A Monoclonal Antibody against Human β-Glucuronidase for Application in Antibody-Directed Enzyme Prodrug Therapy

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ABSTRACT

The selectivity of anticancer agents may be improved by antibody-directed enzyme prodrug therapy (ADEPT). The immunogenicity of antibody–enzyme conjugates and the low tumor to normal tissue ratio calls for the use of a human enzyme and the development of a monoclonal antibody (MAb) against that enzyme for rapid clearance of the conjugate from the circulation. We isolated β-glucuronidase from human liver. BALB/c mice were immunized with the roughly purified human liver β-glucuronidase and we obtained an MAb designated 105. Immunoblotting showed reactivity with native tetrameric human β-glucuronidase. MAb 105 neither bound to enzyme from bovine liver, rat liver, or mouse liver nor reacted with other human lysosomal enzymes. The antibody appeared to be useful to further purify human β-glucuronidase from human liver or human placenta to homogeneity by affinity chromatography. MAb 105 did not inhibit the activity of human β-glucuronidase. When human β-glucuronidase was injected iv into BALB/c mice, the newly generated MAb 105 could indeed accelerate the clearance of the enzyme with a 50% drop in its activity within 5 min.

INTRODUCTION

ANTIBODY-DIRECTED ENZYME PRODRUG THERAPY (ADEPT) aims at the improvement of the therapeutic efficacy of anticancer agents by selective prodrug activation with an enzyme-immunoconjugate at the site of the tumor.1,2 Tumor-selective monoclonal antibodies (MAbs) are used to carry enzymes to tumor cells. The enzyme converts a relatively nontoxic prodrug, which is administered after the conjugate has localized in tumor lesions, into an active cytotoxic drug.

β-Glucuronidase is being used by our group3 and other investigators4,5 for ADEPT. β-Glucuronidase (EC 3.2.1.31) is a tetrameric glycoprotein composed of identical subunits (Mt = 75,000). In man, the enzyme is a lysosomal acid hydrolase that plays a role in the degradation of glucuronic acid-containing glycosaminoglycans. Only minimal amounts of β-glucuronidase can be found in the circulation.

The prodrugs of interest have been prepared by the attachment of a polar glucuronic acid molecule to the drug structure. Such prodrugs are stable in the circulation, are more hydrophilic, and have a reduced cellular accumulation rate.3 Therefore, β-glucuronidase bound to MAbs localized in tumors may specifically activate glucuronic acid prodrugs aiming at an increase in the therapeutic index.

Previously we have studied Escherichia coli-derived β-glucuronidase.6 This enzyme was not stable in human serum and would be immunogenic in patients. Therefore, we continued ADEPT with the use of human β-glucuronidase, which appears to be stable in serum. To produce conjugates of MAbs with human β-glucuronidase, the enzyme needs to be purified to a high specific activity. We successfully developed an MAb directed against human β-glucuronidase that was used to purify the enzyme to homogeneity. Because minimal amounts of circulating enzyme-immunoconjugate will result in systemic activation of prodrug, we also determined whether the MAb could be used to reduce the serum level of human β-glucuronidase administered to mice.

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MATERIALS AND METHODS

Purification of human β-glucuronidase (Step 1)

β-Glucuronidase was purified from human liver that was obtained from patients at autopsy. The tissue was homogenized with a blender at maximum speed in 50 mM acetic buffer at pH 5.2. β-Glucuronidase was precipitated with 30% (w/v) ammonium sulfate and dialyzed against acetate buffer. The enzyme was loaded onto a Mono S column (Pharmacia, Uppsala, Sweden) in acetate buffer and eluted with 0.25 M NaCl. β-Glucuronidase-containing fractions were dialyzed against 50 mM Tris pH 8, loaded onto a Mono Q column (Pharmacia, Uppsala, Sweden), and eluted with a gradient of NaCl (0–0.5 M). The fractions containing enzyme were pooled and stored at 4°C (Preparation A).

Preparation A was used to obtain polyclonal antisera from immunized guinea pigs. Two animals were injected with 75 μg of enzyme diluted in Freund’s complete adjuvant (Gibco, Breda, The Netherlands) at multiple sites. Two weeks later the injections were repeated with enzyme in Freund’s incomplete adjuvant. After another 2 weeks the animals were bled and 17.5 ml guinea pig antisera was obtained.

The anti-human liver β-glucuronidase activity of the guinea pig antisera was measured in an enzyme-linked immunosorbent assay (ELISA) system. Preparation A or normal human serum (50 μl/well, 10 μg/ml) was coated onto microtiter plates (Probind, Becton Dickinson, Ettten Leur, The Netherlands). After incubation for 45 min at 37°C, the plates were incubated with 1% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 30 min at 37°C. The plates were washed with PBS and 50 μl of serial 1:2 dilutions of the guinea pig antisera was added. After incubation for 30 min at room temperature, the plates were washed, incubated with rabbit anti-guinea pig/horseradish peroxidase (HRP) (Dako, Glostrup, Denmark), washed once more, and developed with 3,3′,5,5′-tetramethyl benzidine (TMB) and H₂O₂. Color development was stopped with H₂SO₄, and the absorbance was measured at 450 nm with a Tietertek Multiscan (Organon Teknika, Boxtel, The Netherlands). The titer of the guinea pig antisera was approximately 1:100,000 against the enzyme, whereas little reactivity was observed with normal human serum.

