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Significant accumulations of cathepsin B and prolylendopeptidase in inflammatory focus of delayed-type hypersensitivity induced by *Mycobacterium tuberculosis* in mice☆

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Abstract

To clarify what kinds of proteinases are secreted into the foci of allergic-inflammation involving delayed-type hypersensitivity reaction, we examined the characteristic releases of various proteinases into the foci of *Mycobacterium tuberculosis* (*M. tuber.*)-induced delayed-type allergic-inflammation in mice. The significant activities of cathepsin B and prolylendopeptidase were observed in the washing-fluids of subcutaneous inflammatory foci of *M. tuber.*-induced delayed-type allergic-inflammation, but not *M. tuber.*-induced acute-inflammation. The SDS-resistant complex of cathepsin B and a protein substrate with apparent molecular mass of 74 kDa was observed by Western blot analysis. On the other hand, no significant accumulations of other proteinases, such as matrix metalloproteinases, cathepsin D, and serine proteinases, were determined. CA-074, a specific inhibitor of cathepsin B, suppressed both swelling and cathepsin B activity in the footpad having *M. tuber.*-induced delayed-type allergic-inflammation in vivo. These results suggest that cathepsin B may play an important role in the formation of *M. tuber.*-induced delayed-type allergic-inflammation.
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Keywords: Cathepsin B; Prolylendopeptidase; Delayed-type hypersensitivity; *Mycobacterium tuberculosis*; Inflammation

In inflammatory foci, various proteinases are secreted from cells, such as macrophages, polymorphonuclear leukocytes, fibroblasts, etc., and induce the degradation of protein components in the extracellular matrix. Tissue damages caused by most inflammatory diseases may be due to excessive destruction of extracellular matrix proteins caused by an imbalance following extracellular proteolysis. Previous immunocytochemical studies showed the presence of cathepsins B and D in rhematoid articular cartilage [1,2]. Furthermore, the synovial fluid of rheumatoid arthritis patients has been found to contain significant amounts of cathepsin B and matrix metalloproteinases (MMPs) [3–8]. These proteinases are probably involved in turnover of extracellular matrix proteins in arthritis, since these proteinases are able to degrade extracellular matrix proteins in vitro. Delayed-type hypersensitivity mediated by antigen-specific T-lymphocytes stimulated by antigen participates in various allergic-inflammatory diseases. The antigen-specific T-lymphocytes stimulated in the immune response secrete higher amounts of various cytokines to activate macrophages, granulocytic cells, and lymphocytes [9]. Th1-lymphocytes mainly generate IFNγ and IL-2, and support the activation of macrophages [10–12]. The activated macrophages enhance the inflammatory response in delayed-type hypersensitivity. *Mycobacterium tuberculosis* (*M. tuber.*) is known to be a specific antigen that mediates Th1-lymphocyte-macrophage cooperation system via cytokine excretion to induce typical delayed-type hypersensitivity. In this study,

☆ *Abbreviations:* Z, benzyloxycarbonyl; MCA, methylcoumarylamide; E-64, *N*-(L-3-*trans*-carboxirane-2-carbonyl)-L-leucine-4-amino-butylamine; CA-074, *N*-(L-3-*trans*-propyl-carbamoyl-oxirane-2-carbonyl)-L-isoleucyl-L-proline.

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to clarify what kinds of proteinases are secreted into the foci of allergic-inflammation involving delayed-type hypersensitivity, we examined the activities of various proteinases with synthetic substrates and the identification of molecules of enzymes in the washing-fluids of inflammatory foci of *M. tuber.*-induced allergic-inflammation in mice.

