Autocrine Induction of Collagenase by Serum Amyloid A–Like and β_2-Microglobulin–Like Proteins

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Two autocrine proteins of 14 and 12 kilodaltons that induce the synthesis of rabbit fibroblast collagenase were identified. The proteins were purified from serum-free culture medium taken from rabbit synovial fibroblasts stimulated with phorbol myristate acetate. The amino-terminal sequences of the 14- and 12-kilodalton species were ~60 to 80 percent homologous with serum amyloid A and β_2 microglobulin, respectively. The polyacrylamide gel–eluted proteins retained the ability to induce collagenase synthesis in rabbit and human fibroblasts. These autocrine proteins may provide a means to modulate collagenase synthesis in normal remodeling as well as in inflammation and disease states.

COLLAGENASE IS THE ONLY ENZYME active at neutral *p*H that can degrade collagen (*1, 2*). This enzyme is a major secretory product of fibroblasts stimulated with interleukin-1 (IL-1), tumor necrosis factor (TNF), phorbol esters, and crystals of monosodium urate monohydrate (*1, 2*). The degradative ability of collagenase is most evident in the synovium of patients with inflammatory and proliferative rheumatoid arthritis (*2*). Collagenase production by synovial fibroblasts (synovial cells) that line the joint is responsible for the extensive joint destruction seen in this disease, whereas collagenase produced by chondrocytes plays a major role in the connective tissue destruction seen in osteoarthritis (*1, 2*). We studied the mechanisms controlling induction of collagenase in a model system of rabbit synovial fibroblasts cultured in vitro. Within 4 to 6 hours after addition of a stimulus, there is an increase in collagenase mRNA in the cell (*3*). This is followed by the appearance of collagenase protein in the culture medium (*3*). When these cells are stimulated with the tumor promoter phorbol myristate acetate (PMA) or crystals of monosodium urate monohydrate, they produce autocrine proteins that, by themselves, can act on the fibroblasts to stimulate collagenase production (*4*). These autocrine proteins may represent a mechanism for modulating collagenase synthesis. Now we report the partial amino acid sequence of these proteins, demonstrate their similarity to human serum amyloid A (SAA) (*5*) and human β_2 microglobulin (β_2M) (*6*), and show that they stimulate collagenase production.

We studied the proteins present in the culture medium of either control cells or cells treated with PMA for 48 hours (Fig. 1A). Treatment with PMA induced major proteins of ~57 kD, which correspond to collagenase and a related proteinase, stromelysin (*7*). There were also major proteins induced of ~15 kD or less; it is these proteins that stimulate collagenase synthesis, as measured by immunoprecipitation of [^{3}H]collagenase from culture medium with monospecific antibody (*4, 8*).

Autocrine proteins were purified from serum-free conditioned medium taken from cells stimulated with PMA ($10^{-8}M$; 0.01 μg/ml). The purification scheme (*4*) is a two-step procedure: isoelectric focusing with ampholytes [isoelectric point (*p*I) 3.5 to 10], and subsequent chromatography over a Beckman TSK high-performance liquid chromatography (HPLC) gel filtration column in phosphate buffer. Collagenase-inducing activity was associated with two proteins, of less than 15 kD, and each with a *p*I of 5 or 8 (*4*). When these small molecular size proteins of *p*I 5 were purified and analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) and silver staining, two species were seen, 14 and 12 kD (Fig. 1B) (*9*).

Each protein was electroblotted onto a nylon membrane [polyvinylidene difluoride (PVDF); Immobilon] for amino acid sequencing (*10*). Computer searching of the data base revealed that the first 25 and 18 NH_2-terminal amino acids of the 14-kD protein with a *p*I of 5 or 8 showed ~60% identity with human serum amyloid A, also known as SAA (Fig. 2A) (*5*). Met^{17} and Met^{24}, which are invariant in amyloid of all species, are conserved. The 15 NH_2-terminal amino acids of the 12-kD protein with a *p*I of 5 were also sequenced (Fig. 2B) and were ~80% identical to human β_2 microglobulin (*6*). Until we determine the complete amino acid sequence of these proteins, we will refer to them as SAA-like and β_2M-like.

