Active Immunogene Therapy of Cancer with Vaccine On the Basis of Chicken Homologous Matrix Metalloproteinase-2

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ABSTRACT

Matrix metalloproteinase (MMP) family, in particular MMP-2, may play a key role in angiogenesis and tumor growth. It is conceivable that the breaking of immune tolerance of MMP-2 should be a useful approach to cancer therapy by active immunity. To test this concept, we constructed a plasmid DNA encoding chicken homologous MMP-2 (c-MMP-2) and control vectors. We found that the vaccine based on chicken homologous MMP-2 as a model antigen could induce both protective and therapeutic antitumor immunity. Autointoxtobodies against MMP-2 in sera of mice immunized with c-MMP-2 could be found by Western blotting analysis and ELISA assay. There was the deposition of autoantibodies within the tumor. IgG1 and IgG2b were substantially increased in response to c-MMP-2 immunization. The elevation of MMP-2 in the sera of tumor-bearing mice was abrogated with the vaccination of c-MMP-2. Transmigration of human endothelial cells and tumor cells through gelatin-coated filters was inhibited with immunoglobulins isolated from mice immunized with c-MMP-2. The gelatinase activity of MMP-2, including both latent MMP-2 (M, 72,000) and active MMP-2 (M, 66,000) derived from tumor tissues, was apparently inhibited by the vaccination with c-MMP-2. The antitumor activity and the inhibition of angiogenesis were acquired by the adoptive transfer of the purified immunoglobulins. The antitumor activity and production of autoantibodies against MMP-2 could be abrogated by the depletion of CD4 T lymphocytes. Angiogenesis was apparently inhibited within tumor, and chick CAMs angiogenesis was also inhibited. Thus, our findings may provide a vaccine strategy for cancer therapy through the induction of an autoimmune response against MMP-2 in a cross-reaction by the immunization with the single xenogeneic homologous MMP-2 gene and may be of importance in the additional exploration of the application of other xenogeneic homologous genes identified in human and other animal genome projects in cancer therapy.

INTRODUCTION

ECM degradation is an essential procedure involved in normal physiological actions such as embryo development, wound healing, bone resorption, and mammary involution (1, 2). Inappropriate breakdown of ECM components is thought to play an important role in pathological conditions, including arteriosclerosis, rheumatoid arthritis, and tumor invasive growth and metastasis (3). The MMPs are a family of zinc-dependent endopeptidase responsible for the degradation of the ECM (4). Numerous pathological and clinical studies have shown that the MMPs, associated with an aggressive malignant phenotype and poor prognosis in patients with cancer, are frequently overexpressed in various solid tumor cells and peritumoral stromal cells with higher tumor grade and stage (5–7). In vivo, elevated stromal MMP-2 and MMP-9 activity is highly correlated with increased metastatic potential in most malignant tumor (8, 9). Increased activity of MMPs appears to allow tumor to remodel its surrounding microenvironment, to grow in a permissive space, and to develop supporting stroma, including angiogenesis (10, 11). Although several members of the MMP family are involved, MMP-2 may play a pivotal role in these events (12–19). As the understanding of the multiple effects of MMPs on tumor growth and angiogenesis has increased, therapeutic efforts focused on blocking the enzymes have rapidly developed. The broad spectrum MMP inhibitors, including Marimastat and its analogue Batimastat, have shown their potential role in inhibiting tumor growth and metastases in preclinical experiments. Several MMP inhibitors have been tested for clinical trials in the patients with cancer (20, 21). However, Marimastat and Batimastat have been disappointing in a Phase III clinical trial (22). Clearly there is a need for a new strategy for developing therapeutic regimens by the abrogation of MMPs, in particular searching for more specific agents that will block particular MMPs.

It is conceivable that the breaking of immune tolerance against MMPs involving tumor growth should be used as another useful approach for cancer therapy with active immunity. However, the immunity to MMPs is presumably difficult to elicited with vaccine based on self or syngeneic MMP molecules because of the immune tolerance acquired during the development of the immune system.

