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Development of a Neutralizing Antibody Specific for the Active Form of Matrix Metalloproteinase-13

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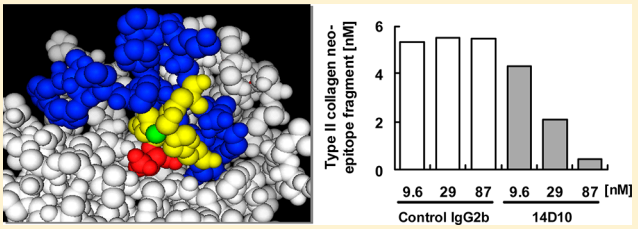
ABSTRACT: Matrix metalloproteinase-13 (MMP-13) is important in the pathology of osteoarthritis (OA). Although MMP-13 is considered a therapeutic target for OA, it is unclear how MMP-13 activity is regulated by the system that comprises various proteinases and their inhibitors. MMP-13 neutralizing antibodies could be a useful tool for investigating the involvement of MMP-13 in cartilage degeneration in OA-affected joints because antibodies possess high affinity and specificity compared with low-molecular weight chemical compounds. On the basis of three-dimensional structure and amino acid sequence information on MMP-13, we selected an appropriate antigen peptide and generated a neutralizing antibody by immunizing mice with the antigen. The most significant property of monoclonal antibody 14D10 was the specific binding to the active form of MMP-13, but not to the latent form, or other MMPs. With this property, active MMP-13 was measured selectively by an enzyme-linked immunosorbet assay. Furthermore, 14D10 suppressed the cleavage of type II collagen in human articular chondrocyte cultures, and 14D10 is thought to inhibit MMP-13 activity effectively. Thus, the highly selective MMP-13 neutralizing antibody (14D10) might be a useful tool for investigating the mechanism of type II collagen degradation in arthritic pathology.



Fibrillar collagens are highly resistant to cleavage by most proteases because of their triple-helical structure. However, specific members of matrix metalloproteinases, namely, collagenase-1, -2, and -3, corresponding to MMP-1, -8, and -13, respectively, have the capacity to cleave fibrillar collagens efficiently at a specific site in the triple-helical domain.[1] Cleavage of the triple-helical domain by collagenases is a critical event in the degeneration of fibrillar collagens, and once this occurs, collagen readily loses its trimeric structure and becomes increasingly susceptible to cleavage by various proteinases.[2] Thus, the initial cleavage by collagenases is a rate-limiting step in the degeneration of collagen in vivo, in both physiological and pathological situations.

Osteoarthritis (OA) is the most common human joint disease in developed countries.[3] Because OA primarily affects elderly people, it is more prevalent in those countries with increasing longevity and is now a large economic and medical burden to society.[3,4] OA is characterized by the gradual loss of articular cartilage over years to decades.[5] Besides aggrecanases, MMP-13 is also considered one of the main enzymes responsible for the loss of cartilage matrix because its level of expression is highly elevated in OA cartilage,[6] and it has a strong cleavage preference for type II collagen, the primary component of articular cartilage.[7] Consistent with this, the induction of enzymatically active MMP-13 could induce joint pathology closely resembling OA in mouse knee joints.[8] Conversely, inhibition of MMP-13 activity had therapeutic effects in animal models of OA.[9,10] Thus, it is expected that the progression of OA could be prevented, or at least delayed, through the inhibition of MMP-13.

For this reason, efforts have been made to develop synthetic inhibitors for MMP-13.[11] Many MMP inhibitors previously developed such as hydroxamic acid inhibited MMP activity by chelation of a zinc ion coordinated at the active center of the enzyme. However, to date, clinical success using MMP-13 inhibitors has not been achieved. This is due in part to the difficulty of generating specific bioavailable inhibitors because of the similarities between the catalytic domains of MMP-13 and other MMPs, a well-conserved enzyme active-site topology and the mobility of residues in the S1′ specificity loop.[12,13] The use of neutralizing antibodies is another strategy for inhibiting the activity of MMPs. Although the problem of specificity could be overcome using a monoclonal antibody whose target specificity was generally higher than that of chemically synthesized inhibitors, it is not easy to develop neutralizing antibodies. Antibodies may be raised by immunization with the entire protein molecule. While this approach can be effective for the generation of neutralizing antibodies, it is difficult to

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exclude cross reactivity to other MMPs, and further studies would be necessary to identify the epitope and the mechanism of inhibition. Alternatively, antibodies may be raised by immunization of a synthetic peptide containing an amino acid sequence unique to the target protein. However, antibodies raised by this method rarely recognize the three-dimensional structure of the target protein.

In this study, we have successfully developed a neutralizing monoclonal antibody for MMP-13 by immunization with a synthetic peptide. The amino acid sequence of the peptide immunogen was specifically determined from the region containing the active site of the enzyme. Antibody 14D10, which recognizes the region around His^{232} on the S1′ specificity loop, inhibits the enzymatic activity of human MMP-13 and orthologs of other animal species, but not other types of MMPs as expected. Using this antibody, which binds specifically to the active form but not the latent form of MMP-13, an enzyme-linked immunosorbent assay (ELISA) was developed, and the amount of active enzyme was determined and compared with that of the total (active and latent) MMP-13 in experimental samples. Thus, the highly selective MMP-13 neutralizing antibody (14D10) might be a useful tool for investigating the mechanism of type II collagen degradation in arthritic pathology.