Experimental

Materials. Z-Phe-Arg-MCA, Z-Arg-Arg-MCA, Arg-MCA, Z-Val-Val-Arg-MCA, Boc-Glu-Lys-Lys-MCA, Boc-Phe-Ser-Arg-MCA, Suc-Ala-Ala-Pro-Phe-MCA, Suc-Leu-Leu-Val-Tyr-MCA, Suc-Ala-Ala-Pro-Val-MCA, Suc-Ala-Pro-Ala-MCA, Suc-Ala-Ala-Ala-MCA, Suc-Gly-Pro-Leu-Gly-Pro-MCA, and MOCAc-Pro-Leu-Gly-Leu-A_2pr(Dnp)-Ala-Arg were purchased from Peptide Institute (Osaka, Japan). Pro-Thr-Glu-Phe-*p*-nitro-Phe-Arg-Leu was purchased from Cosmo Bio (Tokyo, Japan). *M. tuber.* H37RA (heat-killed) was purchased from Difco Laboratories (Detroit, Michigan). E-64 [13] and CA-074 [14–16] were kindly supplied by Taisho Pharmaceutical (Saitama, Japan). CLIK-195 was synthesized in our laboratory [17]. Rabbit anti-rat cathepsins B and L were donated by Dr. E. Kominami (Department of Biochemistry, School of Medicine, Juntendo University, Tokyo). Rabbit anti-cathepsin D was purchased from Upstate (Lake Placid, NY). Sheep anti-MMP-1 and rabbit anti-MMP-2 were purchased from Oncogene Research Products (Boston, MA). Goat anti-MMP-3 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All these antibodies can react with mouse proteins. Goat anti-rabbit IgG, rabbit anti-sheep IgG, and rabbit anti-goat IgG conjugated to alkaline phosphatase were purchased from Chemicon (Temecula, CA). Acid soluble protein kit (Aspro-GP) was purchased from Otsuka Pharmaceutical (Tokushima, Japan). All other chemicals were of analytical grade.

Animal treatments. Male BALB/c mice (18–20 g) purchased from Charles River (Yokohama, Japan) were used. They were housed under conditions of 22 ± 2 °C temperature, 55 ± 5% humidity, and 12 h light from 7 a.m. to 7 p.m., and given commercial pellet diet (MF, Oriental Yeast, Tokyo, Japan) and tap water ad libitum. *M. tuber.* induced delayed-type allergic-inflammation; mouse was injected subcutaneously with emulsion (50 μl) consisting of *M. tuber.* (1 mg/ml) and Freund's incomplete adjuvant (1:1) into regiones cervicalis posterior. After 14 days, the same emulsion (50 μl) was re-injected subcutaneously into the focus of inflammation, and after 6, 24, 48, 72, and 96 h, PBS (5 ml) was injected into the allergic-inflammatory focus. After 1 min, the PBS injected was collected using a syringe. The PBS collected was centrifuged at 500*g* for 10 min at 4 °C and the supernatants were used as washing-fluids in following experiments of Enzyme assay. *M. tuber.*-induced acute-inflammation; mouse was injected subcutaneously with the same emulsion (50 μl) into regiones cervicalis posterior. After 6, 24, 48, 72, and 96 h, the washing-fluids were prepared as described above.

Enzyme assay. The activities of Cathepsin L, B or H were measured with Z-Phe-Arg-MCA, Z-Arg-Arg-MCA or Arg-MCA by the method of Barrett and Kirschke [18], and the individual activity of cathepsins B or L on Z-Phe-Arg-MCA was discriminated with CA-074 or CLIK-195, a cathepsin L specific inhibitor [17]. Cathepsin B activity was calculated by the subtraction of Z-Phe-Arg-MCA-hydrolytic activity in the presence of 1 μM CA-074 from that in the absence of CA-074, and cathepsin L activity was calculated by the subtraction of Z-Phe-Arg-MCA-hydrolytic activity in the presence of 1 μM CA-074 and 0.5 μM CLIK-195 from that in the presence of CA-074. The inhibitors were preincubated with enzyme fraction for 5 min at 37 °C in sodium acetate buffer, pH 5.5, containing 8 mM cysteine and 1 mM EDTA, and the mixture was incubated with substrate for 20 min at 37 °C. The reaction was stopped by addition of 100 mM sodium monochloroacetate, pH 4.3. Cathepsin S activity was measured with Z-Val-Val-Arg-MCA according to the method of Bromme et al. [19] in 50 mM Tris–HCl buffer, pH 7, containing 0.5 μM CLIK-195. The hydrolytic activities of Boc-Glu-Lys-Lys-MCA, Boc-Phe-Ser-Arg-MCA, Suc-Ala-Ala-Pro-Phe-MCA, Suc-Leu-Leu-Val-Tyr-MCA, Suc-Ala-Ala-Pro-Val-MCA, Suc-Ala-Pro-Ala-MCA, Suc-Ala-Ala-Ala-MCA, and Suc-Gly-Pro-Leu-Gly-Pro-MCA were determined according to published methods [20–27]. The substrate was incubated with enzyme fraction for 20 min at 37 °C in 20 mM Tris–HCl buffer, pH 7.5. The reactions were stopped by addition of 100 mM sodium monochloroacetate, pH 4.3. The amount of 7-amino-4-methylcoumarin liberated from substrate was monitored fluorometrically with excitation at 370 nm and emission at 460 nm by a fluorescence spectrometer (Hitachi F-2000). One unit of enzyme activity was defined as the production of 1 μmol of 7-amino-4-methylcoumarin per min from 20 μM of substrate. The activities of matrix metalloproteinases were measured with MOCAc-Pro-Leu-Gly-Leu-A_2pr(Dnp)-Ala-Arg by the method of Knight et al. [28] with some modifications. The substrate was incubated with enzyme fraction for 20 min at 37 °C in 20 mM Tris–HCl buffer, pH 7.5, containing 0.1 M NaCl and 10 mM $CaCl_2$ in the presence or absence of 10 mM EDTA. The activity was calculated by the subtraction of the activity in the presence of EDTA from that in the absence of EDTA. The amount of MOCAc-Pro-Leu-Gly liberated from substrate was monitored fluorometrically with excitation at 328 nm and emission at 393 nm by a fluorescence spectrometer (Hitachi F-2000). One unit of activity was defined as the production of 1 μmol MOCAc-Pro-Leu-Gly per min from 20 μM substrate. The activities of acid proteinases were measured with Pro-Thr-Glu-Phe-*p*-nitro-Phe-Arg-Leu by the method of Dunn et al. [29] with some modifications. The substrate was incubated with enzyme fraction for 20 min at 37 °C in 50 mM sodium acetate buffer, pH 4, in the presence or absence of 10 μM pepstatin. The activity was calculated by subtraction of the activity in the presence of pepstatin from that in the absence of pepstatin. The unit of activity was calculated by specific activity of bovine spleen cathepsin D as standard enzyme.

