1β and 100 u/ml of TNF-α. The conditioned medium was collected and analyzed by immunoblot using anti-stromelysin antibody. A representative example of three similar results.

Cycloheximide inhibited SEA-induced MMP-3 secretion in a dose-dependent manner (Fig. 4A). To determine whther MHC class II molecules are required for this SEA-mediated MMP-3 production, we investigated the effects of anti-MHC class II antibodies on SEA-

stimulated synovial cells. IFN-γ-pretreated synovial cells were stimulated by SEA in the presence or absence of anti-HLA-DR and HLA-DQ monoclonal antibodies. Addition of anti-HLA-DR and HLA-DQ Mo antibodies to IFN-γ-pretreated synovial cells inhibited the SEA-mediated MMP-3 production (Fig. 4B).

Treatment of synovial cells with SEA induces gene expression of inflammatory cytokines, such as IL-1β and TNF-α, which are potent inducers of MMP-3 in rheumatoid synovial cells (21). To determine whether synovial cells secrete MMP-3 directly in response to SEA, or indirectly via these cytokines, the cytokines were blocked by neutralizing antibodies. As shown in Fig. 4C, neither anti-TNF-α nor anti-IL-1β antibodies blocked SEA-mediated MMP-3 secretion from IFN-γ-pretreated synovial cells.

DISCUSSION

Several lines of evidence suggest that dominant T cell clones bearing particular Vβ chains are expanded in the rheumatoid synovium and that Vβ-specific superantigens may play a critical role in triggering synovial inflammation (16,17). Recent studies have demonstrated that superantigens are capable of directly activating synovial cells and inducing the secretion of chemokines, cytokines and proteinases after binding to MHC class II molecules on synovial cells (19, 23, 24). These findings suggest that superantigen-mediated signaling through MHC class II molecules represents a novel mechanism for inflammatory mediators production from synovial cells.

The present results clearly demonstrated that stimulation of synovial cells with bacterial superantigen SEA induced the secretion of a metalloproteinase, stromelysin, which contributes to the articular destruction in RA. Failure of SEA-mediated MMP-3 secretion from IFN-γ untreated synovial cells indicates that SEA binding to MHC class II molecules is necessary for MMP-3 secretion. Studies using the protein synthesis inhibitor, cycloheximide, demonstrated that protein synthesis is also required for SEA-mediated MMP-3 secretion after binding to MHC class II molecules on synovial cells. The secretion of MMP-3 from synovial cells is probably regulated by inflammatory cytokines such as IL-1 and TNF (21). However, the use of neutralizing antibodies against these cytokines in the present study demonstrated that MMP-3 secretion is the direct effect of SEA rather than being mediated by these cytokines.

In contrast, the results of gelatin zymography showed that the secretion of gelatin degrading MMPs was not influenced by SEA treatment. Although the precise mechanisms of articular destruction are not fully understood, rheumatoid synovial fluid contains an excess amount of MMPs. Among these MMPs, stromelysin (MMP-3) is believed to be a key enzyme inducing articular destruction. In this regard, stromelysin is capable of degrading collagens, laminin and fibronectin, and activating other MMPs by cleavage of propeptide domain (9, 10).

More recently, Mehindate et al. (23) reported that SEA induces collagenase gene expression in human synovial fibroblasts. Collagenase is secreted in a latent zymogen form (proenzyme) requiring processing and conversion to the active form by several proteinases and stromelysin (9). The results of these early studies, combined with those of the present study suggest that SEA-mediated stromelysin secretion plays an important role in the perpetuation of synovial inflammation . This effect is mediated directly by stromelysin enzymatic activity and indirectly through the activation of other MMPs secreted by synovial fibroblasts.

Synovial fibroblasts express high levels of MHC class II molecules *in vivo*, as demonstrated recently by Boots et al. (24). Furthermore, MHC class II-mediated signal transduction by superantigen may trigger synovial inflammation in RA. Our data implicate the secretion of superantigen-mediated MMP-3 as a critical event in the process that causes articular destruction in inflammatory joint diseases.

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