Processing of Precursor Interleukin 1β and Inflammatory Disease*

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The processing of precursor interleukin 1β (IL1β) by elastase, cathepsin G, and collagenase, the major proteases released at sites of inflammation, was investigated using recombinant pro-IL1β. Each of these proteases cleaved the 31-kDa inactive precursor to a form similar in size and specific activity (>10⁶ units/mg) to the 17-kDa mature protein isolated from activated monocytes. Elastase, collagenase, and cathepsin G cleaved the IL1β precursor at distinct sites which are amino-terminal to the monocyte-processing site, Ala-117 (Cameron, P., Lumjico, G., Rodkey, J., Bennett, C., and Schmidt, J. A. (1985) J. Exp. Med. 162, 790–801). Amino-terminal sequencing of the products of digestion by elastase and cathepsin G determined that resultant active IL1β proteins contained an additional 13 or 3 amino acids relative to mature IL1β. Synovial fluid collected from patients with inflammatory po-
ticles produced by synovial cells and chondrocytes, degranulation of basophils and eosinophils, and potentiation of neutrophil activation. IL1 is therefore considered to be a key mediator of the inflammatory process (for a review see Refs. 5 and 6). Two different genes for IL1 have been cloned from at least three species and are known to correspond to the two different pl forms of the protein, pl 5 and 7, or IL1α and β, respectively (7–13). Both proteins are synthesized as large precursor proteins of 31 kDa and are processed to the 17-kDa mature form upon secretion (2, 14, 15). In cultured monocytes and monocyte-like cell lines, processing of IL1 is not required for secretion (2–4), and a significant portion of the IL1 that is secreted from activated monocytes is often released as the 31-kDa precursor (2, 3). In fact, the precursor and mature forms of IL1β are released with identical kinetics, suggesting that the two proteins are sec-
tinated via the same mechanism (2).

The IL1β precursor is as biologically active as the mature IL1α protein (16) whereas the IL1β precursor is inactive and processing is required for biological activity (16–18). It has been suggested that IL1β must be processed at or near the site determined to be the amino terminus of morucy-derived 17-kDa IL1β (Ala-117) to exhibit full biological activity (1, 17, 18), and therefore the processing of IL1β may be accomplished via a specific enzyme encoded by the IL1-producing cell. During in uitro experiments using cultured monocytes, the processing of IL1β to 17 kDa is found to occur concomitant with secretion (2), and further studies have shown that the processing activity is associated with the IL1-producing cell (2, 18, 19). In viva, however, it is not known whether the unprocessed IL1β precursor which is released can be processed to an active form after secretion.

We recently purified the 31-kDa IL1β precursor from recombinant Escherichia coli expressing the protein (17). The recombinant precursor did not exhibit full biological activity until treated with chymotrypsin, which generated a 17-kDa protein with 3 additional amino acids at the amino terminus compared to mature IL1β isolated from monocytes (17). This in uitro-generated protein had a specific activity nearly identical to the specific activity of the native protein (17), sug- gesting the possibility that enzymes other than the monocyte-

processing enzyme(s) could produce biologically active mature IL1β.

The tissue degradation that accompanies many inflamma-
tory conditions, such as sarcoidosis and rheumatoid arthritis, is thought to be a direct result of the many proteases that are released at these sites, in particular the neutrophil-derived elastase and cathepsin G, and the synovial cell-derived collagenase (20, 21). In this paper we demonstrate that these proteases can process precursor IL1β to form which are 17–18 kDa and have specific activities comparable to that demonstrated for the monocyte-derived mature IL1β. We also...
show that biological fluids collected from inflammatory sites can provide similar processing activity, suggesting that monocyte convertase (19) may not be exclusively responsible for the conversion of inactive precursor IL1β to the biologically active form(s) in vivo.

MATERIALS AND METHODS

Proteases and Protease Activities—Human leukocyte elastase was obtained from two different commercial sources (Elastin Products and Sigma); pancreatic elastase was obtained from Sigma. Elastase activity was determined according to Bieth et al. (22) using the peptide substrates methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide or succinyl-Ala-Ala-Ala-p-nitroanilide. The amount of p-nitroanilide was calculated from the change in absorbance at 410 nm. A unit was defined as 1 μm of p-nitroanilide liberated per min by 1 ml of lavage fluid. Lavage fluids were diluted 1:10 to 0.5 ml final volume for assay.