A polyclonal antibody affinity column was prepared for further purification of human liver β-glucuronidase. Therefore, the immunoglobulin fraction of the guinea pig antisera was isolated by precipitation with 45% (w/v) ammonium sulfate. After dialysis against PBS, 17.5 ml solution with a protein concentration of 14 mg/ml was obtained. The affinity column was made from 10 ml of the polyclonal anti-β-glucuronidase antibody, which was coupled to 10 ml AffiGel-10 (Bio-Rad, Richmond, CA) in PBS according to the manufacturer’s procedure. A 50% (w/v) ammonium sulfate precipitate of human liver dialyzed against PBS was loaded onto the column. The column was washed with PBS followed by 50 mM acetate buffer at pH 5.2. The enzyme was eluted with 8 M urea in the same acetate buffer, dialyzed against PBS, and stored at 4°C (Preparation B).

Monoclonal antibodies against human β-glucuronidase

Preparation B was used to raise MAbs. BALB/c mice (Harlan/CPB, Zeist, The Netherlands) were immunized with 80 μg of enzyme in Freund’s complete adjuvant. Eight days later the animals were boosted with 20 μg of enzyme in incomplete adjuvant. Three days after the last immunization, popliteal lymph nodes were removed and the lymphocytes were fused with SP2/0 myeloma cells using polyethylene glycol. (6) Hybridomas were grown in RPMI 1640 with 1 mM hypoxanthine, 0.04 mM aminopterin, 1.6 mM thymidine (HAT) (ICN, Zoetermeer, The Netherlands), supplemented with 10% (v/v) fetal calf serum. After 2 weeks the HAT additives were omitted. The supernatants of hybridomas were collected and screened for reactivity with human β-glucuronidase by an ELISA and by an enzyme activity assay. The ELISA was performed as described for the guinea pig antiserum, using Preparation B as the solid phase.

To confirm that the hybridoma supernatants were reactive with native enzyme, and not with a contaminant of Preparation B, an enzyme activity assay was used. Microtiter plates were coated with protein A (Pharmacia, Uppsala, Sweden) at 0.5 μg/well to capture immunoglobulins from the hybridomas or the guinea pig antiserum. The plates were washed with PBS, incubated with 1% (w/v) BSA in PBS, and washed once again. The hybridoma supernatants or the guinea pig antiserum were added to the wells and incubated for 30 min at room temperature. After washing, the wells were incubated for 30 min with β-glucuronidase (Preparation B). The plates were washed and 100 μl substrate (1 mM p-nitrophenylglucuronide in 50 mM acetate buffer pH 4.5) was added to the wells and incubated for 60 min at 37°C. The reaction was stopped by the addition of 100 μl 1 M glycine/NaOH pH 10.5 and the absorbance at 405 nm was read.

The isotypes of the hybridomas with activity in both the ELISA and the enzyme activity assay were determined by reactivity with isotype-specific goat anti-mouse IgG₁, IgG₂a, IgG₃m, IgG₇s, IgM, or IgA (Sigma, St Louis, MO). The specificity of these MAbs was determined with the enzyme activity assay using homogenates of human liver, human placenta, bovine liver, rat liver, and mouse liver (1 mg/ml protein) as enzyme source. Reactivity with human lysosomal enzymes was determined similarly at enzyme concentrations of 0.001 U (μmol/min). To determine the nature of the enzyme recognized by the MAbs, immunoblots were prepared with the polyclonal affinity-purified human liver β-glucuronidase (Preparation B) as follows: enzyme was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) as described by Laemmli. (11) Proteins from gels were transferred to nitrocellulose filters according to Tobin et al. (19) After transfer, the nitrocellulose sheets were incubated for 30 min with PBS containing 1% (w/v) BSA and 1% (w/v) dry milk powder to block protein binding sites. The sheet was cut into strips, which were then incubated with the hybridoma supernatants or the guinea pig antiserum. After 30 min the strips were washed with PBS. After incubation with rabbit anti-mouse/rabbit anti-guinea pig conjugated to horseradish peroxidase (Dako, Glostrup, Denmark) the strips were developed with 3,3′-diaminobenzidine and H₂O₂.
Purification of human β-glucuronidase (Step II)

One hybridoma with high reactivity in both the ELISA and the enzyme activity assay, designated MAB 105, was injected ip into BALB/c mice (Harlan/CPB, Zeist, The Netherlands) to produce ascites. The ascites was loaded onto a protein A-Affigel (Bio-Rad, Richmond, CA) and eluted with 0.1 M citrate buffer pH 3.5. The yield of the antibody was approximately 2 mg/ml ascites. MAB 105 was then coupled to Aminolink gel (Pierce, Rockford, IL) according to the manufacturer's procedure. Purified human β-glucuronidase was obtained by passing a 30% (w/v) ammonium sulfate preparation of human liver in PBS through the column. The column was rinsed with PBS and the enzyme was eluted with 50 mM acetate buffer at pH 5.2 containing 8 M urea. The purified enzyme was dialyzed against PBS and stored at 4°C (Preparation C).