EXPERIMENTAL PROCEDURES

Preparation of Recombinant MMPs. Latent forms of recombinant human collagenases (pro-MMP-1, -8, and -13) were purchased from R&D Systems (Minneapolis, MN) and activated by incubation with *p*-aminophenylmercuric acetate (APMA) (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. The catalytic domain (CD) of rat MMP-13 (UniProt entry P23097, Pro^{99}–Gly^{262}) was cloned into pTrc99AHE, an expression vector modified from pTrc99A (Pharmacia, Uppsala, Sweden) and expressed as an N-terminally His-tagged protein in *Escherichia coli* (XL1-Blue) (Agilent Technologies, Santa Clara, CA). Protein expression was induced by the addition of β-D-thiogalactopyranoside. After an appropriate incubation, the cells were centrifuged and the pellet was stored at −80 °C until it was used. Purification of the protein was performed as follows: the frozen cell pellet was thawed and suspended in ice-chilled 50 mM Tris-HCl (pH 7.9) and lysed by sonication. The crude lysate was centrifuged at 15000*g* for 10 min, and the pellet was solubilized with 8 M urea and then mixed with an equal volume of buffer A [50 mM Tris-HCl (pH 7.9), 8 M urea, 10 mM $CaCl_2$, 300 mM NaCl, and 0.005% Brij35]. This mixture was clarified by centrifugation at 15000*g* for 10 min, and the supernatant was applied to a Ni-NTA agarose column (Qiagen, Hilden, Germany). The column was washed with buffer A containing 20 mM imidazole, and then the protein was eluted with buffer A containing 200 mM imidazole. The purified rat MMP-13 CD was refolded by dialysis against buffer B [50 mM Tris-HCl (pH 7.4), 10 mM $CaCl_2$, 300 mM NaCl, and 0.005% Brij35].

The CDs of human MMP-2 (P08253, Tyr^{110}–Asp^{452}), MMP-3 (P08254, Phe^{105}–Thr^{272}), MMP-9 (P14780, Phe^{107}–Pro^{449}), and MMP-12 (P39900, Gly^{106}–Glu^{267}) were prepared by the same procedure as described above. For the preparation of guinea pig MMP-13 CD, cDNA was amplified by polymerase chain reaction using the primer pair of F1 (TACAATGTTT-TCCCTCGAACACTC) and R1 (TCTAGATTAACCATAG-AGAGACTGGATCC) and cloned into the pTrc99AHE vector. These proteins were purified as described above for recombinant rat MMP-13 CD.

Generation of Antibodies. An immunogen peptide was synthesized by Greiner Bio-One (Kremsmuenster, Austria) and conjugated to maleimide-activated mariculture keyhole limpet hemocyanin (KLH) (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Female, 4–6-week-old A/J mice were given intraperitoneal injection of 100 μg of the KLH-conjugated peptide immunogen emulsified in complete Freund's adjuvant (Difco, Franklin Lakes, NJ). The immunization was repeated with the immunogen emulsified in incomplete Freund's adjuvant three times at 3 week intervals. Ten days after the third immunization, sera were obtained from mice and assessed for binding to recombinant rat MMP-13 CD and the immunogen peptide. Spleen cells were obtained from mice whose serum showed high titers of antibodies to recombinant rat MMP-13 CD, and hybridomas were generated by fusing the spleen cells with P3U1 murine myeloma cells following the standard protocol. The hybridomas were cultured in medium containing hypoxanthine, aminopterin, and thymidine and then cloned by limiting dilution. Supernatants were collected from the cloned hybridoma cultures and examined for reactivity to rat MMP-13 CD by an ELISA as described below. Supernatants showing strong reactivity to rat MMP-13 CD were subjected to further screening using a competitive inhibition ELISA as described below. The isotype of chosen clones was determined with a Mouse Immunoglobulin Isotyping ELISA Kit (BD Bioscience, Franklin Lakes, NJ).

Biotinylation of the Immunogen Peptide. The immunogen peptide was biotinylated using Maleimide-PEG2-Biotin (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions. Briefly, the peptide was dissolved in labeling buffer [100 mM phosphate buffer (pH 6.0) and 5 mM EDTA] and incubated for 2 h at room temperature with a 20-fold molar excess of Maleimide-PEG2-Biotin. The biotinylated peptide was then purified by high-performance liquid chromatography.

Assessment of the Binding Activity of Developed Antibodies to MMP-13. Anti-mouse IgG (Shibayagi, Shibukawa, Japan) was diluted (10 μg/mL) into 50 mM Tris-HCl (pH 7.5), and 35 μL aliquots were added to each well of a 384-well MaxiSorp plate (Nunc, Roskilde, Denmark) and incubated overnight at 4 °C. After being washed, the plate was blocked with 90 μL of BlockAce (DS Pharma Biomedical, Osaka, Japan) and incubated for 2 h at room temperature. Then 15 μL of the sample solution (diluted serum or hybridoma supernatant) and 15 μL of the biotinylated peptide (0.05 ng) and streptavidin–horseradish peroxidase conjugate (2 ng, Thermo Scientific) were placed in each well of the plate and the plates incubated overnight at 4 °C. After being washed, the plate was incubated for 30 min at room temperature with 25 μL of TMB (3,3′,5,5′-tetramethylbenzidine) with Substrate-Chromogen (Dako, Glostrup, Denmark). Finally, the reaction was stopped by the addition of 25 μL of 0.5 M sulfuric acid, and the absorbance at 450 nm was determined by an EnVision multilabel plate reader (PerkinElmer, Waltham, MA).