To demonstrate that these proteins were capable of collagenase induction in rabbit synovial fibroblasts, they were eluted from SDS-polyacrylamide gels (*11*) and tested for biologic activity (Fig. 3A). The gel was divided into four fractions, each of which was tested for bioactivity. Only fraction four, which contained proteins of less than 14 kD, induced collagenase. A positive control of cells treated with IL-1 was included. The autocrine proteins eluted from the gel were as effective as IL-1 in inducing collagenase. We next determined which species of protein could induce collagenase (Fig. 3B). The HPLC-purified proteins and the gel-separated and eluted 12- to 14-kD proteins (1 to 2 μg/ml) induced collagenase synthesis.

We determined whether exogenous human SAA and human β_2M (Sigma) could stimulate collagenase production in rabbit

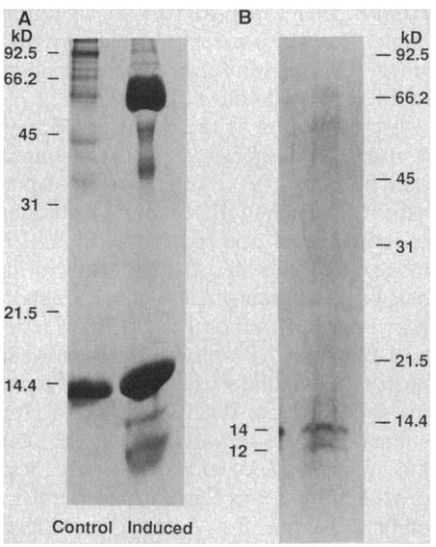

Fig. 1. SDS-PAGE of proteins in culture medium of PMA-stimulated rabbit synovial fibroblasts. (**A**) Monolayer cultures, established from the synovium of young New Zealand White rabbits (*4, 8*), were passaged in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS; Gibco). Cells were grown to confluency in culture dishes (150 mm in diameter), washed three times in Hanks balanced salt solution to remove traces of serum, and then cultured for 48 hours in DMEM supplemented with 0.2% lactalbumin (DMEM-LH) and PMA ($10^{-8}M$). Control cultures received only DMEM-LH. After 2 to 3 days, the medium was tested for collagenase activity (*1, 8*) and medium from collagenase-producing cells was harvested as "induced medium." The proteins were subjected to electrophoresis and silver-stained. (**B**) Silver-stained gel of purified proteins with collagenase-inducing activity. These autocrine proteins were purified as described in (*4*).

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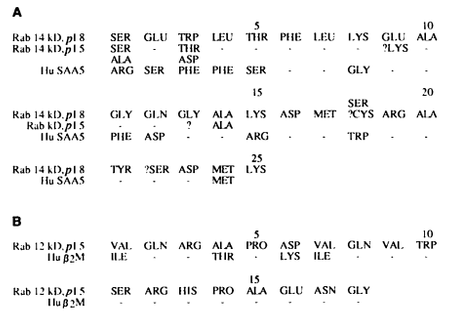

Fig. 2. NH_2-terminal amino acid sequence of collagenase-inducing proteins. Purified rabbit proteins (*4*) were separated by SDS-PAGE with a 15% acrylamide gel (*4, 8*). Approximately 10 μg of protein were electroblotted onto PVDF (Immobilon) and visualized by staining with Coomassie brillant blue (*9*). Each protein was isolated and microsequenced (*9*). (**A**) NH_2-terminus of protein *p*I 5 and 8, 14 kD. (**B**) NH_2-terminus of protein *p*I 5, 12 kD. Sequences were compared with those for human SAA (*5*) and human β_2M (*6*).

synovial fibroblasts (Fig. 3C). The ability of exogenous SAA (*12*) and β_2M (Sigma) to induce collagenase was compared to that of PMA, a stimulus as effective as IL-1 (*1*). Human SAA and β_2M (0.3 to 30 μg/ml) induced collagenase synthesis, whereas bovine serum albumin (BSA) at concentrations of 30 μg/ml did not.