Cathepsin G was obtained from Elastin Products. Cathepsin G activity was measured according to Barrett at 23 to 25 °C using the peptide substrate N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide in an assay volume of 0.5 ml. Unit definition was the same as that for elastase. Lavage fluids were diluted as for elastase activity determination.

Because of the concern that elastase preparations could be contaminated with cathepsin G, commercial lots of pancreatic elastase and cathepsin G were tested on both the elastase and cathepsin G peptide substrates described above. We used pancreatic elastase rather than leukocyte elastase because the two cleave IL1β precursor in an identical fashion but only the former is inhibited by the commercial inhibitor elastatinal. Both the elastase and cathepsin G substrates were cleaved with pancreatic elastase; however cleavage of both substrates was completely inhibited with 90 μg/ml elastatinal, an inhibitor specific for pancreatic elastase (24). In contrast, cathepsin G cleavage of its specific substrate methoxysuccinyl-Ala-Ala-Pro-Phe-p-nitroanilide was not affected by elastatinal, as expected (24). Thus, the elastase cleavages observed are not due to contaminating cathepsin G. In addition, cleavage of precursor IL1β to 17-18 kDa products with either protease (see below) was completely inhibited by phenylmethylsulfonyl fluoride, indicating that they did not arise from contaminating proteases outside of the serine protease family.

Two different kinds of collagenase were used: bacterial collagenase from Clostridium (Sigma) and human-derived collagenase from HES-9 fibroblasts treated with conditioned medium from lipopolysaccharide-stimulated P388D1 cells. Collagenase activity was assayed following the method of Caswton and Barrett (25) using 55°-labeled rat skin, acid-soluble type I collagen. One unit of activity was defined as the digestion of 10 μg of collagen in 16 h.

Purification, Processing, and Immunoblot Analysis of Pro-IL1β—The purification and characterization of recombinant precursor IL1β has been described in some detail earlier (17). Purified recombinant precursor was treated with commercial proteases or lavage fluids as described in the figure legends. Immunoblot analysis on the resultant products was performed as described previously (17) using a polyclonal antiserum which reacts with mature IL1β.

EL4 Biological Assay—The specific activity of precursor IL1β or protease-treated precursor IL1β samples were determined in the EL4 biological activity assay (26). This assay measures the ability of IL1 to stimulate the production of interleukin 2 in a cultured EL4 murine T-cell line. IL1 dose-dependent interleukin 2 production is quantitated by following the incorporation of tritiated thymidine into an interleukin 2-dependent mouse cytotoxic T-cell line as described previously (26). Samples assayed in duplicate and/or at different times typically exhibited specific activities within 50%.

Synovial Fluids—Synovial fluids were obtained by aspiration of affected knee joints and were clarified by centrifugation and frozen as described previously (27).

Bronchoalveolar Lavage—Bronchoalveolar lavage fluid was obtained during bronchoscopy from the right middle lobe of patients or volunteers described in the figure legend. Sixty ml of sterile saline solution was instilled with a syringe through the bronchoscope into the lobe and immediately suctioned back into the same syringe. This procedure was repeated three times. The total yield of bronchoalveolar lavage fluid obtained was typically 120-190 ml from a total 240 ml of fluid instilled.

Amino-terminal Sequencing—The site of cleavage by elastase, cathepsin G, and lavage fluid from the sarcoid patients on precursor IL1β was determined by sequencing the amino terminus of each of the resultant, stable carboxy terminal-derived fragments. Precursor IL1β was incubated with protease or lavage fluid as described in the figure legends. The digests were fractionated by reversed-phase high-performance liquid chromatography. The digest was made 0.1% in trifluoroacetic acid and chromatographed on a Brownlee RP500 column (5-μm particle size, C-8, 1 × 250 mm) equilibrated in 24% acetonitrile, 0.1% trifluoroacetic acid at a flow rate of 50 μl/min. The column was developed with a linear gradient to 60% acetonitrile, 0.1% trifluoroacetic acid over 45 min. The absorbance of the effluent of the column at 214 and 280 nm was monitored with a Hewlett-Packard model 1040A diode array detector. Each digest gave a major peak which eluted between 20 and 26 min. This peak was collected manually, lyophilized, and the polypeptide redissolved in 0.1% SDS. The polypeptides were subjected to automated Edman degradation in an Applied Biosystems model 470A gas-phase sequencer. The resultant phenylthiohydantoin derivatives were identified and quantitated by reversed-phase high-performance liquid chromatography.