Assessment of the Specificity of Developed Antibodies Using a Competitive Inhibition ELISA. Anti-mouse IgG (Shibayagi) was diluted (10 μg/mL) into 50 mM Tris-HCl (pH 7.5), and 150 μL aliquots were added to each well of a 96-well MaxiSorp plate and incubated overnight at 4 °C. After being washed, each well of the plate was blocked with 300 μL of BlockAce and incubated for 2 h at room temperature. Then 50

μL of anti-MMP-13 antibody and 50 μL of the biotinylated peptide (0.05 ng) and streptavidin−horseradish peroxidase conjugate (2 ng) were added. Simultaneously, 50 μL of graded concentrations of the CD of human MMP-2, MMP-3, MMP-9, or MMP-12, rat MMP-13, guinea pig MMP13, or activated human MMP-1, MMP-8, or MMP-13 was added as a competitor and incubated overnight at 4 °C. After being washed, the plate was incubated for 30 min at room temperature with 100 μL of TMB with Substrate-Chromogen. Finally, the reaction was stopped by the addition of 100 μL of 0.5 M sulfuric acid, and the absorbance at 450 nm was determined with an EnVision plate reader.

Assessment of the Binding Affinity of the Developed Antibody by Surface Plasmon Resonance. The binding affinity of the developed anti-MMP-13 antibody was measured with a BIAcore T100 surface plasmon resonance instrument (GE Healthcare, Little Chalfont, U.K.) and expressed as an equilibrium constant (K_D). Anti-mouse IgG was immobilized on a CM5 chip using a Mouse Antibody Capture Kit (GE Healthcare). The antibody of interest was injected over the anti-mouse IgG-immobilized surfaces followed by the injection of recombinant rat MMP-13 CD. Three kinetic parameters, the association rate constant (k_a), the dissociation rate constant (k_d), and the equilibrium dissociation constant (K_D), were obtained with BIAevaluation version 3.1 (GE Healthcare), using a 1:1 binding model. The K_D value ($K_D = k_d/k_a$) was used to evaluate the binding affinity of the antibody.

Evaluation of the MMP-13 Neutralizing Activity of the Anti-MMP-13 Antibody. The neutralizing activity of the developed antibody was determined by measuring the inhibitory effect of the antibody on the activity of recombinant MMP-13 using a synthetic peptide or native type II collagen as the substrate. For the assay using a peptide substrate, a fluorescence-quenching substrate for MMPs, (7-methoxycoumarin-4-yl) acetyl-Pro-Leu-Gly-Leu-[3-(2,3-dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-Arg-NH_2, was purchased from Peptide Institute (Osaka, Japan). This peptide, once cleaved by MMPs at the Gly−Leu bond, emits a strong fluorescence.[14] This peptide substrate was suspended in assay buffer [50 mM Tris-HCl, 10 mM $CaCl_2$, 300 mM NaCl, and 0.005% Brij35 (pH 7.6)], and 10 μL aliquots of this solution were placed in each well of a 384-well low binding plate (Corning, Acton, MA) together with 10 μL of the rat MMP-13 CD, which was preincubated for 2 h at 25 °C with anti-MMP-13 antibodies or control IgG. The plate was incubated for 1 h at room temperature; then the samples were excited at 340 nm, and the fluorescence emission intensity was measured at 405 nm with an EnVision plate reader.

For the assay using native type II collagen, bovine type II collagen (Chondrex, Redmond, WA) was immobilized in each well of a MaxiSorp plate by overnight incubation at 4 °C. After being blocked with BlockAce, the plate was incubated for 4 h at 37 °C with 50 μL of rat MMP-13 CD or the activated human pro-MMP-13. Then the reaction was stopped by the addition of EDTA at a final concentration of 5 mM. Collagenolytic activity was evaluated by the concentration of the type II collagen neoepitope in the supernatant, which was determined using a sandwich ELISA developed by our laboratory.[15]

Kinetic Analysis. Recombinant rat MMP-13 CD (2 pM) was incubated for 1 h at 25 °C with one of four graded concentrations (1.9−50 nM) of the anti-MMP-13 antibody in a 384-well low binding plate. The intramolecular fluorescence-quenching substrate (10 μL) was prepared in assay buffer at four graded concentrations (0.8−6.4 μM) and mixed with the treated rat MMP-13 CD described above (10 μL). After incubation at 25 °C for 1 h, the catalytic activity of the pretreated rat MMP-13 CD was evaluated by the fluorescence generated by the cleavage of the substrate.