Characterization of β-glucuronidase preparations

The total protein concentration of the samples from different purification steps was determined with the Micro BCA protein assay, according to the manufacturer's instructions using BSA as standard (Pierce, Rockford, IL). The enzyme activity of the samples was determined with p-nitrophenylglucuronide (10 mM in 0.1 M acetate pH 4.5, 30 min at 37°C) as a substrate. The reaction was stopped by the addition of 1 M glycine/NaOH pH 10.5 and the absorbance at 405 nm was read with a Titertek Multiscan (Organon Teknika, Boxtel, The Netherlands). The specific activity was expressed as units (1 μmol/min)/mg protein. To check the purity of the enzyme preparations, samples were subjected to SDS-PAGE under reducing conditions according to Laemli.7

To determine whether MAB 105 had any effect on the activity of β-glucuronidase, protein A-purified MAB 105 was incubated with enzyme (Preparation C) in PBS for 1 hr at 37°C at ratios ranging from 0.1 to 75-fold excess antibody. p-Nitrophenylglucuronide (1 mM in 0.1 M NaAc at pH 4.5) was then added and the mixture was incubated for 30 min at 37°C. The reaction was stopped by the addition of 1 M glycine/NaOH pH 10.5 and the absorbance at 405 nm was read.

In vivo studies

The pharmacokinetics of human β-glucuronidase and the effects of coadministration of MAB 105 were studied in BALB/c mice (Harlan/CPB, Zeist, The Netherlands). Two groups of three mice each were injected iv with 100 μg enzyme (Preparation C). After 1 hr, one group received 100 μg MAB 105 iv. The other group, which served as a control, was injected with PBS. Blood samples (50 μl) were obtained with heparinized hematocrit tubes from the orbital plexus at 5 min after enzyme administration, just prior to and 5 min after MAB 105 or PBS administration, and at 2, 4, and 24 hr after the enzyme injection. The blood samples were spun at 10,000g for 5 min, and the β-glucuronidase concentrations in the plasma were determined with p-nitrophenylglucuronide as described above.

RESULTS

Monoclonal antibodies against human β-glucuronidase

Human liver β-glucuronidase obtained after purification by polyclonal antibody affinity chromatography (Preparation B) was used for immunization of BALB/c mice. After fusion of lymph node cells from one immunized mouse with SP2/0 cells, approximately 1000 hybridomas were generated. Eighty percent of the wells contained antibodies against the enzyme, as determined by ELISA, whereas 40% of the wells contained antibodies with reactivity in the enzyme activity assay. Thirty hybridomas with high reactivity in both assays were selected and subcloned by limited dilution. After 1 week, supernatants were tested again and five clones with the highest reactivity in the enzyme activity assay were selected for further recloning.

One MAB with the best reactivity in the ELISA and the enzyme activity assay was further characterized. The clone, designated 105, was of the IgG, isotype. The tissue specificity and species reactivity of the hybridoma were determined and reactivity was observed with human β-glucuronidase from placenta and liver homogenates. MAB 105 did not react with homogenates of bovine, murine, or rat liver indicating the specificity for the human enzyme. No reactivity was observed with human lysosomal enzymes other than β-glucuronidase (Table 1). The nature of the binding of MAB 105 with the enzyme was analyzed in immunoblots of human placental or liver β-glucuronidase. When the enzyme preparations were solubilized in 0.1% SDS at room temperature, the MAb reacted with a single protein of approximately 280 kDa (Fig. 1). When the solubilized samples were boiled for 5 min before electrophoresis, reactivity was lost. The 280-kDa protein band was not visible on protein-stained gels, but a major band of enzyme monomer at 70 kDa could be seen. These data indicate

<table>
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<tr>
<th>Tissue</th>
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*β-Glucuronidase activity (absorbance at 405 nm of p-nitrophenylglucuronide) as determined with the enzyme activity assay using homogenates of human liver, human placenta, bovine liver, rat liver, and mouse liver (1 mg/ml protein) as enzyme source.

†Reactivity with human lysosomal enzymes (absorbance at 405 nm of p-nitrophenylglucuronide) in the enzyme activity assay. Input enzyme concentrations were 0.001 U (μmol/min).
Effect of MAb 105 on β-glucuronidase activity

The effect of MAb 105 on the enzyme activity of human β-glucuronidase was determined. MAb 105 and β-glucuronidase (Preparation C) were incubated at ratios ranging from 0.1 to 100. After 1 hr at 37°C, the specific activity of β-glucuronidase was measured with p-nitrophenylglucuronide. There was no decrease in activity of the enzyme in the presence of up to 75-fold excess MAb (Fig. 3) indicating the lack of interference with the active site of the enzyme.