A combination of the rabbit proteins, *p*I 8, induced collagenase secretion of human skin fibroblasts in a concentration-dependent manner (Fig. 4). Nanomole amounts of protein were almost as active as IL-1 in inducing collagenase of secretion from human cells, suggesting a lack of species specificity. When the rabbit proteins were tested on rabbit cells, 10 ng of purified autocrine proteins per milliliter induced collagenase to the same extent as PMA stimulation (*13*).

Finally, we tested whether a cDNA clone for human SAA could hybridize with mRNA from control and PMA-stimulated rabbit cells (Fig. 5). A cDNA probe from human SAA (*5*) hybridized to a 0.6-kb mRNA, the size of the mRNA for SAA (*5*), from PMA-stimulated rabbit cells more strongly than to mRNA from control cells (*14*). We also hybridized these mRNAs with cDNA probes from human and mouse β_2M. Although the human cDNA hybridized to human mRNA from synovial cells, neither the human (*6*) nor mouse (*15*) probes hybridized with rabbit mRNA, even under conditions of reduced stringency (*16*). Thus, the overall homology among rabbit, mouse (*12*), and human (*6*) β_2M mRNAs is insufficient for cross-species hybridization.

Various cytokines can induce collagenase synthesis by fibroblasts (*1*). The monocyte product IL-1, TNF (*11, 17*), substance P from neural tissue (*18*), epidermal growth factor (*19*), fibroblast growth factor (*20*), and platelet-derived growth factor (*21*) can increase collagenase synthesis. In addition, corneal epithelial cells produce a cytokine (20 kD by gel filtration) that will stimulate collagenase synthesis by corneal fibroblasts (*22*), and synovial fibroblasts synthesize a small molecular size protein (also unidentified) that induces collagenase production in chondrocytes (*23*). To this list, we can now add SAA-like and β_2M-like proteins, which unlike the others, can behave as autocrines. Their function, however, is not confined to an autocrine role, for human SAA, produced by the liver and purified from serum (*12*), can stimulate collagenase synthesis by rabbit fibroblasts, and rabbit proteins were active on human cells (Fig. 4).

The ability of these SAA-like and β_2M-like proteins to induce collagenase was somewhat unexpected. However, β_2M (1 to 2 μg/ml) isolated from rat calvariae increases bone resorption (*24*). Originally named "bone-derived growth factor," this protein was shown by NH_2-terminus amino acid sequencing and RNA gel blot analysis to be identical to β_2M. In addition, β amyloid, a 42-residue peptide that is the product of a 3.2- to 3.7-kb transcript and that shows no similarity in amino acid sequence with the first 25 NH_2-terminal residues of the SAA-like protein we describe, inhibits serine proteases (*25*). Thus, we can now assign possible functions for the abundant but poorly understood amyloid proteins.

β_2M is present in cells and tissues throughout the body, and at least in the mouse, it is encoded by a single gene (*12*). In contrast, SAA was thought to be exclusively produced by the liver (*26*). Recent studies, however, detected SAA and mRNA in a number of extrahepatic sites, including macrophages, but fibroblasts were not mentioned (*26*). In our system, fibroblasts are the source for abundant quantities of SAA (Figs. 1A and 5A), and there are two species of protein produced, one with a *p*I of 5 and one with a *p*I of 8. We do not yet know whether these differences are transcriptional or reflect post-translational modifications, although several studies suggest that there is more than one gene for SAA (*27*).