Many genes were highly conserved during the evolutionary process, which was characterized by the gene similarity to varying degrees among different species. Many counterparts of the genes of humans and mice can be identified from the genome sequence of the fruit fly Drosophila melanogaster and of the other animals such as the chicken (23). For example, MMPs have been cloned from a wide variety of species such as mammals, birds, frogs, hydra vulgaris, and plants (10, 24, 25). The ubiquitous nature of MMP family results from their conservative evolution. MMP-2 in mouse has been known to be homologous with those in humans and other species to varying extent (10). Sequence comparison analysis by searching the SwissProt database in this study indicates that the primary sequence of mouse MMP-2 at the amino acid level is 82% and 91% identical with chicken and human homologues, respectively. In addition, it has been reported that the abrogation of MMP-2 alone inhibits the transition from the prevascular to the vascular stage during tumor development and subsequently inhibits tumor growth (14). Moreover, tumor-induced angiogenesis and the invasion and metastasis of tumor cells were suppressed in MMP-2-deficient mice (19). These findings indicated that MMP-2 alone plays an important role in angiogenesis and tumor growth.
growth. Thus, the current studies explore the feasibility of immuno- 
gene therapy of tumors with the vaccine based on chicken homolo-
gous MMP-2 as a model antigen by the breaking of immune tolerance 
among MMP-2 in a cross-reaction between the xenogeneic homolo-
gous and self-molecules.

To test this concept, we constructed a c-MMP-2. At the same time, 
the plasmid DNA encoding the corresponding mouse self-MMP-2 
(m-MMP-2) and e-p were also prepared and used as controls. The 
vaccines were tested for the ability to induce antitumor immunity in 
tumor models in mice.

MATERIALS AND METHODS

**Vaccine Preparation.** The chick MMP-2 cDNA was amplified from total 
RNA isolated from primary cultures of chicken embryo fibroblast by RT-PCR 
using primers derived from the published sequence of chick MMP-2 (GenBank accession no. U0775; Ref. 24). The mouse MMP-2 cDNA was amplified from mouse kidney cDNA library (Clontech Laboratories, Inc., Palo Alto, CA) using primers derived from the published sequence of mouse MMP-2 (GenBank accession no. NM 008610; Ref. 25). When the products of the expected size were obtained, they were inserted into pT-Adv plasmids (Clontech Laboratories, Inc., and subcloned into pcDNA3.1 (Invitrogen, San Diego, CA). MMP-2 of chick and mouse inserted into pcDNA3.1 was named c-
MMP-2 and m-MMP-2, respectively. As a control, pure plasmid was used as 
e-p. The full-length sequence of chick and mouse MMP-2 was confirmed by 
dideoxy sequence to be identical to those reported previously (24, 25). Plas-
mids for DNA vaccination were purified by using two rounds of passage over 
Endo-free columns (Qiagen GmbH, Hilden, Germany), as reported previously 
(26). The expression of plasmid DNA was confirmed in the transfected cells by 
RT-PCR and by anti-MMP-2 antibodies in Western blotting analysis and ELISA.

**Tumor Models and Immunization.** Meth A fibrosarcoma and H22 hep-
atoma models were established in BALB/c mice (26, 27). LLC model was 
established in C57BL/6 mice. These tumor cell lines expressed MMP-2, as 
confirmed by Western blot analysis with commercially available antibody and 
RT-PCR. Mouse were immunized with different doses (5–150 μg/mouse) of 
DNA vaccine in 0.9% NaCl solution by i.m. injection once a week for 4 weeks. 
Additional control animals were injected with 0.9% NaCl solution. Tumor 
dimensions were measured with calipers, and tumor volumes were calculated 
according to the formula: width^2 x depth/2 (8). All studies involving mice were 
approved by the institute’s animal care and use committee.

**Metastasis Assays.** Spontaneous metastasis of LLC was established as 
described previously (28). Briefly, C57BL/6 mice received i.m. injections of 
with 10^7 LLC cells in 100 μl of PBS. Then animals were randomly 
divided into four groups (10 mice/group). Treatment with 100 μl of c-MMP-2, 
m-MMP-2, or e-p or 100 μl of 0.9% NaCl solution alone were administered 
i.m. weekly, beginning on day 14 before tumor implantation. Mice were 
sacrificed on day 21 after inoculation, and in each animal, lungs were removed 
and surface lung metastases were also measured and scored as being ≤ or >3 
mm (8). The lungs of animals were also fixed in 10% buffered formalin followed by 
counting of metastatic nodules for histological investigation.