Plasma acid soluble protein level. Plasma acid soluble protein (ASP) level was determined by kit.

INFγ level. The washing-fluids of inflammatory foci of *M. tuber.*-induced allergic-inflammation were 50 times concentrated and then INFγ levels in the washing-fluids were measured by ELISA kit.

Western blot analysis. Ten milliliters of the washing-fluid prepared at 72 h after second injection of emulsion as described above was supplemented with 1 ml of 10% SDS and concentrated to 1 ml. The fluid was applied to a Superdex 75 HR10/30 equilibrated with 20 mM Tris–HCl buffer, pH 7.5. All proteins eluted were collected and the eluents (approximately 14 ml) were concentrated to 500 μl. The gel-filtration fraction was then applied to a Mono-Q HR5/10 equilibrated with 20 mM Tris–HCl buffer, pH 7.5, and the eluents were 5 times concentrated. The eluents concentrated were subjected to SDS–PAGE with a 15–25% polyacrylamide gradient gel in the presence of 5% of 2-mercaptoethanol. SDS–PAGE was performed by the method of Laemmli [30]. The proteins were transferred electrophoretically to an Immobilon transfer membrane (Millipore, Bedford, MA) and immunoblotting was developed with a first antibody for cathepsins B, L, and D, matrix metalloproteinases 1, 2, and 3, respectively, and a corresponding second antibody conjugated alkaline phosphatase. The alkaline phosphatase reaction was performed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma) on the membrane.

Effects of CA-074 and indomethacin on footpad swelling in the M. tuber.-induced allergic-inflammation. Mice were subcutaneously injected with 50 μl of the emulsion consisting of *M. tuber.* (1 mg/ml saline) and Freund's incomplete adjuvant (1:1) into back neck, and after 14 days, the emulsion (30 μl) consisting of saline extract (0.5 mg/ml proteins) of *M. tuber.* and Freund's incomplete adjuvant (1:1) was