RESULTS

Cleavage of IL1β Precursor to Biologically Active Forms by Proteases Present at Inflammatory Sites—We have previously described the purification and biological characterization of recombinant precursor IL1β (17). The 31-kDa recombinant precursor has a specific activity of less than 10² units/mg in the EL4 IL1 activity assay compared to 5 × 10³ units/mg for 17-kDa mature IL1β from monocytes or from recombinant E. coli (28). Under conditions where the 31-kDa precursor is cleaved to produce a 17-kDa COOH-terminal fragment, the recombinant protein gains full biological activity. The activity of the precursor is therefore an intrinsic property of the protein and not an artifact of expression, isolation, or assay conditions (17).

Cathepsin G, collagenase, and elastase are the three major proteases produced at sites of inflammation (21). We have used the recombinant IL1β precursor in proteolysis experiments to investigate the processing of precursor IL1β by these proteases. The immunoblot shown in Fig. 1 demonstrates that elastase (lanes 5 and 6), collagenase (lane 3), and cathepsin G (lane 1) cleaved the IL1β precursor to smaller forms which migrate on SDS-polyacrylamide gel electrophoresis with apparent sizes ranging from 17 to 18 kDa. The 18-kDa leukocyte elastase-generated product was converted to a smaller 17-kDa form upon further addition of enzyme (compare lanes 5 and 6) or upon prolonged incubation (data not shown). In each case, greater than 50% of the precursor was stably processed upon incubation with protease. Because the antiserum used in these experiments reacts with epitopes on mature IL1β, the proteolytically derived proteins represent COOH-terminal fragments of the precursor and contain the mature, active region of the protein.

The processing sites for cathepsin G and elastase were determined by sequencing the amino termini of the products shown in Fig. 1. The product of collagenase digestion could not be sequenced due to interfering contaminant proteins.) The results are summarized in Fig. 2. Cathepsin G and elastase cleave precursor IL1β after amino acids Tyr-113 and Ile-103, respectively, generating proteins which have 3 and 13 additional residues at the amino terminus compared to the mature IL1β isolated from lipopolysaccharide-activated monocytes. These cleavage sites are consistent with the known specificities of these enzymes, and were reproduced using proteases obtained from several commercial sources (see “Materials and Methods”). Both pancreatic and leukocytic elastases gave the same cleavage pattern. In the case of collagenase, similar results were obtained using either the human enzyme or the Clostridium equivalent, and the larger size of the product indicates that these enzyme preparations are not contaminated with cathepsin G or elastase.
6320 Processing of Precursor IL1β by Cathepsin G, Collagenase, and Elastase. Purified recombinant precursor IL1β (1 μg) was incubated with cathepsin G (1.0 unit), collagenase (10 units), and leukocyte elastase (10 or 100 units) in a final volume of 20 μl of phosphate-buffered saline. After incubation at 37 °C for 15 min, the reactions were terminated by the addition of SDS-loading buffer and the samples were electrophoresed on 15% SDS-polyacrylamide gel electrophoresis and then analyzed by Western analysis as described previously (17). Samples of precursor IL1β and mature IL1β were treated identically without added protease as controls and are indicated.

FIG. 1. Processing of precursor IL1β by cathepsin G, collagenase, and elastase. Purified recombinant precursor IL1β (1 μg) was processed with cathepsin G, elastase, or bronchoalveolar lavage (BAL) cathepsin G. It should be noted that we did not detect any cathepsin G activity in our elastase preparations (see "Materials and Methods"). However, in control experiments using peptide substrates, we observed that the cathepsin G substrate could be cleaved by elastase. Therefore it is not unexpected that this enzyme would cleave precursor IL1β at the cathepsin G-cleavage site.

The IL1 activity of each of the four processing products was tested in the EL4 interleukin 2-induction assay (26) and compared to both precursor and mature IL1β. The results are shown in Table I. Although slightly larger than the monocyte-derived 17-kDa protein, the specific activity of each of the proteolytically derived proteins approximated the monocyte-derived product. Cathepsin G, collagenase, and elastase processed the inactive IL1β precursor to forms which exhibit biological activity greater than or equal to 10⁶ units/mg, compared to 5 x 10⁶ units/mg for mature IL1β (17, 28). Thus processing of the inactive IL1β precursor by any of the three proteases increased the specific activity of the IL1β molecule by more than six orders of magnitude, generating comparably active IL1β proteins.