**MMP-2 Level in Serum Determined by ELISA.** The level of MMP-2 in serum 
in serum after i.m. administration of DNA plasmid was assayed by ELISA using 
a protocol similar to one previously described by us (29). Briefly, sera from 
mice in each group were collected just before the immunization and every 
3 days for 4 weeks after the first immunization. Ninety-six-well microtiter plates 
(Falcon) were coated with recombinant MMP-2 (Santa Cruz Biotechnology) 
in 50 mM carbonate buffer (1/200 dilution) at 4°C overnight, blocked, and washed. Sera diluted serially (100 μl/well) were added and 
incubated for 1 h at 37°C. Mouse monoclonal anti-MMP-2 (Santa Cruz 
Biotechnology) diluted 1/400 was added (100 μl/well) and incubated for 1 h at 
37°C. Alkaline phosphatase-conjugated rabbit antirabbit IgG (Sigma-Aldrich, 
St. Louis, MO) diluted 1/3000 was added (100 μl/well) and incubated for 1 h at 
37°C. After washing four times in washing buffer, phosphatase substrate 
P-nitrophenyl phosphate (Sigma-Aldrich) was added for 30 min, and absorb-
ance was measured at 405 nm with an ELISA reader (Bio-Rad, Hercules, CA). 
Recombinant MMP-2 was used as a standard. The concentration of MMP-2 in 
each sample was determined by interpolation from the standard curve. The 
antibodies used above showed positive reaction not only to the purified mouse 
MMP-2, but also to chicken MMP-2, as confirmed in our experiments.

**Immunglobulin Subclass Response to c-MMP-2 Immunization in ELISA.** The immunoglobulin subclass was determined by ELISA as 
described previously (30, 31). Briefly, microtiter plates were coated with recom-
binant chicken or mouse MMP-2 protein (0.5 μg/well) in 100 μl of coating 
buffer [sodium carbonate (pH 9.6)] and incubated at 4°C overnight. Nonspe-
cific binding was blocked with 2% milk powder in PBST for 2 h. Murine sera 
diluted serially were added to the wells. Plates were incubated for 2 h at 37°C, 
washed, and then incubated with horseradish peroxidase-conjugated antiguinea pig IgG1, IgG2a, IgG2b, IgM, or IgA. The ELISA was developed using 2,2’-
azino-di-[3-ethyl-benzthiazolinesulfonate (6) as the substrate (Roche Diagnos-
tics, Mannheim, Germany), and absorbance was measured at 405 nm with an 
ELISA reader (Bio-Rad). The recombinant chicken or mouse MMP-2 and MMP-9 were expressed, refolded, and purified from *Escherichia coli*, as detailed previously (32). The purified mouse latent MMP-2 was also obtained from Calbiochem (La Jolla, CA).

**Purification of Immunglobulin and Adoptive Transfer in Vivo.** Immunglobulins were purified from the pooled sera derived from the mice at day 
7 after the fourth immunization or from control mice by affinity chromatog-
raphy (CM Affi-gel Blue Gel Kit; Bio-Rad). To assess the efficacy of immunglobulins in antitumor activity in vivo, the purified immunoglobulins (10– 
300 μg/mouse) were adoptively transferred i.v. 1 day before mice were challenged with 1 x 10^6 to 1 x 10^7 tumor cells and then treated twice per week for 
3 weeks as described previously (26). As a control, immunoglobulins before 
adoptive transfer were adsorbed by the immunoabsorption method as detailed 
previously (33). Briefly, immunoglobulins were incubated with Talon metal 
affinity resin (Clontech Laboratories, Inc.)-bound recombinant mouse MMP-2 
or MMP-9 at 4°C overnight with gentle rocking. The removal of the antitumor 
activity of MMP-2 was confirmed by ELISA against MMP-2.

**In Vivo Depletion of Immune Cell Subsets.** Immune cell subsets were 
depleted as described previously (26, 34). Mice received i.p. injections of 500 
μg of either the anti-CD4 (clone GK1.5, rat IgG), anti-CD8 (clone 2.43, rat 
IgG), anti-NK (clone PK136) mAb, or isotype controls 1 day before the 
immunization and then immunized with 100 μg of plasmid once a week for 4 
weeks. Mice were challenged with tumor cells (LLC and Meth A, 2 x 10^6) 
after the fourth immunization. These hybridomas were obtained from Ameri-
can Type Culture Collection. The depletion of CD4^+ , CD8^+ , and NK cells was 
consistently >98%, as determined by flow cytometry (Coulter Elite Esp, 
Coulter, Hialeah, FL).

**Western Blot Analysis.** Western blot was done as described previously 
(30). Briefly, cells were lysed in 1 ml of lysis buffer. Proteins (50 μg/lane) were 
separated by SDS-PAGE. Gels were electrophoretically transferred onto 
polyvinylidene fluoride membrane. The membrane blots were blocked, washed, then washed and incubated with a biotinylated secondary antibody (Biotinylated horse antirabbit IgG or IgM), followed by Vectorstain ABC Vector 
Laboratories, Burlingame, CA). To ensure equivalent loading of each cell 
ylate by the Western Blot analysis, we measured protein content in the 
cell lysates by a colorimetric assay (Bio-Rad) before loading. Also, 0.5 μg of 
the purified mouse MMP-2 protein was used as positive control.