Evaluation of the Neutralizing Activity of MMP-13 Released from Human Articular Chondrocytes. Type II collagen (Chondrex) was immobilized on each well of a polystyrene culture plate (Sumitomo Bakelite, Tokyo, Japan). After being extensively washed, 4×10^4 cells of normal human articular chondrocytes (Lonza, Basel, Switzerland) were placed in each well of the plate, and the cells were cultured at 37 °C with DMEM (Life Technologies) supplemented with 0.1 mg/mL BSA, 1× ITS+3 Liquid Media Supplement (Sigma-Aldrich), and 50 μM ascorbic acid in humidified air containing 5% CO_2. Twenty-four hours later, the medium of each well was replaced with fresh medium containing recombinant human IL-1β (1 ng/mL), Oncostatin M (10 ng/mL) (both from Sigma-Aldrich), and graded concentrations of the developed anti-MMP-13 antibody. Three days later, the enzymatic activity of MMP-13 was inhibited by the addition of EDTA (final concentration of 5 mM), and the amount of collagenolysis occurring during the culture period was evaluated by the concentration of type II collagen neoepitopes in the culture supernatant, measured by an ELISA.

Quantification of MMP-13 Released from Human Chondrocytes. The amount of MMP-13 released from human articular chondrocytes was determined as follows. Normal human chondrocytes were cultured as a monolayer until they reached full confluence. Growth medium was replaced with fresh medium in the presence or absence of IL-1β (1 ng/mL). Twenty-four hours later, the supernatants were collected and clarified by centrifugation. The concentration of MMP-13 was determined using a commercially available ELISA (R&D Systems) following the manufacturer's instructions. The amount of active MMP-13 in the supernatant was determined as follows. A MaxiSorp plate was coated with the anti-MMP-13 antibody (14D10) and blocked with BlockAce. Then the culture supernatants were added to each well of the plate and incubated at room temperature for 2 h. After being washed, the plate was incubated overnight at 4 °C with another anti-MMP-13 monoclonal antibody (Daiichi-fine Chemical, Toyama, Japan) biotinylated using Maleimide-PEG2-Biotin and europium-labeled streptavidin (PerkinElmer). The next day, the plate was washed and then treated with an enhancement solution (PerkinElmer), and the time-resolved fluorescence emission intensity was measured at 620 nm with a 340 nm excitation light using an EnVision plate reader.

Epitope Mapping. To determine the epitope of the developed anti-MMP-13 antibody, six subfragments of the immunogen peptide were generated using a 431A Peptide Synthesizer (Life Technologies, Carlsbad, CA). The epitope was determined by a competitive inhibition ELISA using these peptides as competitors.

RESULTS

Determination of the MMP-13 Peptide Sequence for Use as an Immunogen. In this study, we raised anti-MMP-13 neutralizing antibodies using a synthetic peptide as an immunogen. Determination of the peptide sequence is a critical point for successful antibody development. We carefully chose the peptide sequence considering the following points: (i) a unique sequence to MMP-13 to minimize cross reactivity

Table 1. Amino Acid Sequences of the Immunogen Peptide and the Corresponding Regions of Other Human MMPs

	amino acid sequence																				
MMP-13[a]	G	H	S	L	G	L	D	H	S	K	D	P	G	A	L	M	F	P	I	Y	T	Y
MMP-1	·	·	·	·	·	·	S	·	·	T	·	I	·	·	·	·	Y	·	S	·	·	F
MMP-2	·	·	A	M	·	·	E	·	·	Q	·	·	·	·	·	·	A	·	·	·	·	·
MMP-3	·	·	·	·	·	·	F	·	·	A	N	T	E	·	·	·	Y	·	L	·	H	S
MMP-7	·	·	·	·	·	M	G	·	·	S	·	·	N	·	V	·	Y	·	T	·	G	N
MMP-8	·	·	·	·	·	·	A	·	·	S	·	·	·	·	·	·	Y	·	N	·	A	F
MMP-9	·	·	A	·	·	·	·	·	·	S	V	·	E	·	·	·	Y	·	M	·	R	F
MMP-10	·	·	S	·	·	·	F	·	·	A	N	T	E	·	·	·	Y	·	L	·	N	S
MMP-11	·	·	V	·	·	·	Q	·	T	T	A	A	K	·	·	·	S	A	F	·	·	F
MMP-12	·	·	·	·	·	·	G	·	·	S	·	·	K	·	V	·	·	·	T	·	K	·
MMP-14	·	·	A	·	·	·	E	·	·	S	·	·	S	·	I	·	A	·	F	·	Q	W
MMP-15	·	·	A	·	·	·	E	·	·	S	N	·	N	·	I	·	A	·	F	·	Q	W
MMP-16	·	·	A	·	·	·	E	·	·	N	·	·	T	·	I	·	A	·	F	·	Q	·
MMP-17	·	·	A	I	·	·	S	·	V	A	A	A	H	S	I	·	R	·	Y	·	Q	G
MMP-19	·	·	A	·	·	·	G	·	·	R	Y	S	Q	·	·	·	A	·	V	·	G	·
MMP-20	·	·	A	·	·	·	A	·	·	T	·	·	S	·	·	·	Y	·	T	·	K	·
MMP-21	·	·	V	·	·	·	P	·	T	Y	R	T	·	S	I	·	Q	·	N	·	I	P
MMP-23	·	·	A	·	·	·	M	·	·	Q	H	G	R	·	·	·	L	N	A	·	L	R
MMP-24	·	·	A	·	·	·	E	·	·	S	·	·	S	·	I	·	A	·	F	·	Q	·
MMP-25	·	·	A	·	·	·	G	·	·	S	A	·	N	S	I	·	R	·	F	·	Q	G
MMP-26	·	·	·	·	·	·	Q	·	·	G	N	Q	S	S	I	·	Y	·	T	·	W	Y
MMP-27	·	·	A	·	·	·	S	·	·	N	·	Q	T	·	·	·	·	·	N	·	V	S
MMP-28	·	·	T	·	·	·	T	·	·	P	A	·	R	·	·	·	A	·	Y	·	K	R

[a]Sequence for the peptide immunogen (Gly^{214}–Tyr^{250}).