The mechanisms by which these autocrines induce collagenase synthesis is unclear. Possibly, that they act on fibroblasts via specific receptors. Possibly, too, addition of an exogenous stimulus first induces these proteins, which then effect an increase in collagenase mRNA. Indeed, an SAA gene is inducible with IL-1 (*28*). Perhaps the autocrines are induced in fibroblasts by the products of activated macrophages (*29*).

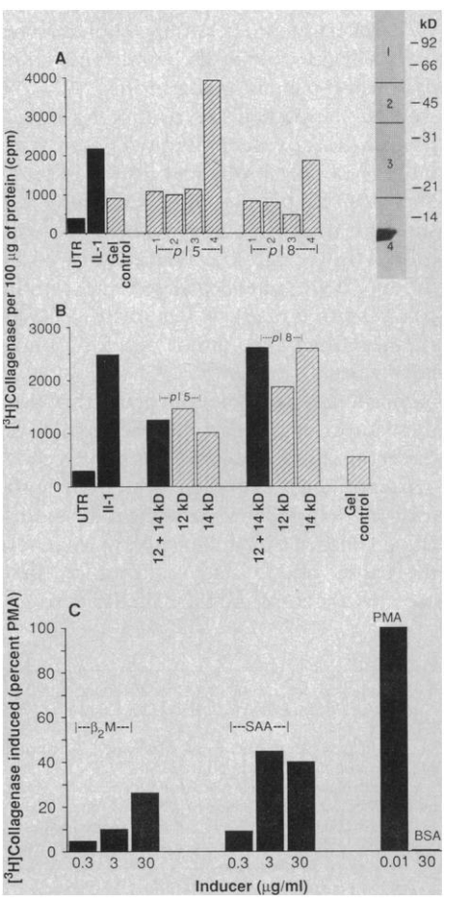

Fig. 3. Ability of SAA-like and β_2M-like proteins to induce collagenase. The purified proteins (*4*) were separated by SDS-PAGE and eluted from the gel (*11*). After dialysis versus ammonium bicarbonate and lyophilization (*4*), the proteins were reconstituted at 1 to 2 μg/ml and tested for their ability to induce collagenase. (**A**) Localization of collagenase-inducing activity to proteins of less than 14 kD. The gel was sliced as shown, processed, and tested for collagenase induction, as was a nonprotein containing portion of the gel (Gel control). The black bars indicate the negative control of untreated cells and a positive control of cells treated with IL-1 (60 ng/ml). (**B**) Collagenase-inducing activity of the gel-eluted 12- and 14-kD proteins. The black bars indicate the negative control of untreated cells and the positive controls of cells treated with IL-1 (60 ng/ml) or with the autocrine proteins before elution from acrylamide gel. (**C**) Ability of exogenous human SAA (*11*) or β_2M (Sigma) to induce collagenase. Various concentrations of human SAA or β_2M were compared to that of a positive control, PMA (0.01 μg/ml), or a negative control, BSA (30 μg/ml). For collagenase induction, we measured the ability of rabbit protein to stimulate the biosynthesis of [^{3}H]collagenase. Purified proteins were added to cultures of rabbit synovial fibroblasts in leucine-free medium along with [^{3}H]leucine (25 μCi/ml). After incubation at 37°C for 30 hours, radiolabeled collagenase was immunoprecipitated from the culture medium with monospecific antibody (*4, 8*). [^{3}H]Collagenase was quantified by SDS-PAGE, autoradiography, and measuring the counts per minute of the immunoprecipitated collagenase (*4, 8*). All experiments were performed a minimum of three times. Hatched bars, eluted from gel.