**Immunohistochemistry.** To identify the autotransfused deposited in tumor 
tissue and its surrounding stroma, immunofluorescent staining was performed 
as described previously (26); briefly, frozen sections were fixed in acetone, 
washed with PBS, and incubated with FITC-conjugated goat antibody against 
mouse IgG, IgA, or IgM (Sigma-Aldrich). Slides were examined by fluores-
cence microscopy. For the determination of vessel density in tumor tissue, the 
sections were processed and stained with rat antimonuice CD31 antibody 
(Pharmingen, San Diego, CA) as described previously.

**Elution of Autotransfused from the Tumor Tissues.** IgG deposited in the tumor 
tissue was collected by immunoprecipitation and by acid elution, as 
described previously (35). Briefly, tumor extracts isolated from mice immu-
nized with c-MMP-2, m-MMP-2, or e-p or from nonimmunized mice were 
precipitated with protein G-beads; the precipitates were eluted with 0.1 M citric 
acid (pH 3.0), followed immediately by neutralization [3 μl NaCl, 1.5 M glycine (pH 8.9)]. Immunoreactivity of the eluates was examined against mouse recombinant MMP-2 protein in Western blot analysis.

**Cell Transmigration Assay in Vivo.** Tumor cells and HUVEC transmig-
ration assays were performed in 8-μm pore Transwell chambers (Costar,
Badhoevedorp, The Netherlands) as described previously (36). Briefly, cells were resuspended in serum-free medium and seeded at 15,000 cells/well on gelatin-coated filters in the presence of the purified immunoglobulins (10–200 μg/ml) from mice vaccinated with c-MMP-2, m-MMP-2, e-p, or 0.9% NaCl solution on the upper chamber. As a control, cells were treated with the immunoglobulins adsorbed by mouse MMP-2 as described above. Lower wells of the chambers were filled with medium supplemented with 2% BSA and 10% FBS. Migration assays were performed for 8 h at 37°C.

Gelatin Zymography. Gelatinase activity was determined by gelatin zymography as described previously (8, 36). Briefly, tumor tissues were homogenized in cold lysis buffer. Proteins (100 μg/lane) were separated, without prior denaturation, by electrophoresis through SDS/polyacrylamide gels containing 1 mg/ml gelatin. The gel was then incubated in 2.5% Triton X-100 for 2 h to remove SDS and then incubated for development of enzyme activity, stained with Coomassie brilliant blue, and destained in methanol/acetic acid. Gelatinase activity was detected as unstained bands on a blue background. Protein content in the cell lysates was determined by a colorimetric assay (Bio-Rad). Densitometric analysis for the quantitative determination of gelatinase activity was performed on scanned images with the Multi-Analyzer software from Bio-Rad, as described previously (36).

Chick CAM Assay. CAM assays were carried out as described previously (37). Three-day-old fertilized eggs were cut a window in the eggshell and sealed with transparent tapes. After 3 days of incubation, filter discs (5-mm diameter) containing various doses of immunoglobulins (0–300 μg) isolated from experimental mouse sera were applied to the CAM of individual embryo. Forty-eight h later, CAMs were harvested and observed under stereomicroscope.

RESULTS

Tumor Growth Delay. Mice were immunized i.m. once weekly continuously for 4 weeks with different doses (5–150 μg/mouse) of c-MMP-2, m-MMP-2, e-p, or 0.9% NaCl solution alone and then challenged with 1 × 10^5 10^5 Meth A fibrosarcoma, H22 hepatoma and LLC cells at day 7 after the fourth immunization. As shown in Fig. 1, tumor grew progressively in all nonimmunized mice and mice immunized with m-MMP-2 or e-p, but there was apparent protection from tumor growth in mice vaccinated with c-MMP-2 (Fig. 1, A–C). Furthermore, the survival of the tumor-bearing mice treated with c-MMP-2 was also longer than that of the nonimmunized mice or mice immunized with m-MMP-2 or e-p (Fig. 1, D–F). To explore the therapeutic efficacy of c-MMP-2 vaccine, we treated the mice on day 4 after tumor cells implant. Treatment with c-MMP-2 once weekly resulted in significant antitumor activity. The survival of the tumor-bearing mice treated with c-MMP-2 was also significantly greater than those of the controls (Fig. 1, G–I). The dose (100 μg/mouse) used in Fig. 1 is an optimal one selected for immunization in several preliminary experiments. Treatment with a 150-μg dose did not show greater effect than that with 100 μg. Treatment with a 5–15-μg dose shows little effect.