In vivo studies

The pharmacokinetics of purified human β-glucuronidase and the effect of coadministration of MAb 105 directed against the enzyme was studied in BALB/c mice. The serum concentration of human β-glucuronidase, measured as enzyme activity at various time points after administration, is depicted in Figure 4. Human β-glucuronidase showed a rapid disappearance from the circulation with a t_{1/2} of approximately 0.5 hr. When MAb 105 was injected 1 hr after the enzyme, the activity in the serum dropped to 50% within 5 min. One hour after administration of MAb 105, the enzyme level in the blood was

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**FIG. 1.** Immunoblot of human β-glucuronidase with MAb 105. Human placenta was solubilized in 0.1% SDS and run on a 10% SDS–PAGE gel. Proteins were transferred to nitrocellulose and incubated with MAb 105 or control MAb (323/A3) followed by rabbit anti-mouse/HRP. The bands were visualized with diaminobenzidine.

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**FIG. 2.** SDS–PAGE analysis of β-glucuronidase purified from human placenta. Purified enzyme was solubilized in 0.1% SDS, boiled for 2 min, and applied to a 10% SDS–PAGE gel. The gel was stained with Coomassie blue. (A) Salt fractionation and ion-exchange chromatography, (B) affinity chromatography with polyclonal antibody, and (C) affinity chromatography with monoclonal antibody 105.
Human β-gluconidase is an attractive enzyme for use in ADEPT because very low levels of the enzyme are present in the circulation. Previous work in our laboratory has already shown that a conjugate between Escherichia coli-derived β-gluconidase and MAB 323/A3 induced selective cytotoxicity upon enzymatic activation of the prodrug Epirubicin-gluconide. We expect the human enzyme to be less immunogenic in patients when compared to the bacterial enzyme.

We generated MAB 105 to purify human β-gluconidase. The purification from human liver and placenta by affinity chromatography was more efficient than the ion-exchange procedure, and resulted in a homogeneous enzyme preparation (Fig. 1) with high specific activity (10–30 U/mg). This procedure should be applicable for the large-scale production of purified human β-gluconidase to be used in the preparation of enzyme-immunoconjugates.

MAB 105 could successfully be applied in the reduction of the level of human β-gluconidase in BALB/c mice. Within 5 min after administration of the MAB, enzyme levels dropped by 50% (Fig. 4). A similar acceleration in the clearance was observed in the case of an antibody-β-gluconidase conjugate (data not shown). Apparently, the antibody binds to circulating β-gluconidase and the complexes formed are rapidly removed by the reticuloendothelial system. It has been shown earlier that MABS directed against bacterial or yeast enzymes can accelerate the clearance of enzyme-immunoconjugates. Sharma et al. described an MAB, SB43, against bacterial carboxypeptidase G2 that could be used to remove enzyme-immunoconjugates from the circulation, but the MAB rapidly inactivated the enzyme. This necessitated the coupling of galactose to MAB SB43 to ensure fast clearance from the circulation and thus to avoid inactivation of the enzyme already present in the tumor. The MAB 102-26 described by Kerr et al. that is reactive with yeast cytosine deaminase was also used to reduce circulating enzyme levels. This MAB inhibited the enzyme activity by 30%. Both studies showed dramatically improved tumor-to-blood ratios, despite the reduction of enzyme activity by binding to the MABS. However, the absolute amount of enzyme activity in the tumors was reduced, which may reflect the partial inhibition of enzyme at the tumor site.

Our study is the first report on a mouse MAB reactive with a human enzyme that was used to clear the enzyme from the circulation. Of great importance is that MAB 105 did not inactivate the enzyme, even in the presence of a 75-fold excess of antibody (Fig. 2). This advantage may allow MAB 105 to circulate for a long period of time and remove any human β-gluconidase or conjugate reentering the circulation.

The use of human or humanized MAB-enzyme conjugates in combination with a noninactivating MAB to clear the conjugate from the circulation prior to administration of prodrgu should improve the therapeutic potential of ADEPT.

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Monoclonal Antibodies against Human Collagenase and Stromelysin

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ABSTRACT

Mouse monoclonal antibodies against recombinant human fibroblast procollagenase and prostromelysin have been generated and characterized. The epitope-containing domains for the antibodies have been assigned based on their immunoreactivities against recombinant proenzymes, mature enzymes, truncated collagenases, proteolytic fragments of stromelysin, and chimeric molecules constructed from different domains of the two enzymes. These antibodies can be divided into four groups: (1) antibodies that recognize the truncated 19-kDa NH2-terminal collagenase, (2) antibodies that recognize the C-terminal domain of collagenase and stromelysin, (3) antibodies that recognize a 31-kDa NH2-terminal collagenase fragment, and (4) antibodies that recognize the 19-kDa NH2-fragment of stromelysin. The prostromelysin-specific antibody 11N13 is of particular interest; it neutralizes stromelysin activity in a stromelysin peptide substrate assay, with an IC50 value of 75 nM. MAb 11N13 may be useful for in vivo and in vitro studies to validate the roles of stromelysin in tumor cell invasion, metastasis, and connective tissue disorders.