subcutaneously re-injected into footpad, and the footpad swelling was measured at 6, 24, 48, and 72 h. CA-074 (50 mg/kg) or indomethacin (5 mg/kg) was intraperitoneally administered 30 min prior to re-injection of the emulsion and at 6, 20, 32, 44, 56, and 68 h after re-injection of the emulsion. Footpad swelling in the *M. tuber.*-induced acute-inflammation in mice was also measured. The emulsion (30 μl) consisting of saline extract (0.5 mg/ml proteins) of *M. tuber.* and Freund's incomplete adjuvant (1:1) was subcutaneously injected into footpad, and the footpad swelling was measured at 6, 24, 48, and 72 h. The volumes of hind paw were measured using a plethysmometer (TK-101, Unicom, Chiba, Japan) and swelling percentage was calculated according to $(V_t - V_n)/(V_n) \times 100$, where V_t expresses the volume of hind paw before injection of *M. tuber.* into footpad, and V_n expresses the volume of hind paw after injection of the antigen. After measurement of footpad swelling at 72 h, the inflammatory foci were excised from footpad and homogenized in a cold 50 mM sodium acetate buffer, pH 5.5, containing 1 mM EDTA with a Dounce tissue grinder (Wheaton Science Products, NJ). After sonication, the homogenates were centrifuged at 21,000*g* for 10 min at 4 °C. Cathepsin B activity in the supernatants was measured with Z-Arg-Arg-MCA as described above.

Results and discussion

Determination of various proteinase activities in the inflammatory foci of M. tuber.-induced delayed-type inflammation

Plasma acid-soluble protein (ASP) levels in the *M. tuber.*-induced allergic-inflammatory mice were determined, and the results are shown in Fig. 1. Plasma ASP, which mainly consists of α1-acid glycoprotein and α1-antitrypsin, is a useful diagnostic marker for inflammatory diseases [31,32]. The plasma ASP levels were time-dependently increased, and the maximum level was observed at 72 h after the second antigen challenge (Fig. 1C), suggesting the induction of a typical delayed-type hypersensitivity reaction. We next examined various proteinase activities in the washing-fluids of