The mice immunized with these vaccines have been, in particular, investigated for the potential toxicity. No adverse consequences were indicated in gross measures such as weight loss, ruffling of fur, life span, behavior, and feeding. Furthermore, no pathologic changes of liver, lung, kidney, spleen, brain, heart, and so on were found by the microscopic examination.

Fig. 1. Induction of protective antitumor immunity and induction of therapeutic antitumor immunity. Mice (10 mice in each group) were immunized i.m. with 100 μg of c-MMP-2 (●), m-MMP-2 (□), e-p (▲), or 0.9% NaCl solution alone (▲) once a week for 4 weeks and then challenged with 2 × 10^6 Meth A (A and D) or H22 (B and E) and LLC (C and F) cells s.c. 1 week after the fourth immunization. Mice (10 mice in each group) treated with i.m. injection of 100 μg of c-MMP-2 (●), m-MMP-2 (□), e-p (▲), or 0.9% NaCl solution alone (▲) once weekly for 4 weeks starting at day 7 after 2 × 10^6 Meth A (G) or H22 (H) and LLC (I) cells were introduced s.c. into mice. Significant difference was found in tumor volume (P < 0.05) between c-MMP-2-treated and control groups. A significant increase in survival in c-MMP-2-treated mice, compared with the control groups (P < 0.01, by log rank test), was found with three tumor models.

Fig. 2. Reduction in pulmonary metastases with the vaccination of c-MMP-2. Treatment with 100 μg of c-MMP-2, m-MMP-2, e-p, or 0.9% NaCl solution (NS) alone were administered i.m. weekly, beginning on day 14 before the tumor implantation. Mice were sacrificed on day 21 after inoculation, and in each animal, lungs were removed and surface lung metastases were also measured and scored as being ≤ or > 3 mm. Spontaneous metastasis of LLC was established as described in “Materials and Methods” section. Treatment with c-MMP-2 reduces lung metastases. Mean numbers of lung tumor nodules in each group are shown. The percentage of metastatic foci > 3 mm are marked as solid bars (■).
Reduction of Pulmonary Metastases. The murine LLC model was used to determine whether immunization with chicken MMP-2 vaccine in mice might also suppress the implantation and growth of metastatic tumor. We examined the lungs of mice injected i.m. with $2 \times 10^5$ LLC cells for evidence of metastasis. Most of the mice treated with control plasmids or 0.9% NaCl solution developed macroscopic lung metastases, whereas only 2 of 10 mice developed tumor nodules in the lungs when treated with c-MMP-2. The mean number of lung metastases per affected mouse vaccinated with c-MMP-2 was reduced to 33–50% of control groups (Fig. 2). The percentage of metastatic nodules $>3$ mm was assessed as an indicator of angiogenesis because tumors $>3$ mm are thought to require an effective vasculature (38). The percentage of lung tumor nodules $>3$ mm was decreased from 60 to 20% in mice treated with c-MMP-2. Lung weight, which correlates with total tumor burden, was also reduced to 40–60% of control groups.

Serum MMP-2 Levels in Response to the Vaccination with c-MMP-2. Tumor-bearing mice received i.m. injection of 100 $\mu$g of c-MMP-2 or control plasmid DNA or 0.9% NaCl solution alone once a week for 4 weeks. Serum was collected, and MMP-2 levels in serum were determined by ELISA. Treatment with c-MMP-2 or m-MMP-2 resulted in slight elevation of MMP-2 in serum on days 6 and 12, compared with the controls ($P < 0.01$), but MMP-2 levels in mice treated with c-MMP-2 were apparently reduced after day 18 (Fig. 3; $P < 0.01$). In contrast, treatment with m-MM-2 showed persistent and more apparently elevated level of MMP-2 after day 18. In addition, MMP-2 levels in sera were higher in tumor-bearing mice than in normal mice.

Characterization of Autoantibodies against Mouse MMP-2. Sera from mice immunized were assayed for the presence of the autoantibodies against MMP-2 and immunoglobulin subclass by ELISA. Sera obtained from mice immunized with c-MMP-2 showed substantial increases in IgG1 and IgG2b reacting specifically to mouse MMP-2 or chicken MMP-2 protein, compared with the control samples (Fig. 4A). Furthermore, Sera from mice immunized were also assayed for the presence of autoantibodies against MMP-2 in Western blotting analysis. Sera from mice immunized with c-MMP-2 recognized a single $M_r 72,000$ band in LLC, Meth A, H22, and HUVEC cell, with the same size as purified mouse MMP-2 (Fig. 5A), but the sera isolated from control groups showed the negative staining (Fig. 5B). As a control, commercially available rabbit antimonouc MMP-2 antibody (Fig. 5C), also recognized a single $M_r 72,000$ band, with the same molecule size as sera from mice immunized with c-MMP-2 did.