INTRODUCTION

THE MATRIX METALLOPROTEINASES (MMPs) are a family of zinc endoproteases involved in connective tissue remodeling. Members of the family include the collagenases, stromelysins, and gelatinases.1) MMPs are secreted as zymogens in response to a variety of stimuli, such as growth factors, inflammatory cytokines, and tumor promoters.2 4) The propeptide contains a conserved cysteine that ligates the active site zinc to maintain latency. Catalytically active enzymes can be generated by oxidation of this cysteine or by limited proteolysis of the propeptide.5 6) The active form of these enzymes generally possesses a two-domain structure consisting of (1) an N-terminal region that is ~170 amino acids (19 kDa) in length and contains the Zn2+ active site, and (2) a C-terminal hemopeixin-like domain that contains ~210 residues that is thought to have a role in binding the enzymes to the target substrates.7) The N-terminal 19-kDa domain contains the inherent catalytic activity of the enzyme. This fragment has the same activity toward peptide substrates as the full length enzyme, and it also has the capacity to bind low-molecular-weight inhibitors with the same affinity as the full length enzyme.8)

There is increasing evidence that MMPs play a pathological role in connective tissue diseases and cancer. Elevated levels of both interstitial collagenase (MMP-1) and stromelysin (MMP-3) have been found in the joints of many patients suffering from active rheumatoid arthritis.9 11) The disregulated expression of these enzymes leads to the eventual destruction of the cartilage and supporting connective tissue. A role for MMPs in cancer has also been suggested by the high frequency of MMP gene expression detected in biopsies of head and neck carcinomas, as well as from lung carcinoma samples.12 13) Enzymes found at elevated levels include PUMP (MMP-7),14) 72-kDa gelatinase (MMP-2),15) 92-kDa gelatinase (MMP-9),16) and the recently described stromelysin III (MMP-11).17) Inhibition of MMP’s enzymatic activity repre-
sents a point for therapeutic intervention in each of these pathologies and, indeed, MMP inhibitors have been shown to reduce tumor cell invasion in vitro and metastasis in vivo.\(^{116-20}\)

Here we report the production and characterization of mouse monoclonal antibodies raised against recombinant human fibroblast procollagenase and prostromelysin. We have used a series of truncated and chimeric constructs to localize epitopes for each of these antibodies. In addition, we have identified one monoclonal antibody, 11N13, that is capable of neutralizing stromelysin's catalytic activity. This antibody could be a valuable tool for examining the functional activity of stromelysin as well as its role in the activation of other MMPs in vitro and in vivo.

### MATERIALS AND METHODS

**Preparation of recombinant, truncated and chimeric metalloproteinases**

Expression and purification of recombinant human fibroblast procollagenase (antigen), collagenase, truncated collagenase molecules, prostromelysin (antigen), stromelysin, and chimeric enzymes have been described elsewhere (Becherer et al.,\(^{20}\)). The chimeric enzymes, which are called prostromelase and collysin, were made by swapping the C-terminal domains of the procollagenase and prostromelysin cDNAs (Fig. 1).

**Immunization and fusion**

BALB/c mice were immunized with 10 μg of either recombinant human fibroblast procollagenase or prostromelysin emulsified in RIBI adjuvant system (RIBI, Hamilton, MT). After three immunizations at 2 week intervals, mice were rested for 3 weeks. Antigens (10 μg without adjuvant) were then injected intravenously each day for 3 days before fusion. The fusion protocol was the standard method of Kohler and Milstein\(^{21}\) with minor modifications.\(^{22-25}\) Briefly, 10⁶ spleen cells were fused with 5 × 10⁶ mouse myeloma cells (P3X63Ag8.653) using polyethylene glycol 1450 (ATCC, Rockville, MD). Fused cells were grown in 100 μM hypoxanthine, 0.4 μM Aminopterin, 16 μM Thymidine (HAT) selection medium for 2 weeks, followed by 1 week in HT medium. The hybridomas were then grown in RPMI-1640 containing 10% fetal bovine serum, 50 μg/ml endothelial cell growth supplement (Collaborative Research, Bedford, MA), and 50 μg/ml gentamycin. Hybridoma supernatants were screened for specific antibodies by ELISA and then selected cells cloned by limiting dilution.

**Ascites production and purification**

A total of 10⁷ cloned cells were washed, resuspended in phosphate-buffered saline (PBS), and injected into the peritoneal cavity of pristane-primed BALB/c mice (Harlan Bioproduct, Indianapolis, IN). Ascitic fluids were collected 10–14 days after injection. Ascites of IgG class antibodies were delipidated using 1,1,2-trichlorotrifluoroethane and diluted with PBS (pH 7.0) containing 10 mM EDTA before loading onto a precalibrated protein G Sepharose CL-6B (Pharmacia, Piscataway, NJ) column. After washing with 10 column volumes of buffer, antibodies were eluted with 0.5 M acetic acid at pH 3.0 and neutralized with 1 M Tris, pH 8.0. IgM class antibodies were purified using an ImmunoPure kit.