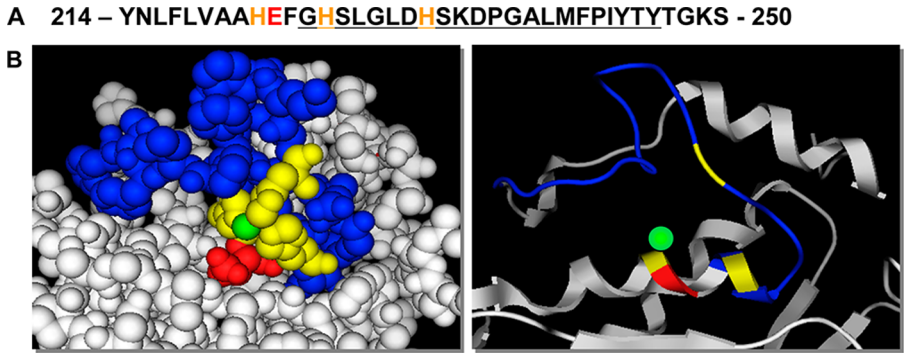

Figure 1. Catalytic region of human MMP-13. (A) Amino acid sequence surrounding the active site of human MMP-13. Amino acids at positions 214–250 are shown. Underlining indicates the amino acid sequence of the immunogen peptide. The catalytic center is colored with red, and the three histidines that bind the zinc ion are colored orange. (B) Three-dimensional structure of the catalytic region of MMP-13 (Protein Data Bank entry 1YOU) shown as a space-filling model (left) and a ribbon model (right). The zinc ion, catalytic center Glu^{223}, and His^{222}, His^{226}, and His^{232} that coordinate with the zinc ion in the catalytic region are colored green, red, and yellow, respectively. The region synthesized as the immunogen peptide is colored blue.

Table 2. Amino Acid Sequences of Human MMP-13 Chosen for Immunization and Corresponding MMP-13 Sequences of Other Animal Species

	amino acid sequence																					
human[a]	G	H	S	L	G	L	D	H	S	K	D	P	G	A	L	M	F	P	I	Y	T	Y
canine	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
rabbit	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
mouse	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
rat	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
guinea pig	·	·	·	·	·	·	E	·	·	·	·	·	·	·	·	·	·	·	V	·	·	·

[a]Sequence for the peptide immunogen (Gly^{214}–Tyr^{250}).

with other MMPs (Table 1), (ii) located close to the active site of the enzyme to obtain neutralizing antibodies (Figure 1A), (iii) located on the surface of the MMP-13 molecule that allows antibodies to have easier access (Figure 1B), (iv) should not form a secondary structure (Figure 1B), and (v) conserved sequence among species so that the antibody could be widely used for animal experiments (Table 2). On the basis of these points, a peptide sequence (GHSLGLDHSKDPGALMFPIY-

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TY) corresponding to the end of the active-site helix hB and the former half of the S1′ specificity loop of human MMP-13 was chosen as the immunogen.

Generation of Monoclonal Antibodies. Cell fusion was performed using spleen cells from mice immunized with the immunogen peptide−KLH conjugate, and seven monoclonal antibodies that bound to MMP-13 CD were obtained. These antibodies were tested for neutralizing activity and specificity besides the affinity for MMP-13 (Table 3). All seven antibodies did not react with MMP-1, -2, -3, -9, or -12 (data not shown), and five showed less than 10% cross reactivity with MMP-8, which had the highest degree of homology to MMP-13. Of four antibodies that had MMP-13 neutralizing activity, 14D10 (IgG2b/κ) showed the strongest neutralizing activity and minimal cross reactivity with other MMPs. Thus, antibody 14D10 was further characterized.

Table 3. Characteristics of Different Anti-MMP-13 Monoclonal Antibody Clones

	14D10	8D5	1D9	8G6	8H12	3D11	2G2
affinity for MMP-13, K_D (nM)	0.33	1.4	1.2	0.36	10	2.2	1.3
neutralizing activity[a]	++	+	++	+	−	−	−
cross reactivity with MMP-8 (%)[b]	1.2	6.2	110	9.4	250	9.7	6.6

[a]Legend: ++, IC_{50} of <100 nM; +, IC_{50} of <400 nM; −, no inhibition. [b]Cross reactivity was determined by a competitive ELISA and calculated by the relationship (IC_{50} of MMP-13/IC_{50} of MMP-8) × 100.