To identify possible deposition of autoantibodies in tumor and its surrounding stroma, we investigated the tissues by immunofluorescence staining. There was the deposition of IgG in the tumor tissues and its surrounding stroma from mice immunized with c-MMP-2 but not in those from mice immunized with m-MMP-2, e-p, or the nonimmunized (Fig. 6A). No IgM- or IgA-specific fluorescence was found. In addition, there was no immunoglobulin deposition in the immunized or nonimmunized mice in normal stroma in the major organs such as kidney, liver, spleen, and brain. To investigate whether the deposited IgG within tumor tissues is anti-MMP-2 antibody, we have isolated IgG from the tumor tissue extract by the immunoprecipitation with protein G-beads and acid elution. Eluate from the mice immunized with c-MMP-2 contained abundant IgG relative to that from the mice immunized with m-MMP-2 or e-p or from nonimmunized mice. The isolated IgG recognized the purified MMP-2 in Western Blotting analysis (Fig. 6B), indicating that the deposited IgG is anti-MMP-2 antibody.

![Fig. 3. MMP-2 levels in serum in response to the vaccination of c-MMP-2. Mice (10 mice in each group) treated with i.m. injection of 100 $\mu$g of c-MMP-2, m-MMP-2, e-p, or 0.9% NaCl solution (NS) alone once weekly for 4 weeks starting at day 7 after $2 \times 10^5$ Meth A cells were introduced s.c. into mice. Sera from mice in each group were collected just before the immunization and every 3 days for 4 weeks after the first immunization. MMP-2 level in each serum sample was determined using an ELISA kit. Data were expressed as mean $\pm$ SD.](image)

![Fig. 4. Abrogation of immunoglobulin subclass response and antitumor activity by the depletion of immune cell subsets. A, sera obtained from mice immunized with c-MMP-2 were tested against mouse MMP-2 or chicken MMP-2 by ELISA. Immunization with c-MMP-2 showed an apparent elevation of IgG1 and IgG2b reacting specifically to mouse MMP-2 or chicken MMP-2 protein, compared with the control samples (Fig. 4A). Furthermore, Sera from mice immunized were also assayed for the presence of autoantibodies against MMP-2 in Western blotting analysis. Sera from mice immunized with c-MMP-2 recognized a single $M_r 72,000$ band in LLC, Meth A, H22, and HUVEC cell, with the same size as purified mouse MMP-2 (Fig. 5A), but the sera isolated from control groups showed the negative staining (Fig. 5B). As a control, commercially available rabbit antimonouc MMP-2 antibody (Fig. 5C), also recognized a single $M_r 72,000$ band, with the same molecule size as sera from mice immunized with c-MMP-2 did.](image)
Transmigration of human endothelial cells and tumor cells through gelatin-coated filters was inhibited by immunoglobulins isolated from mice immunized with c-MMP-2 by an average of 62 and 51%, respectively, compared with control groups (Fig. 7A). The adsorption of the immunoglobulins with purified c-MMP-2 antigen could abrogate their inhibitory effect on the cell transmigration.

Furthermore, protective antitumor effect was tested with immunoglobulins isolated from mice immunized with c-MMP-2 and control vaccines or from nonimmunized mice. The purified immunoglobulins were adoptively transferred into mice 1 day before mice were challenged with tumor cells and then mice were treated for 3 weeks. Adoptive transfer of immunoglobulins isolated from mice vaccinated with c-MMP-2 into naïve mice was effective in affording protection from tumor challenge. Adsorption of immunoglobulins with recombinant MMP-2 protein before adoptive transfer could abrogate its antitumor activity (Fig. 7B).

Role of CD4+ T Lymphocytes in the Induction of Antitumor Immunity by the Immunization with c-MMP-2. The mice depleted of CD4+ T lymphocytes did not develop detectable antibodies against the lysates of MMP-2-positive tumor cells (Fig. 4A). In contrast, treatment with anti-CD8 or anti-NK mAb had no effect. Also, the mice depleted of CD4+ T lymphocytes were not protected from tumor challenge in c-MMP-2-vaccinated mice (Fig. 4B). These data suggest that CD4+ T cells may be required for the antitumor immunity by the vaccination with c-MMP-2.