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**FIG. 1.** Summary of the immunoreactivity of monoclonal antibodies to stromelysin and collagenase. The topological structures of procollagenase, prostromelysin, truncated, and chimeric forms of the enzymes are shown. The zinc catalytic site is shown in domain I (black shading) while domain II represents the noncatalytic carboxy-terminal half of the enzymes. Relative immunoreactivities of the antibodies in ELISA are indicated by the number of ‘+’. 
COLLAGENASE AND STROMELOYSIN MONOCLONAL ANTIBODIES

(Pierce, Rockford, IL) according to the manufacturer’s instructions. This system is based on IgM binding to mannan-binding protein. The purities of the IgM and IgG antibodies, assessed by SDS-PAGE, were approximately 90% and greater than 95%, respectively. Antibodies used for neutralization studies were concentrated over Amicon centrifprep filters (Amicon, Beverly, MA).

**Enzyme-linked immunosorbent assay (ELISA)**

Immunoplates (96-well) were coated overnight with 50 μl of antigens at 0.25 μg/ml in TBS (50 mM Tris, 150 mM NaCl, pH 8.0). After blocking nonspecific protein binding sites with blocking buffer (2% normal goat serum, 1 mg/ml of polyethylene glycol, and 0.05% Tween 20 in TBS), hybridoma supernatants (50 μl) were added to the wells for 1 hr at room temperature. After washing with TBST (TBS with 0.05% Tween-20), an optimal concentration (1/4000) of goat anti-mouse IgG conjugated with alkaline phosphatase (Promega, Madison, WI) was added and allowed to bind for 1 hr before plates were washed and alkaline phosphatase substrate (p-nitrophenyl phosphate, Sigma, St. Louis, MO) added. Immunoreactivity was detected by color development, measured at 405 nm in a microplate reader (UV max™, Molecular Devices, Menlo Park, CA). Immunoglobulin classes and subclasses were determined by ELISA typing kits (Fisher, Pittsburgh, PA).

**Western blotting**

Proteins (antigen preparations) were separated by electrophoresis on 12% SDS–polyacrylamide gels and transferred onto nitrocellulose. The blots were blocked as above and incubated with hybridoma supernatants or purified antibody (1 μg/ml). Bands were detected with goat antimouse IgG conjugated with alkaline phosphatase using NBT-BCIP substrates (nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, Promega, Madison, WI).

**Preparation of proteolytic fragments of stromelysin**

Purified recombinant human stromelysin was reduced and alkylated by incubating the protein in 6 M guanidine–HCl, 0.5 M Tris–HCl, 10 mM EDTA, and 20 mM dithiothreitol (pH 8.6) for 1 hr at 37°C under nitrogen, followed by addition of 4-vinylpyridine to 50 mM for 30 min at room temperature. The pyridylethylated material was desalted by HPLC with a BU300 column (2.1 x 30 mm, Brownlee, Foster City, CA) using a linear gradient of acetonitrile (16–64%) in 0.1% trifluoroacetic acid (TFA) over 30 min. The eluted protein was then digested with sequencing grade Lys-C (Wako, Richmond, VA) in 0.1 M Tris–HCl (pH 8.5) for 16 hr at room temperature, with an enzyme:substrate ratio of 1:100. The Lys-C-generated peptides were then separated and isolated on the BU300 column using a linear gradient of acetonitrile (8–64%) in 0.1% TFA over a 40-min period. Peptide fragments were dried by flushing with nitrogen and then resuspended in TBS.

Automated Edman degradations were performed using the Applied Biosystems 477A liquid-pulse sequencer (Applied Biosystem, Foster City, CA) equipped with a 120A PTH analyzer for the identification of phenylthiohydantoin amino acids.

**Stromelysin peptide substrate assay (SPSA)**

The stromelysin fluorogenic peptide substrates dinitrophenol (DNP)-Pro-Gln-Gln-Phe-Trp-Lys-Arg-Lys,N-methylantranilic acid (NMA) or DNP-Pro-Lys-Pro-Gln-Gln-Phe-Lys (NMA) were used to assay stromelysin enzyme activity. Cleavage of the Gln-Phe linkage liberates NMA-containing fragments generating a fluorescent signal detectable at 450 nm. The assay was performed at 23°C with 50 nM stromelysin, 10 μM substrate, and either chemical inhibitors or antibodies in a final volume of 300 μL SPSA buffer (200 mM NaCl, 50 mM Tris–HCl, 5 mM CaCl₂, 20 μM ZnSO₄, and 0.05% Brij at pH 7.6). Fluorescence readings were made with a plate reader using Ex 360/Em 450 (LS-5B Luminescence Spectrometer, Perkin-Elmer, Norwalk, CT).