Other enzymes have been suggested to process IL1 in vivo, e.g. trypsin or plasmin (29). Both trypsin and plasmin cleave the protein further away from the monocyte-processing site than the proteases studied above. Trypsin cleaves the precursor after Lys-76 (18), generating a molecule with increased IL1 activity, but not a fully active IL1β (17). Although the resultant trypsin product is only 25 amino acids longer than the larger elastase-generated product, this protein is 10,000-fold less active. Together these data suggest that the region responsible for down-modulating the activity of the IL1β precursor resides predominantly within the residues from methionine-77 to isoleucine-103.

Processing of the IL1β Precursor by Synovial Fluid—Elastase, cathepsin G, and collagenase are present at substantially elevated levels in arthritic joints (30–33). Since fluids from diseased joints are found to have increased levels of IL1 activity (34–36), we examined the possibility that precursor IL1β could be processed in the synovial fluid isolated from diseased joints of arthritic patients.

Purified recombinant IL1β precursor was added to the synovial fluid obtained from several patients diagnosed with a variety of different disease states (27). As shown in Fig. 3, the synovial fluid from a patient diagnosed with severe inflammatory polyarthritis cleaved the exogenously added IL1β precursor to several smaller fragments, the most prominent of which was 17 kDa (Fig. 3, lane 2). A control sample, obtained from a patient diagnosed with gout exhibited no detectable processing of the precursor (Fig. 3, lane 3). Osteoarthritis patients did not elaborate IL1β processing activity, and one patient diagnosed with septic arthritis demonstrated a barely detectable level of activity (data not shown). When samples were run in the absence of added precursor, no 17-kDa IL1β was detected. The 17-kDa protein which results when the

TABLE I

<p>| Activities of Cleaved and Uncleaved Forms of IL1β |
|-------------|-------|------------------|</p>
<table>
<thead>
<tr>
<th>Sample</th>
<th>Mass</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mature IL1β</td>
<td>17</td>
<td>5.0 x 10⁶ units/mg</td>
</tr>
<tr>
<td>Pre-IL1β</td>
<td>31</td>
<td>&lt;10⁶ units/mg</td>
</tr>
<tr>
<td>+ Elastase</td>
<td>18</td>
<td>0.8 x 10⁶ units/mg</td>
</tr>
<tr>
<td>+ Collagenase</td>
<td>17</td>
<td>1.0 x 10⁶ units/mg</td>
</tr>
<tr>
<td>+ Cathepsin G</td>
<td>17</td>
<td>4.0 x 10⁶ units/mg</td>
</tr>
</tbody>
</table>

*IL1 activity determined by the EL4 assay described previously (20).
Processing of Precursor IL1β

exogenous 31-kDa IL1β precursor was incubated in the synovial fluid of patients with inflammatory arthritis was therefore a product of processing by proteases present in the diseased synovium. The bands seen in lanes 2 and 3 which do not correspond to precursor IL1β or processed products of IL1 were present in all synovial fluid samples tested either in the presence or absence of added recombinant precursor.

Although less than 10% of the added precursor was processed in these experiments, this corresponds to 10–50 ng of IL1β in 20 μl of synovial fluid. This amount of processing activity would, therefore, be more than sufficient to account for the 7–10 ng/ml IL1 which is typically detected in the synovial fluid of rheumatoid arthritis patients (34–36). It is not clear which enzyme(s) is responsible for the observed processing activity. Similarly, we do not know what form or forms of IL1β exist in vivo since we have failed to detect IL1β in synovial fluid either by immunoblotting or immunoprecipitation. However, the amount of IL1 protein predicted by activity assay (34–36) would be below the limit detectable by these methods.

Processing of the IL1β Precursor in Bronchoalveolar Lavage Fluid—As in rheumatoid arthritis, lymphokine production is characteristic of several pulmonary diseases of chronic inflammation, including sarcoidosis (37). In sarcoid patients, the increased number of T-lymphocytes in bronchoalveolar lavage fluid is responsible for the release of a soluble chemotactic factor for peripheral blood monocytes (38). Monocytes from these patients display an increased ability to produce IL1 (39). It was therefore of interest to examine the processing of precursor IL1β in the bronchoalveolar lavage fluid of these patients.