Decrease in Gelatinase Activity in the Tumors. We next examined the MMP-2 activity in tumor tissues from mice immunized with c-MMP-2, m-MMP-2, or e-p or nonimmunized mice using gelatin zymography (Fig. 8A) and densitometric analysis for the quantitative determination of gelatinase activity (Fig. 8B). As shown in Fig. 8A, the gelatinase activity of MMP-2, including both latent MMP-2 (M, 72,000) and active MMP-2 (M, 66,000), derived from three murine tumor models was apparently inhibited by the vaccination with c-MMP-2, compared with control groups (P < 0.01). However, the vaccination did not inhibit the gelatinase activity of MMP-9. The inhibition of MMP-2 was also confirmed by Western blot analysis (Fig. 8C). These findings indicate that the activity of MMP-2 is impaired by the immunization with c-MMP-2 in mice.

Inhibition of Angiogenesis. Because MMP-2 may play an important role in the degradation of basement membrane in microvessel walls and penetration of neovascular sprouts during tumor angiogen-
esis, we suppose that the reduction of tumor growth and metastasis induced by immunization with plasmid encoding chicken MMP-2 may be involved in the inhibition of angiogenesis. Angiogenesis within tumor tissue was estimated counting the number of microvessels on the section staining with an antibody reactive to CD31. The vaccination with c-MMP-2 resulted in apparent inhibition of angiogenesis in tumors, compared with control groups (Fig. 9). Similar findings were confirmed in three tumor models. Also, angiogenesis was inhibited using immunoglobulins isolated from mice vaccinated with c-MMP-2 in chick CAM assay, whereas those from control groups had no effect on neovascularization. In addition, the pretreatment of the immunoglobulins from c-MMP-2-immunized mice with recombinant MMP-2 protein could abrogate their inhibitor effect on neovascularization (Fig. 10).

DISCUSSION

Among the many different immunotherapeutic strategies that are currently being evaluated, active specific immunotherapies with cancer vaccines based on tumor antigens represent very promising approaches for cancer therapy (39). However, to date, with the few exceptions of melanoma tumor antigens, there is still limited information on the identity and density of antigenic peptides and CTL epitopes presented by human solid tumors (39). Efforts are therefore continuing to develop new strategy for cancer vaccines.

Remodelling of the ECM, mediated in part by MMPs, is important for the entire process of cancer etiology, progression, and metastasis (1, 2). MMPs facilitate tumor invasion and metastasis by several distinct mechanisms. It has been established that tumor cells must have the ability to degrade components of the ECM, to attach and detach from ECM, to expand through defects in the ECM, and to stimulate the generation of new blood vessels during cancer progression (10). Recent evidence supports the view of the role of MMPs not only in breakdown of physical barriers but also in modulating cell migration, promoting blood vessel penetration, and uncovering cryptic sites of other proteins with special biological activities (10). Tumor...
cells are believed to use the increased activity of these enzymes to invade locally and metastasize systemically. Stromal therapy emerged as a viable approach to cancer prevention, and intervention can be applied at multiple points in the treatment cascade (40). Antiproteolytic by the abrogation of the activity of MMPs, particularly in the context of angiogenesis, has become a promising target for anti-invasion treatment (21, 22).

Several observations have been made in this study concerning the vaccine based on chicken homologous MMP-2 as a model antigen and antitumor immunity. The vaccine based on chicken homologous MMP-2 as a model antigen could induce both protective and therapeutic antitumor immunity. Autoimmune response against MMP-2 may be provoked in a cross-reaction by the immunization of chicken MMP-2, and the autoantibody targeting to MMP-2 is probably responsible for the antitumor activity. These suggestions are supported by our findings in this study. There was the deposition of autoantibodies against MMP-2 within tumor. Autoantibodies against MMP-2 could be found by Western blot analysis and ELISA assay. The elevation of MMP-2 in the sera of tumor-bearing mice was abrogated with the vaccination of c-MMP-2. Transmigration of human endothelial cells and tumor cells through gelatin-coated filters in vitro was inhibited. The inhibition of the gelatinolytic activities in tumors of mice immunized with c-MMP-2 was found using gelatin zymography. The antitumor activity and the inhibition of angiogenesis were acquired by the adoptive transfer of the purified immunoglobulins. IgG1 and IgG2b were substantially increased in response to c-MMP-2. There were the antitumor activity and production of autoantibodies against MMP-2 that could be abrogated by the depletion of CD4+ T lymphocytes. Angiogenesis was apparently inhibited within tumors, and chick CAMs angiogenesis was also inhibited. On the basis of our findings mentioned above, we may rule out the possibility that the antitumor activity with chicken homologous c-MMP-2 may result from the nonspecifically augmented immune response against the tumor growth in host mice.