Specificity of 14D10 for MMP-13. The cross reactivity of 14D10 with other MMPs was determined by a competitive ELISA. The results demonstrated that 14D10 had strong binding activity for human, rat, and guinea pig MMP-13 with similar affinity (Figure 2A), but a much lower binding activity for MMP-1, -3, or -8 and no binding activity for MMP-2, -9, or -12 (Figure 2B). We also investigated the binding activity of 14D10 with human proMMP-13 before and after activation by APMA (Figure 2C). Interestingly, this antibody bound to the active form of MMP-13, but not to its latent form.

Neutralizing Activity of 14D10. The neutralizing activity of 14D10 was evaluated using the synthetic peptide or bovine type II collagen as a substrate. When the peptide substrate was used for the assay, the activity of rat MMP-13 CD was efficiently inhibited by 14D10 with an IC_{50} (concentration causing half-maximal inhibition) of 12 nM (Figure 3A). We investigated the kinetics of cleavage of the peptide substrate by rat MMP-13 CD, which had been treated with various concentrations of 14D10 prior to analysis. Treatment with 14D10 significantly reduced the V_{max} (maximal velocity) but had little effect on the K_m (Michaelis constant of kinetics), indicating that 14D10 likely inhibits the activity of MMP-13 in a noncompetitive manner (Figure 3B).

Next, the neutralizing activity was tested using native bovine type II collagen instead of the peptide substrate. As shown in Figure 4A, 14D10 inhibited the collagenolytic activity of rat MMP-13 CD with an IC_{50} of approximately 20 nM, similar to that for the peptide substrate shown in Figure 3A. Furthermore, 14D10 had an equivalent IC_{50} in the collagenolysis inhibition assay with APMA-activated human proMMP-13 (Figure 4B).

The neutralizing activity of 14D10 was evaluated in another experiment using primary cultured human articular chondrocytes. In this experiment, native type II collagen coated on the plastic plate was subjected to collagenolysis by collagenases released by chondrocytes. As assessed by the concentration of type II collagen neoepitopes, 14D10 suppressed collagenolysis in the human chondrocyte culture (Figure 5A). The IC_{50} was approximately 10-fold higher than that of the cell-free experiment (Figure 4B).

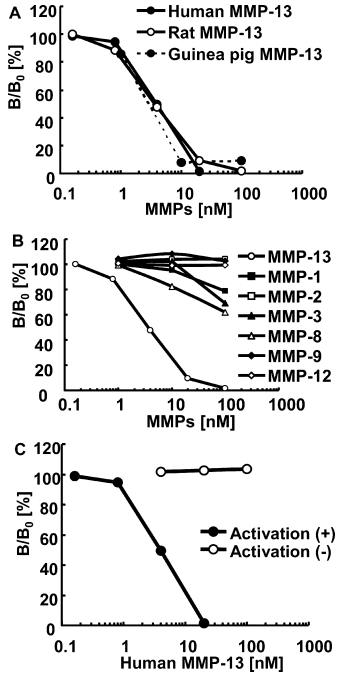

Figure 2. Binding characteristics of the MMP-13-neutralizing antibody 14D10. Binding (B/B_0 %) of 14D10 to various MMPs was evaluated using a competitive inhibition ELISA. (A) The assay was performed using activated human proMMP-13 and rat and guinea pig MMP-13 CDs as competitors at various nanomolar concentrations. (B) Competitive inhibition ELISA with activated human proMMP-1 and proMMP-8 with those of various human MMP CDs. (C) Competitive inhibition ELISA using recombinant human proMMP-13, before and after activation of the enzyme.

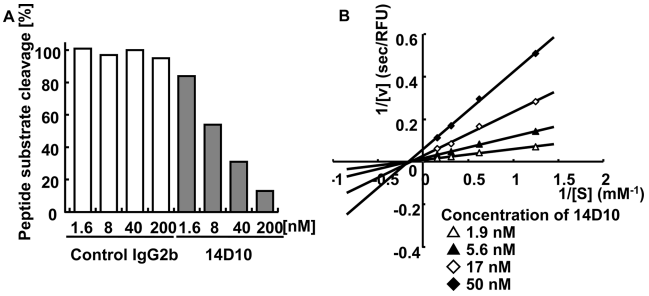

Figure 3. Evaluation of MMP-13 neutralizing activity of 14D10 using a peptide substrate. (A) Recombinant rat MMP-13 CD was incubated with the indicated concentrations of 14D10 or control IgG2b, and enzymatic activity was measured using a fluorescence quenching peptide substrate. MMP-13 neutralizing activity was measured as the percentage of peptide substrate cleavage. (B) Kinetic analysis of rat MMP-13 CD treated with 14D10. The catalytic activity was evaluated using a fluorescence quenching peptide substrate prepared at concentrations of 0.8, 1.6, 3.2, and 6.4 μM. Results are shown in Lineweaver−Burk double-reciprocal plots. The experiment was repeated twice, and the means are shown. RFU stands for relative fluorescence units.

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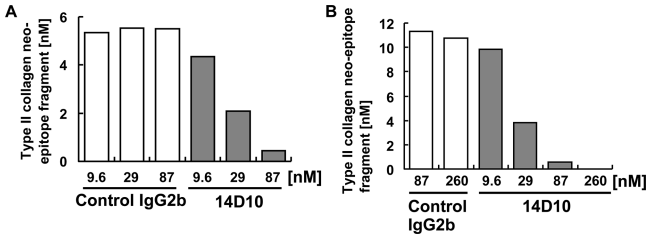

Figure 4. Evaluation of the MMP-13 neutralizing activity of 14D10 using type II collagen as a substrate. Rat MMP-13 CD (A) or APMA-activated human proMMP-13 (B) was preincubated with 14D10 or control IgG2b, and the collagenolytic activity was measured using bovine native type II collagen as a substrate.