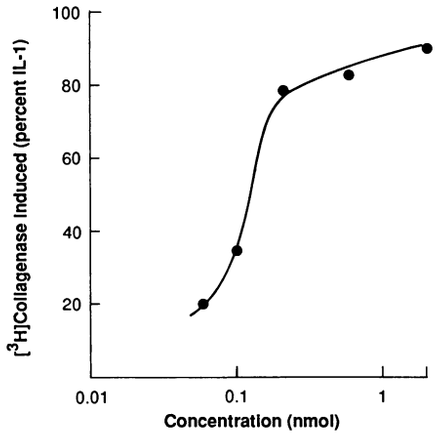

Fig. 4. Concentration dependence of rabbit proteins on collagenase induction of human skin fibroblasts. Various concentrations of purified 12-kD and 14-kD proteins, *p*I 8, were tested for their ability to induce collagenase (Fig. 2). IL-1 (60 ng/ml) represents a positive control.

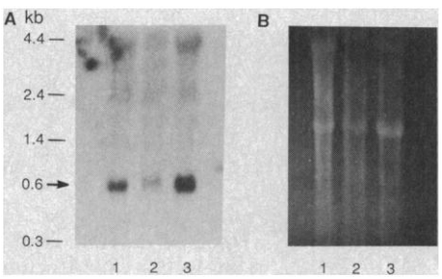

Fig. 5. RNA blot analysis of rabbit mRNA hybridizing with a human SAA probe. Whole-cell RNA (25 μg) from untreated control or rabbit fibroblasts stimulated with PMA for 48 hours was separated on a 1% agarose gel containing 2.2*M* formaldeyhyde (*16*). After electrophoresis in phosphate buffer (*16*), the gel was transferred to a GeneScreen plus filter (Du Pont, Biotechnology Systems) and hybridized with a [^{32}P]deoxycytidine triphosphate–oligolabeled cDNA probe for human SAA (*5*) under stringent conditions (*16*). Lanes 1 and 3, RNA from PMA-stimulated cells; lane 2, RNA from control cells. (**A**) Hybridization with human SAA probe and (**B**) total cellular RNA stained with ethidium bromide.

Alternatively, they may amplify the signal for collagenolysis from low levels of Il-1, TNF, or substance P. We suggest that these proteins may play a role in modulating connective tissue breakdown in both normal and disease states.

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14. This increased hybridization is not an artifact of gel-loading for three reasons. (i) Figure 5B shows that approximately equal amounts (25 μg) of whole-cell RNA were loaded in each lane; (ii) when this same blot was probed with a cDNA clone for stromelysin-proactivator (*3*, *7*), a metalloproteinase whose transcription is increased by PMA (*3*), large increases in stromelysin mRNA were seen in lanes 1 and 3, which contain RNA from PMA-stimulated cells (*3*); and (iii) Fig. 1A shows increased proteins corresponding in molecular size to SAA-like proteins in PMA-stimulated cells compared to control cells.
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A cAMP-Regulated Chloride Channel in Lymphocytes That Is Affected in Cystic Fibrosis

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A defect in regulation of a chloride channel appears to be the molecular basis for cystic fibrosis (CF), a common lethal genetic disease. It is shown here that a chloride channel with kinetic and regulatory properties similar to those described for secretory epithelial cells is present in both T and B lymphocyte cell lines. The regulation of the channels by adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase in transformed B cells from CF patients is defective. Thus, lymphocytes may be an accessible source of CF tissue for study of this defect, for cloning of the chloride channel complex, and for diagnosis of the disease.

AN UNDERLYING MOLECULAR BASIS for CF involves an unusual Cl^- selective channel with distinctive properties in the apical membrane of the epithelial cells (*1–6*). Activation of the Cl^- channel by cAMP-dependent phosphorylation is defective in CF, implying an altered phosphorylation of the ion channel or of a regulatory protein or an altered response to the phosphorylation. Our observations in lymphocyte cell lines initiated a specific study of whether the CF-affected Cl^- channel also resides in these cells.

We first used Jurkat E6-1 human T lymphocytes (*7*). Single-channel Cl^- currents were recorded by the extracellular patch-clamp technique (*8*) from inside-out and cell-attached patches. Activity of the Cl^- channel was first identified by excising

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