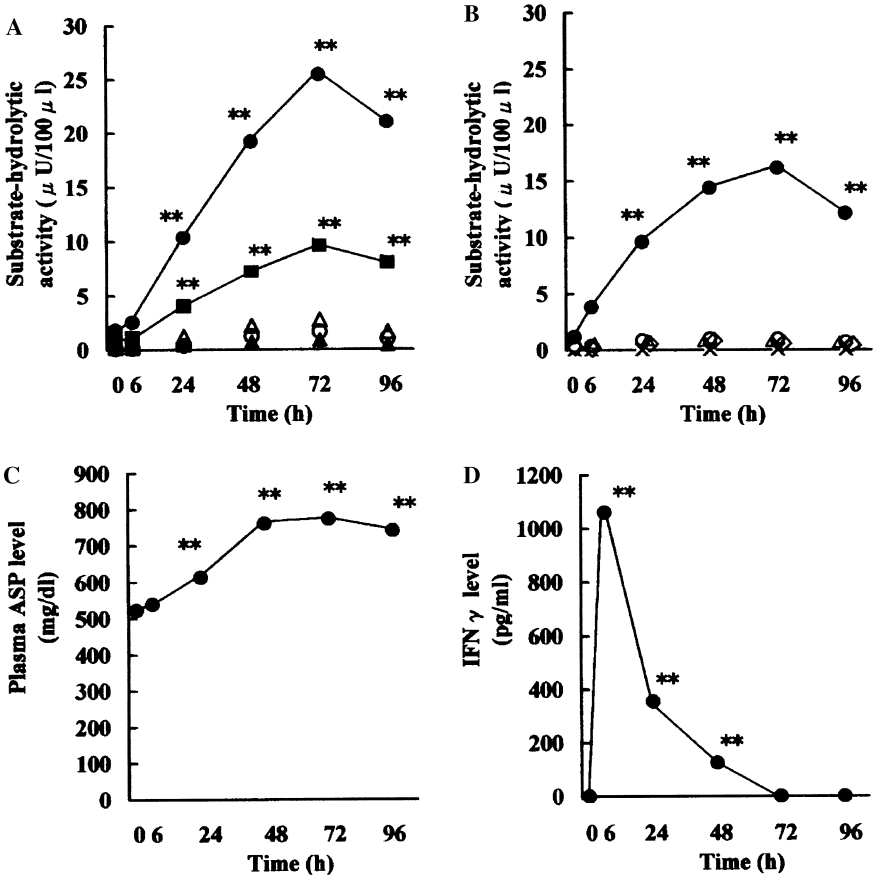

Fig. 1. (A) Time course of the changes of various proteinase activities in the foci of *M. tuber.*-induced allergic-inflammatory mice. ●, Cathepsin B activity by Z-Phe-Arg-MCA ‡. ■, Cathepsin B activity by Z-Arg-Arg- MCA. ▲, Cathepsin L activity by Z-Phe-Arg-MCA ‡. ◯, Cathepsin H activity by Arg-MCA. Δ, Cathepsin S activity by Z-Val-Val-Arg-MCA. ‡, The individual activity was measured by the differential assay using CA-074 and CLIK-195. $^{**}p < 0.01$; significant difference from 0 h group (Student's *t* test). Each value indicates the mean of 4–5 experiments. (B) Time course of the changes of various proteinase activities in the foci of *M. tuber.*-induced allergic-inflammatory mice. ●, Prolylendopeptidase activity by Suc-Gly-Pro-Leu-Gly-Pro-MCA. Δ, The activities of tryptases, plasmin, etc., by Boc-Glu-Lys-Lys-MCA. ◇, The activities of chymases, cathepsin G, etc., by Suc-Ala-Ala-Pro-Phe-MCA. ×, The activities of elastases by Suc-Ala-Ala-Pro-Val-MCA. ◯, The activities of elastases by Suc-Ala-Ala-Ala-MCA. $^{**}p < 0.01$; significant difference from 0 h group (Student's *t* test). Each value indicates the mean of 4–5 experiments. (C) Time course of the changes of ASP levels in the serum of *M. tuber.*-induced allergic-inflammatory mice. $^{**}p < 0.01$; significant difference from 0 h group (Student's *t* test). Each value indicates the mean of 4–5 experiments. (D) Time course of the change of IFNγ level in the subcutaneous foci of *M. tuber.*-induced allergic-inflammatory mice. $^{**}p < 0.01$; significant difference from 0 h group (Student's *t* test). Each value indicates the mean of 4–5 experiments.

subcutaneous non-inflammatory tissues in *M. tuber.*-induced delayed-type allergic-inflammatory mice. Among the proteinase activities measured with various synthetic substrates, the activities of cathepsin B and prolylendopeptidase were significantly high in the washing-fluids of inflammatory foci in the *M. tuber.*-induced delayed-type allergic-inflammaton (Fig. 1A). Consistent with the plasma ASP levels, the activities of cathepsin B and prolylendopeptidase in the washing-fluids of inflammatory foci in the *M. tuber.*-induced delayed-type allergic-inflammation were time-dependently elevated at 6, 24, 48, and 72 h after the second challenge of *M. tuber.* The activities of other lysosomal cysteine proeinases, such as cathepsins L, S, and H, in the washing-fluids of inflammatory foci of the *M. tuber.*-induced allergic-inflammation were weak. Moreover, no significant hydrolytic activities of Boc-Glu-Lys-Lys-MCA, Boc-Phe-Ser-Arg-MCA, Suc-Ala-Ala-Pro-Phe-MCA, Suc-Leu-Leu-Val-Tyr-MCA, Suc-Ala-Ala-Pro-Val-MCA, Suc-Ala-Pro-Ala-MCA, Suc-Ala-Ala-Ala-MCA, MOAc-Pro-Leu-Gly-Leu-A_2pr(Dnp)-Ala-Arg, and Pro-Thr-Glu-Phe-*p*-nitro-Phe-Arg-Leu were observed under the present experimental conditions (Figs. 1A and B). The purification, identification, and characterization of the prolylendopeptidases secreted are important and are now in progress.

We measured IFNγ levels in the washing-fluids of inflammatory foci in the *M. tuber.*-induced allergic-inflammation in mice. A remarkable IFNγ secretion was detected at 6 and 24 h after the second challenge of antigen (Fig. 1D), suggesting the activation of Th1-lymphocytes during the early-stage of the *M. tuber.*-induced delayed-type allergic-inflammation. It was reported that Th1-lymphocytes activated in an immune response caused a delayed-type hypersensitivity reaction [9]. Activated Th-1-lymphocytes have been known to synthesize and excrete cytokines, such as IFNγ, tumor necrosis factor (TNF), and granulocyte monocyte-colony stimulating factor (GM-CSF), which strongly stimulates macrophages [10–12]. The macrophages activated by cytokines mainly participate in the formation of delayed-type allergic-inflammation. The findings of the present study show that cathepsin B and prolylendopeptidase are significantly released into the local inflammatory foci from the macrophages activated by cytokines during the *M. tuber.*-induced delayed-type hypersensitivity.