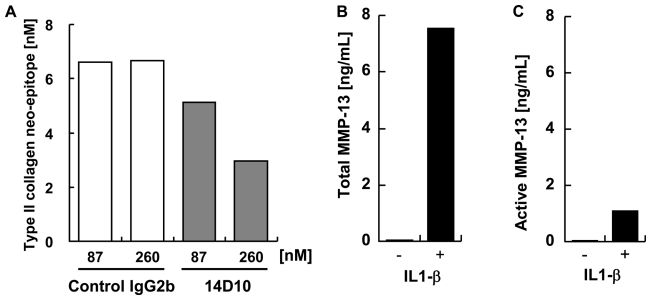

Figure 5. Inhibition of the collagenolytic activity of human chondrocyte culture by 14D10. Human articular chondrocytes were cultured as a monolayer on a plate coated with native type II collagen and stimulated by IL-1β and Oncostatin M to release collagenases, in the presence of the indicated concentrations of 14D10 or control IgG2b. Three days later, cleavage of type II collagen was estimated by the amount of type II collagen neoepitopes in the culture media. Human articular chondrocytes were cultured in the presence or absence of IL-1β for 24 h, and the amount of total MMP-13 (B) or active MMP-13 (C) in culture medium was determined by a conventional ELISA or a newly developed ELISA using 14D10, respectively.

Quantification of Active MMP-13 in Culture Supernatants of Human Articular Chondrocytes. Human articular chondrocytes were cultured for 24 h in the presence or absence of IL-1β, and the amount of total (latent and active) MMP-13 in the culture supernatants was determined by a conventional ELISA. Concentrations of MMP-13 in the medium increased dramatically following IL-1β treatment (Figure 5B). Next, we measured the amount of active MMP-13 in the medium using the ELISA developed with 14D10, which specifically recognized the active form of MMP-13 (Figure 5C) and might bind to MMP-13 regardless of the substrate concentration (implied by the noncompetitive mode of inhibition shown in Figure 3B). These results demonstrated that the active form comprised only 15% of the total MMP-13 released in the medium, underscoring the importance of measuring the amount of active enzyme, as well as the total enzyme.

Determination of the Epitope Recognized by 14D10. To determine the epitope for 14D10, we generated six subfragments of the immunogen peptide (Figure 6A) and evaluated the reactivity of 14D10 with respect to these peptides by a competitive inhibition ELISA. Consequently, 14D10 showed binding to peptide 3, 4, or 5 with an affinity similar to that for the immunogen peptide (peptide 6), a significantly weaker binding to peptide 2, and virtually no binding to peptide 1 (Figure 6B). These results indicated that the region around

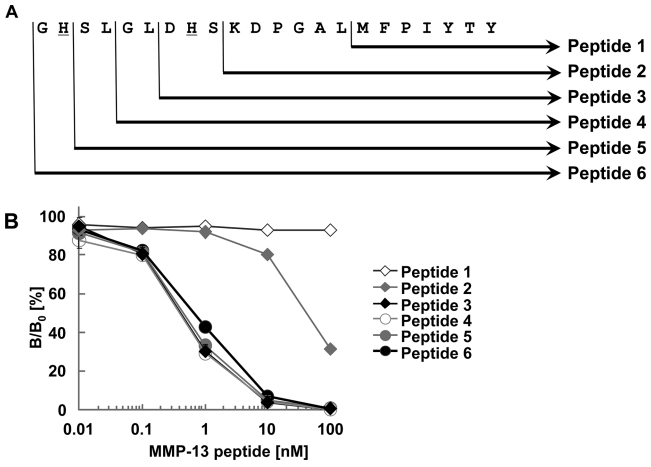

Figure 6. Determination of the epitope for 14D10. (A) Amino acid sequences of the six subfragments (peptides 1−6) used to determine the epitope recognized by 14D10. His^{226} and His^{232}, which bind to the zinc ion in the catalytic region, are underlined. (B) Results of the competitive inhibition ELISA using the indicated concentrations of the six subfragments.

His^{232} of the immunogen peptide is the most important for binding of the neutralizing antibody 14D10. Histidine at position 232 is involved in the coordination of zinc, which is essential for the catalytic activity of the enzyme.[16] As shown in Figure 2A, Asp^{231}, which is different from Glu^{231} of guinea pig MMP-13 (Table 2), might be less involved in the binding of 14D10. In addition, the adjacent Lys^{234}−Leu^{239} region may also contribute to the binding of 14D10 to MMP-13 to some extent.