**Antibody binding inhibition assay**

A sandwich ELISA assay system, using 29N5 as solid phase antibody and biotinylated 11N13 as detecting antibody, was used to assess the catalytic binding site reactivity of 11N13. Immunoplates (96-well) were coated overnight at 4°C with capturing antibody 29N5 (25 nM) in TBS. After blocking nonspecific binding sites, different concentrations (0.2–50 nM) of stromelysin were added to the wells for binding to 29N5 for 1 hr. After washing with TBST, the activity of stromelysin bound to the solid phase was measured using the stromelysin peptide substrate assay described above, in the presence or absence of inhibitors at 1 μM. After stromelysin enzymatic activity was measured, biotinylated 11N13 (25 nM, biotinylation performed using NHS-LC-Biotinylation kit, Pierce, Rockford, IL) was added without washing to the mixture for a further 1 hr incubation. Bound biotinylated 11N13 was then assessed after washing with alkaline phosphatase-derivated strepavidin (Pierce, Rockford, IL).

**RESULTS**

**Establishment and screening of hybridomas**

As members of the MMP family, procollagenase and stromelysin share many regions of amino acid homology and may also share common epitopes. We therefore adopted a common screening strategy for the production of monoclonal antibodies to these proteins. Mice were immunized with purified recombinant human procollagenase or stromelysin and spleen cells fused with P3X63Ag8.653 myeloma cells. Out of 180 positive growth wells, a total of 17 antigen-reactive wells were identified by ELISA. All uncloned antigen-reactive hybridomas were further screened by ELISA against the following panel of recombinant human antigens: procollagenase and stromelysin (the immunogens), truncated collagenase molecules (19, 25, 31, 37, and 43 KDa), and two chimeric enzymes that we have termed collysin (N-terminal collagenase, C-terminal stromelysin) and prostremolase (N-terminal prostromelysin, C-terminal collagenase). The topological structures of these proteins are shown in Figure 1. Hybridomas showing different cross-reactivity profiles against this panel of antigens were then cloned by limiting dilution and clones
rescreened by ELISA. Four monoclonal antibodies (3M18, 42M10, 29N5, and 11N13) exhibiting different reactivities, and active in both ELISA and Western blotting assays, were chosen for further study.

**Epitope specificity**

The immunoreactivity of monoclonal antibodies 3M18, 42M10, 29N5, and 11N13 was examined. The ELISA results are summarized in Figure 1 and Western blots illustrated in Figure 2. Two of the antibodies, 42M10 and 11N13 (both IgG1 isotype), were found to react with epitopes specific to their immunizing antigens—procollagenase and prostromelysin, respectively. 42M10 recognizes all collagenase constructs except the 19- and 25-kDa fragments. Because it also binds the chimeric prostromelase, 42M10 appears to recognize an epitope localized within a 6-kDa region of collagenase at the C-terminal end of the 31-kDa species. Similarly, 11N13 reacts with prostromelase but not colllysin, indicating that in this case the epitope is localized in the NH₂-terminal region of prostromelysin, which includes the catalytic site and zinc binding region (see Fig. 1).

As predicted, immunizing with either procollagenase or prostromelysin produced cross-reactive antibodies recognizing epitopes shared by these two MMPs. 3M18 (IgM) derived from procollagenase immunization and 29N5 (IgG1) derived by prostromelysin immunization provide examples of this type of antibody (Fig. 1). The cross-reactivity of 3M18 with all the antigens tested indicates that this antibody recognizes an epitope located within the 19-kDa NH₂-terminal region of collagenase and stromelysin containing the catalytic region. In contrast, the 29N5 reactive epitope appears to be located at the C-terminal end of collagenase and stromelysin, but we cannot rule out the possibility that the epitope is conformation dependent and is generated only within mature MMPs, not their fragments.

**Further characterization of stromelysin epitopes**

To further define the stromelysin epitopes recognized by 3M18, 29N5, and 11N13, stromelysin was digested with the endopeptidase Lys-C to produce 14 fragments that were separated by reverse-phase HPLC (Fig. 3a). The immunoreactivity of 3M18, 29N5, and 11N13 against these 14 fragments was examined by ELISA. The results clearly showed that 3M18 reacts specifically with a single fragment corresponding to peak 10 of the HPLC profile (Fig. 3b), whereas 11N13 reacted with the fragment in peak 12 (Fig. 3c). In contrast, 29N5 did not react with a discrete peptide fragment. One explanation is that the 29N5 epitope is a conformational one. Fragments 10

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**FIG. 2.** Western blot analysis of intact, truncated, and chimeric MMPs. The MMP preparations (2 µg/lane) were electrophoresed, transferred onto nitrocellulose, and blotted as described in Materials and Methods. Antibodies used for blotting are (a) 3M18, (b) 42M10, (c) 29N5, and (d) 11N13.
FIG. 3. Reactivity of antibodies with stromelysin fragments. (a) The profile of Lys-C-generated fragments separated by HPLC using a BU300 column with fragments 10 and 12 marked. (b) Reactivity of stromelysin (strom) and Lys-C fragments 1–14 with 3M18 (○) and IgM control (■). (c) Reactivity with 11N13 (○) and IgG1 control (■), both purified and used at 1 μg/ml.