To find out that cathepsin B and prolylendopeptidase were characteristically accumulated in the inflammatory foci of *M. tuber.*-induced delayed-type allergic-inflammation, the activities of these enzymes in the washing-fluids of subcutaneous inflammatory foci in the *M. tuber.*-induced acute-inflammation were investigated. As a result, no significant activities of these enzymes were observed, suggesting that the secretions of cathepsin B and prolylendopeptidase into inflammatory foci are likely due to the immune response involving delayed-type hypersensitivity, but not acute-inflammation (data not shown).

Identification of proteinases in inflammatory foci in the M. tuber.-induced delayed-type allergic-inflammation

Using Western blot analysis with a specific antibody against cathepsin B, L, and D, matrix metalloproteinases (MMPs)-1, 2 or 3, identification of molecules of these proteinases secreted into the inflammatory foci in the *M. tuber.*-induced delayed-type allergic-inflammation in mice was performed.

The washing-fluid prepared at 72 h after second injection of antigen was treated with 1% SDS to inactivate all enzymes in the washing-fluid. The mixture was

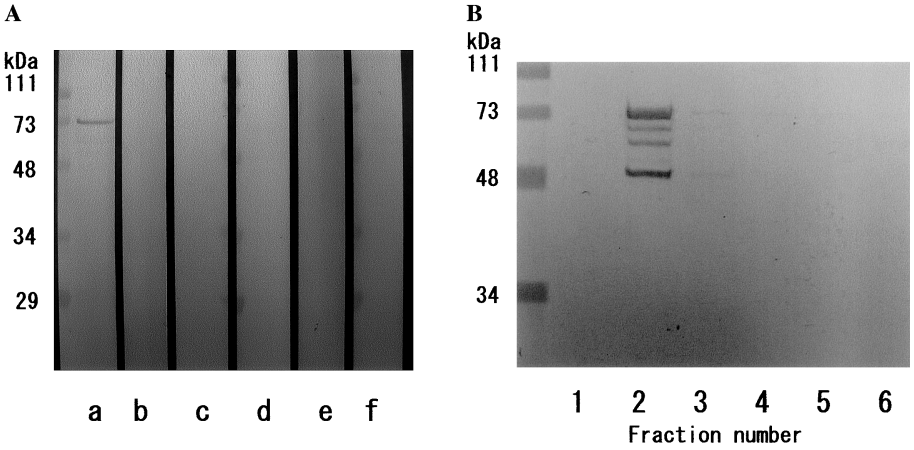

Fig. 2. Identification of molecules of various proteinases in the inflammatory foci in the *M. tuber.*-induced delayed-type hypersensitivity mice by Western blot analysis. (A) Western blot analysis for various proteinases was carried out after a gel filtration chromatography with Superdex 75. (a) Cathepsin B, (b) cathepsin L, (c) cathepsin D, (d) MMP-1, (e) MMP-2, and (f) MMP-3. (B) The proteins eluted from a gel filtration chromatography with Superdex 75 were applied to an ion-exchange column chromatography with Mono-Q. Western blot analysis for cathepsin B was carried out after an ion-exchange column chromatography with Mono-Q.

concentrated and applied to a gel filtration chromatography with Superdex 75 to remove non-specific proteins, which make irregular developments on Western blot analysis. All proteins eluted from a Superdex 75 were concentrated and then subjected to Western blot analysis.

As shown in Fig. 2A, a single molecular band specifically reacted with an anti-cathepsin B antibody was observed at an apparent molecular mass of 74 kDa. It was thought that cathepsin B secreted into the inflammatory foci in the *M. tuber.*-induced delayed-type allergic-inflammation might bind to extracellular proteins, and partially make a SDS-resistant complex with an unknown protein substrate as an active enzyme.

The proteins eluted from the gel filtration using Superdex 75 were then applied to an ion-exchange column chromatography with Mono-Q, and all proteins eluted were subjected to Western blot analysis for cathepsin B. As a result, some degraded products still bound to cathepsin B were observed at apparent molecular masses from 48 to 74 kDa (Fig. 2B). This result obviously indicates that the 74 kDa protein determined on Western blot analysis after gel filtration is a SDS-resistant protein complex consisting of the active form of cathepsin B and its soluble unknown protein substrate. The enzyme denatured by 1% SDS was thought to be renatured partially during the ion-exchange column chromatography with Mono-Q to degrade the protein substrate.