Incubation of the 31-kDa IL1β precursor with lavage fluid of patients with inflammatory pulmonary disease resulted in processing as shown in Fig. 4. No processing of pro-IL1β was detected in lavage samples from healthy control subjects (lanes 3–5 and 9), sarcoid patients with no active disease (lanes 2, 6, and 7), or a lung transplant patient who did not exhibit infection (lane 8). However, the lavage fluid of patients with emphysema or infection following lung transplant processed a fraction of the precursor to an 18-kDa form (Fig. 4B, lanes 10 and 11) which may correspond to either the form generated upon elastase or collagenase treatment (Fig. 1). Both elastase and collagenase activities have been detected in the lavage fluid of patients with severe emphysema. In other experiments (data not shown), we can demonstrate that cleavage of precursor IL1β by elastase is completely inhibited by both α1-antitrypsin and α2-macroglobulin. Thus the processing that we have observed in these lavage fluids corresponds to free elastase and not elastase complexed with protease inhibitors.

More striking, however, was the result obtained with the lavage fluid of sarcoid patients shown in Fig. 4A, lanes 12 and 13. The IL1β precursor was completely processed to 17-kDa by incubation with the lavage fluid of sarcoid patients with active disease. Only one processed product was detectable when pro-IL1β was incubated with the lavage fluid of sarcoid patients, indicating processing by a single, specific protease. These results were confirmed by amino-terminal sequencing of the product shown in lane 12. A single amino terminus was detected which corresponded to processing at Tyr-113, identical to the results obtained with purified cathepsin G and elastase (Fig. 2). To investigate further which enzyme(s) could be responsible for the processing of pro-IL1β in the lavage samples, these samples were assayed for the presence of cathepsin G and elastase. Both samples 12 and 13 contained cathepsin G activity (4.1 and 1.95 units, respectively), but only sample 13 had elastase activity (2.8 units). Based on these results, it seems likely that cathepsin G is responsible for the processing of pro-IL1β in samples 12 and 13.

**DISCUSSION**

We have shown that the IL1β precursor, which is secreted from monocytes in vitro, can be cleaved by extracellular proteases present at inflammatory sites. Not only do collagenase, elastase, and cathepsin G cleave purified IL1β precursor in vitro, but there is evidence for processing activity in the synovial fluids and lung lavages of patients diagnosed with various inflammatory conditions. Specifically in bronchoalveolar lavages we find evidence for processing of exogenous IL1β precursor by both elastase and cathepsin G. Such processing activity is absent from noninflamed tissue fluids.

Several studies have demonstrated that IL1 may induce neutrophil accumulation and possible degradation with subsequent release of elastase and cathepsin G (6, 41–44). IL1 is also known to stimulate the release of collagenase from synovial cells (42). Therefore the results reported here suggest that the fraction of IL1β which is secreted in the active form from activated monocytes at an inflammatory site may induce the release of proteases which we have shown to be competent to process the secreted, inactive 31-kDa IL1β precursor. Additional extracellular processing of IL1β might augment the...
release of proteases and could stimulate further production of IL1 (46), resulting in an amplification of the inflammatory response.

Proof of this model requires isolation and identification of the amino terminus of IL1β released at inflammatory sites to determine if the processing site(s) corresponds to any of the alternative amino termini we have observed. Unfortunately, we have been unable to isolate and sequence IL1β derived in vivo due to the low amounts of protein involved, and there are no published reports presumably for the same reason. Active IL1β from monocytes stimulated in vitro has been sequenced (1) and the 17-kDa protein isolated is probably the product of a monocyte-specific convertase (19). Although monocytes release precursor IL1β in vitro (2), the precursor has not been detected in vivo most likely due to its inactivity, protease sensitivity (17), and/or the lack of precursor-specific antisera. Furthermore, since IL1 is only produced in substantial quantities at inflammatory sites, we might expect from the present studies that most of the IL1β would exist in some processed form.

The suggestion that precursor IL1β may be processed after secretion by any of several proteases present in rheumatoid arthritis, pulmonary sarcoidosis, and emphysema may have important implications in the development of therapeutic agents. In particular, the action of drugs which inhibit the production of IL1 activity from monocytes by interfering with the monocyte-mediated processing of IL1β (19) might not be able to inhibit the production of IL1β activity in vivo. Clearly the development of such inhibitors will help in addressing this question.

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