A number of studies have shown that synthetic MMP inhibitors can inhibit angiogenesis and tumor growth (20, 21). However, few of these inhibitors were specific to a particular MMP (40). Furthermore, the broad-spectrum MMP inhibitors Marimastat, and its analogue Batimastat have been disappointing in a Phase III clinical trial (22). Clearly there is a need for a new strategy for blocking particular MMPs. The important role of MMP-2 alone in angiogenesis and tumor growth is supported by a number of important experimental observations (12–19). For example, it has been shown that tumor-induced angiogenesis, and the invasion and metastasis of tumor cells were suppressed in MMP-2-deficient mice (19). The suppression of MMP-2 alone by antisense oligonucleotides has a profound effect on the angiogenic phenotype of tumor and subsequently inhibits tumor growth (13). Prevention of liver metastasis of human colon cancer can be achieved by selective inhibition of MMP-2 (16). Endothelial cells have been shown to produce MMP-2 during differentiation into capillary tube-like structures, and exogenous addition of MMP-2 was shown to enhance this process (14). In addition, MMP-2 is associated with the risk for a relapse in postmenopausal patients with node-positive breast carcinoma treated with antiestrogen adjuvant therapy (18). The progression of plasma cell tumors with an increase in the angiogenic and invasive potential of bone marrow plasma cells may be involved in MMP-2 production (15). These findings mentioned above may help to explain the inhibitory effect of angiogenesis and antitumor activity with c-MMP-2 vaccine targeting to MMP-2 alone in this study.

We found in this study that mice depleted of CD4+ T lymphocytes by the injection of anti-CD4 mAb and vaccinated with c-MMP-2 were not protected from tumor challenge. At the same time, the mice depleted of CD4+ T lymphocytes did not develop detectable autoantibodies against c-MMP-2. By contrast, treatment with anti-CD8 or anti-NK mAb failed to abrogate the antitumor activity. These data suggest that the induction of the autoantibody response against mouse MMP-2, which is responsible for c-MMP-2-induced antitumor activity, may involve CD4+ T lymphocytes. It is known that CD4+ T lymphocytes can steer and amplify immune responses through secretory of cytokines and expression of surface molecules (30, 41, 42). CD4+ T lymphocytes have been reported to be required for the induction of the antitumor immunity by the vaccination with a recombinant vaccinia virus encoding self-TRP-1 in mouse melanoma model (30). Also, the antitumor immunity could be induced by DNA immunization against human gp75/TRP-1 or TRP-2 (the slaty locus protein) and has depended on CD4+ T lymphocytes in melanoma mouse models (43). Furthermore, it has been reported that CD4+ T lymphocytes play a prominent role in classic mouse models of autoimmune such as experimental allergic encephalitis, systemic lupus erythematosus, and autoimmune gastritis (41). These findings may help explain the requirement for CD4+ T lymphocytes in the induction of autoimmune response against mouse MMP-2 in a cross-reaction.

Taken together, autoimmune response against mouse MMP-2 may be provoked in a cross-reaction by the immunization of chicken MMP-2, and the autoantibody targeting to MMP-2 is probably responsible for the antitumor activity. The vaccine based on chicken homologous MMP-2 as a model antigen in this study may provide a strategy for the immunogene therapy of cancer through the induction of autoimmune response against the self-molecules associated with tumor growth in a cross-reaction by the immunization with the single xenogeneic homologous gene. Furthermore, the induction of antitumor immunity by overcoming immune tolerance to self-molecules with xenogeneic counterparts may circumvent the facts that few tumor-specific antigens have been identified in human solid tumors and that the host usually shows immune tolerance to self-molecules as antigens. Many genes were highly conserved during evolutionary process, which was characterized by the gene similarity to varying degrees among different species (23, 44). Many counterparts of the genes of human and mouse can be identified from the genome sequence of the Drosophila melanogaster and of the other animals such as avian and Xenopus laevis (24, 44). Thus, the breaking of immune tolerance to the self-molecules involving tumor growth with xenogeneic counterparts may be of importance for the additional exploration of xenogeneic homologous genes identified in human and other animal genome projects in cancer therapy.

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Active Immunogene Therapy of Cancer with Vaccine On the Basis of Chicken Homologous Matrix Metalloproteinase-2

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