The active form of the enzyme, but not proform of the enzyme, can bind to protein substrates, because the propeptide region of proform of the enzyme completely folds on the surface of the enzyme domains and blocks access of substrate [33]. Therefore, the enzyme accumulated in the inflammatory foci of *M. tuber.*-induced delayed-type allergic-inflammation might be processed active forms of cathepsin B. The significant accumulation of active cathepsin B in the inflammatory foci of the *M. tuber.*-induced delayed-type hypersensitivity suggests reactive secretion from the cells, possibly macrophages activated by cytokines, during delayed-type hypersensitivity.

Corresponding to the activities in the washing-fluids, no significant molecules of other proteinases, such as cathepsin L, cathepsin D, MMP-1, MMP-2, and MMP-3, were observed on the Western blot analysis (Fig. 2A), suggesting the specific secretion of cathepsin B into the

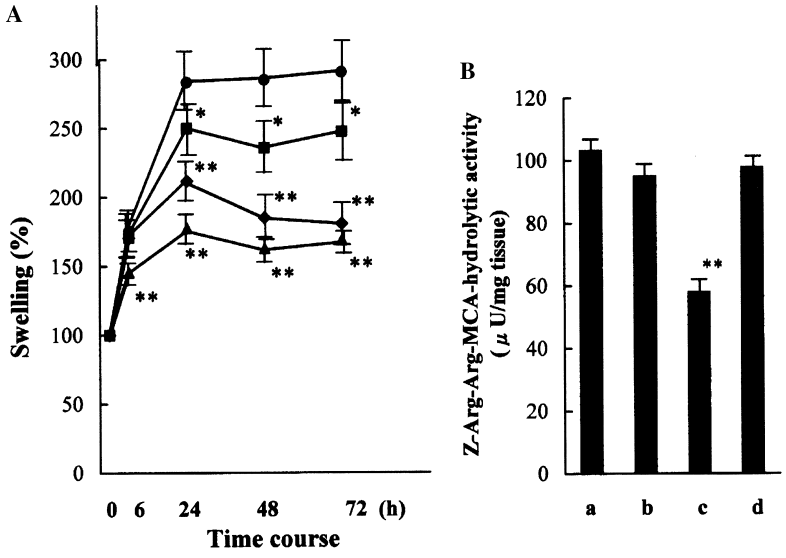

Fig. 3. (A) Inhibitory effects of CA-074 and indomethacin on the footpad swelling in the *M. tuber.*-induced allergic-inflammation in mice. ●, footpad swelling in the *M. tuber.*-induced allergic-inflammatory mice. Mice were injected with 0.05 ml of the emulsion consisting of *M. tuber.* (1 mg/ml saline) and Freund's incomplete adjuvant (1:1), and after 14 days, the emulsion (0.03 ml) consisting of saline extract (0.5 mg/ml proteins) of *M. tuber.* and Freund's incomplete adjuvant (1:1) was subcutaneously injected into the footpad, and the footpad swelling was measured at 6, 24, 48, and 72 h. ■, The effect of CA-074 on the formation of footpad swelling in the *M. tuber.*-induced allergic-inflammation in mice. CA-074 (50 mg/kg) was intraperitoneally administered 30 min prior to the injection of emulsion consisting of saline extract of *M. tuber.* and Freund's incomplete adjuvant (1:1) and at 6, 20, 32, 44, 56, and 68 h after the injection of the emulsion. ◆, The effect of indomethacin on the formation of footpad swelling in the *M. tuber.*-induced allergic-inflammation in mice. Indomethacin (5 mg/kg) was intraperitoneally administered as the same manner of CA-074 administration described above. ▲, footpad swelling in the *M. tuber.*-induced acute-inflammation in mice. The emulsion (0.03 ml) consisting of saline extract (0.5 mg/ml proteins) of *M. tuber.* and Freund's incomplete adjuvant (1:1) was subcutaneously injected into the footpad, and the footpad swelling was measured at 6, 24, 48, and 72 h. $^{**}p < 0.01$, $^{*}p < 0.05$; significant difference from the footpad volume before injection of the emulsion into footpad (Student's *t* test). Each value indicates the mean ± S.E. of 5–6 experiments. (B) Cathepsin B activity in subcutaneous inflammatory tissue homogenates of the footpads of the *M. tuber.*-induced allergic-inflammatory and the *M. tuber.*-induced acute-inflammation. (a) The activity in the footpads of the *M. tuber.*-induced acute-inflammation. (b) The activity in the footpads of the *M. tuber.*-induced allergic-inflammation. (c) The activity in the footpads of the *M. tuber.*-induced allergic-inflammation treated with CA-074. (d) The activity in the footpads of the *M. tuber.*-induced allergic inflammatory mice treated with indomethacin. One unit (U) corresponds to the production of 1 μmol of 7-aminomethylcoumarin per min from 20 μM Z-R-R-MCA. $^{**}p < 0.01$; significant different from the activity in the footpads of the *M. tuber.*-induced allergic-inflammation.