FIG. 4. (a) Amino acid sequence of human stromelysin. The number is based on the reported sequence\(^{(10)}\) after subtracting the signal and propeptide. The sequence of Lys-C digested fragment #10 is shown boxed; fragment #12 is shown in italic and underlined. Assignment of the fragments was based on Edman sequencing. The arrow indicates the swap site (position 153) used to make the chimeric enzymes collysin and prostromelase. (b) A schematic representation of prostromelysin and procollagenase showing the position of the epitope-containing domains for MMP monoclonal antibodies. Domain assignment for 3M18 and 11N13 is based on the immunoreactivities of antibodies with Lys-C fragments. All other domain assignments are based on immunoreactivities with truncated and chimeric enzymes.
and 12 were analyzed by Edman sequencing to assign the epitopes recognized by 3M18 and 11N13 to the regions of stromelysin (Fig. 4a). Since 11N13 does not react with collagen that contains the C-terminal of stromelysin, our results suggest that the 11N13 recognizes an epitope within residues 117 to 153.

Based on the results discussed above, we assign the epitope-containing domains for 3M18, 42M18, 29N5, and 11N13 antibodies as shown in Figure 4b.

Inhibition of stromelysin activity

We next examined the capacity of the four antibodies to inhibit the enzyme activity of collagenase and stromelysin in fluorogenic peptide substrate assays. None of the antibodies had any effect on collagenase activity (data not shown). However, purified 11N13 significantly inhibited stromelysin activity when compared to isotype-matched controls (42M18, another procollagenase antibody, and the src-specific antibody 327) (27) (Fig. 5a). The 29N5 antibody was also partially inhibitory at high concentrations (Fig. 5a), whereas 3M18 and an IgM control had no effect. 11N13 inhibition of stromelysin activity was dose dependent, with 50% inhibition achieved at approximately 75 nM. The inhibitory activity of 11N13 was not due to quenching since addition of exogenous antibody had no effect on fluorescence. These results clearly demonstrate that 11N13 is a neutralizing antibody for stromelysin. Two synthetic inhibitors, GI130085 and GR129574, (28) which were designed to target the Zn$^{2+}$ binding site of MMPs, were included as controls and showed the expected IC$_{50}$ values of 25 and 100 nM, respectively (Fig. 5b).

To determine if the neutralization activity of 11N13 was due to its reactivity with the Zn$^{2+}$ binding catalytic site, we examined the effect of stromelysin inhibitors on 11N13 binding to stromelysin. A sandwich ELISA format was used, with 29N5 antibody bound to the solid phase and biotinylated 11N13 antibody as the detecting reagent. Stromelysin bound to the solid phase 29N5 antibody retained enzymatic activity, which was inhibited by the addition of excess (1 μM) GI130085 and GR129574, and unlabeled 11N13 antibody (Fig. 6a). The profiles of inhibitory activity by 11N13 or synthetic inhibitors in solid phase were similar to those in soluble phase (see Fig. 5b). Following assay for enzyme activity, biotinylated 11N13 antibody was still able to detect bound stromelysin, and this was completely blocked by the presence of unlabeled 11N13. In contrast, the catalytic site-specific inhibitors GI130085 and GR129574 had no effect on biotinylated 11N13 binding, indicating that this antibody does not bind directly to the catalytic site of stromelysin (Fig. 6b). Neutralization of the enzyme activity may, therefore, be due to a steric block of access for peptide substrates or perhaps to allosteric effects on the enzyme.

DISCUSSION

In this paper, we report the production and characterization of four monoclonal antibodies raised against procollagenase and prostromelysin that recognize different epitopes within these enzymes. Epitopes recognized by the antibodies were partially characterized by analyzing their reactivity with a panel of recombinant enzymes, including truncated and chimeric molecules, as well as peptide fragments of stromelysin. The stromelysin-specific antibody 11N13 is of particular interest because it is able to neutralize stromelysin enzyme activity, at least against the synthetic peptide substrates used in this study (whether or not 11N13 can neutralize stromelysin activity against natural protein substrates remains to be investigated). Since 11N13 does not compete with the synthetic catalytic site inhibitors, its neutralizing activity could be explained by steric hindrance of substrate binding to stromelysin.
Cooksley, et al.\(^{(29)}\) in using a pair of monoclonal antibodies recognizing different epitopes.

The development of specific inhibitors of the individual MMPs will be useful for evaluating the role each of these enzymes plays in disease. Neutralizing monoclonal antibodies that inhibit specific MMPs have previously been prepared for 92-kDa gelatinase\(^{(20,33)}\) and collagenase.\(^{(22)}\) Monoclonal antibody 11N13 is a unique reagent for analyzing the catalytic activities attributed to stromelysin. Specifically, stromelysin has been reported to be an activator of procollagenase \textit{in vitro}\(^{(33-35)}\) and to have a role in metastasis and connective tissue disorders \textit{in vivo}. This antibody will be a useful tool for testing these hypotheses.

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**REFERENCES**

28. Compounds GR129574 and GI130085 are low molecular weight competitive inhibitors of collagenase, gelatinase, and stromelysin. Both are dipeptide analogues containing a zinc chelating group that binds at the active site of the MMPs. GR129574 is covered in U.S. Patent 55,252,560 issued on 11/2/93.

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