DISCUSSION

This study developed an anti-MMP-13 neutralizing antibody using a synthetic peptide as the immunogen. The peptide sequence was selected on the basis of requirements for the immunogen: uniqueness to MMP-13, location adjacent to the active site, location on the molecular surface, no secondary structure formation, and conservation among animal species. The immunogen Gly^{225}−Tyr^{250} peptide sequence corresponded to the unstructured loop region between helices and was located downstream adjacent to the active center Glu^{223} (shown in Figure 1). Splenocytes from a mouse showing the highest antibody titers to rat MMP-13 CD after repetitive immunization with the peptide immunogen were used for hybridoma generation. In general, antibodies raised against peptides having partial sequences of target proteins may not have high affinities for the entire protein.[17] While the efficiency of antibody generation might be somewhat low, all seven antibodies obtained bound to rat MMP-13 CD with a high affinity as shown in Table 3. Furthermore, four antibodies (57%) had neutralizing activity for MMP-13. Finally, 14D10 was chosen as the best clone with a high binding affinity (K_D = 0.33 nM), neutralizing activity (IC_{50} = 12 nM), and specificity for MMP-13 (cross reactivity with MMP-8 = 1.2%). Furthermore, 14D10 recognizes the active form of human and animal MMP-13 but not the latent form. The epitope for 14D10, His^{232}−Leu^{239}, is located in the most exposed part of the S1′ specificity loop on the surface of the CD (Figure 1B).

A reasonable explanation for the mechanism of the neutralizing activity is that MMP-13 is inhibited from substrate binding by the antibody blocking the active site in a competitive manner. However, as shown in Figure 3B, the result of kinetic

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analysis revealed that 14D10 inhibited the catalytic activity of MMP-13 in a noncompetitive manner. Alternatively, the antibody may modify the active site. The epitope for 14D10 contains His[232], one of three histidine residues coordinating a zinc ion at the active site. Hence, antibody binding might change the spatial position of the zinc ion in the active site, resulting in abrogation of the catalytic capability of the enzyme. Although the precise mechanism remains to be elucidated, this antibody is likely to inhibit MMP-13 activity by modification of the CD but not by substrate competition.

Similar to other MMPs, MMP-13 is produced in vivo in an inactive latent form. To become enzymatically active, MMP-13 undergoes proteolytic processing to cleave the propeptide region.[18] Currently, the amount of active MMP-13 can be estimated only by indirect methods, for example, by measuring the collagenolytic activity.[19] In this study, we took advantage of the properties of 14D10 and developed a unique ELISA that can directly determine the amount of active MMP-13 present. The measurement of culture supernatants of human articular chondrocytes using this ELISA revealed that the active form comprised only ~15% of the total MMP-13 measured by the conventional ELISA (Figure 5B,C).

The activity of MMPs in vivo is precisely regulated by a number of critical steps, including synthesis and secretion, activation of the proenzyme, inhibition of the active enzyme, and localization and clearance of the enzyme.[9] Among these, activation of the proenzyme could be the most critical in regulating enzymic activity in vivo.[20] However, despite its importance, it has been difficult to determine the amount of the active form of MMPs, and there have been no reports of anti-MMP antibodies that selectively bind the active form. Here we describe neutralizing antibodies to the active form of MMP-13 that can be used as a tool to directly determine the amounts of the active form. We expect that the developed antibody would significantly facilitate our understanding of the role of MMP-13 under physiological and pathological conditions.

Neutralizing antibodies have previously been developed against MMP-9 or MMP-14/MT1-MMP.[21−23] REGA-3G12, a monoclonal antibody raised against human MMP-9 purified from neutrophils, binds with high affinity and inhibits the biological activity of MMP-9 but not that of MMP-2.[24] Although REGA-3G12 demonstrated that specific binding and neutralizing activity might be attained by the amino-terminal part of the CD surface but not the zinc ion binding part, where the precise epitope is or how the antibody inhibits MMP-9 activity is still unclear. DX-2400, a potent and highly selective MMP-14/MT1-MMP neutralizing antibody, was developed by Dyax Corp. using phage display technology with MMP-14 CD as the target.[21] This antibody inhibited MMP-14 with a K_i in the subnanomolar range in a competitive manner, indicating direct blockage of the catalytic site. However, it would be difficult to raise antibodies having the expected characteristics by immunization with the whole enzyme or the CD. Thus, a potent and selective neutralizing antibody should be separated from large quantities of nonspecific antibodies by appropriate screening, and the modes of inhibition and epitopes of the obtained antibodies would be unpredictable.

In another approach for developing neutralizing antibodies, an anti-gelatinase (MMP-2/MMP-9) antibody SDS3 was raised against the Zn tripod, a synthetic zinc−histidine complex mimicking the catalytic center of MMPs.[23] As expected, SDS3 bound to the zinc chelating region of the active form of MMPs. However, the MMP against which the antibody titer was raised was not predicted before immunization. Thus, although the synthetic Zn tripod might be a useful immunogen for the effective generation of MMP-neutralizing antibodies, it might not be suitable for generating neutralizing antibodies against a specific target.

In conclusion, we have developed a neutralizing monoclonal antibody against MMP-13 using a selective strategy in designing a peptide immunogen. This approach is very effective and can reproducibly make neutralizing antibodies against MMP-13 and, we are hopeful, other proteases or enzymes, and the significant findings of this work are the characteristics of the novel anti-MMP-13 antibody (14D10), which would be of great benefit for research of the pathology of MMP-13-related diseases such as OA.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

MMP-13, matrix metalloproteinase-13; OA, osteoarthritis; CD, catalytic domain; TMB, 3,3′,5,5′-tetramethylbenzidine.

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