foci of the *M. tuber.*-induced delayed-type allergic-inflammation.

Inhibitory effect of CA-074 on the M. tuber.-induced delayed-type allergic-inflammation

To confirm whether cathepsin B participates in the formation of *M. tuber.*-induced delayed-type allergic-inflammation, the influence of cathepsin B inhibitor on footpad swelling in the *M. tuber.*-induced allergic-inflammation in mice. We investigated the influences of CA-074 and indomethacin, an anti-inflammatory agent that inhibits the generation of prostaglandins, on footpad swelling in the *M. tuber.*-induced allergic-inflammation in mice was analyzed. Footpad swelling in the *M. tuber.*-induced delayed-type allergic-inflammation was greater than that in the *M. tuber.*-induced acute-inflammation (Fig. 3A). CA-074 or indomethacin was administered intraperitoneally 30 min prior to re-challenge with *M. tuber.* and at 6, 20, 32, 44, 56, and 68 h after re-challenge. The administration of CA-074 or indomethacin significantly reduced footpad swelling in the *M. tuber.*-induced delayed-type allergic-inflammation. Cathepsin B activity in the footpad of the *M. tuber.*-induced delayed-type allergic-inflammation was approximately 40% inhibited by treatment with CA-074, but not indomethacin (Fig. 3B). Indomethacin, an anti-inflammatory agent that strongly inhibits biosynthesis of prostaglandins, was not able to inhibit cathepsin B (data not shown), indicating that the inhibitory mechanisms of these agents on footpad swelling were completely different. The total activities of cathepsin B in a, b, and d were almost similar, suggesting that secretion of cathepsin B, but not biosynthesis of the enzyme, might be enhanced in the *M. tuber.*-induced delayed-type allergic-inflammation. CA-074 might inhibit total cathepsin B in the tissue of footpad swelling in the *M. tuber.*-induced delayed-type allergic-inflammation. On the other hand, cathepsin B activity in the liver of the mice treated by CA-074 was more than 95% inhibited, indicating the low bioavailability of CA-074 in the footpad. If more potent inhibitors of cathepsin B are developed, they may express stronger inhibitory effect on footpad swelling mediated by delayed-type hypersensitivity. These results suggest an important role of cathepsin B in the formation of *M. tuber.*-induced delayed-type allergic-inflammation. In correlation, it was reported that peptidyl fluoromethyl ketones as inhibitors of cathepsin B suppressed the severity of inflammation in experimental arthritis in rats [34].

Previous biochemical and immunocytochemical studies have shown that a significantly large amount of cathepsin B is present in the articular cartilage and inflammatory synovial fluids of patients with rheumatoid arthritis [1–4]. We also reported the significant accumulation of cathepsin B and prolylendopeptidase in the synovial fluids of patients with rheumatoid arthritis, but not osteoarthritis, a non-allergic inflammatory disease [35]. We found that cathepsin B accumulated in the inflammatory focus of *M. tuber.*-induced delayed-type hypersensitivity strongly bound to an unknown soluble protein. To clarify what is this protein and why cathepsin B specifically binds to this protein, further experiments are required. Cathepsin B secreted into the inflammatory focus of *M. tuber.*-induced delayed-type allergic-inflammation may also bind to extracellular insoluble protein substrates [36], such as fibronectin, collagens, and laminin, to degrade these structural proteins in extracellular matrix.

We report here that cathepsin B and an unknown prolylendopeptidase are specifically secreted into the inflammatory focus during the process of *M. tuber.*-induced delayed-type allergic-inflammation. The findings of this study suggest that cathepsin B may play an important role in the formation of delayed-type allergic-inflammation and furthermore, specific cathepsin B inhibitors seem to be useful therapeutic tools for treatment of diseases involving delayed-type hypersensitivity.

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