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Adenovirus-mediated Gene Transfer of MMP-2 into Cultured Porcine Trabecular Meshwork Cells

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Summary. This study aimed to use adenoviral gene transfer to express matrix metalloproteinase (MMP)-2 in cultured porcine trabecular meshwork cells and to evaluate the duration of adenovirus-mediated MMP-2 expression and its enzymatic activity. MMP-2 cDNA was synthesized by ligating three segments of MMP-2 cDNA obtained by reverse transcription-polymerase chain reaction (RT-PCR) with mRNA extracted from mouse lungs. MMP-2 cDNA was inserted into replication-deficient adenoviral vectors. Western blotting revealed that MMP-2 was highly expressed by adenoviral gene transfer in cultured porcine trabecular meshwork cells. Zymography confirmed that the expressed MMP-2 possessed enzymatic activity and that MMP-2 activity increased dose-responsively with the viral titer. MMP-2 expression was detected two days after the additional virus preparation and continued for at least three weeks. Adenoviral vectors could efficiently deliver MMP-2 cDNA to cultured trabecular meshwork cells, with MMP-2 gene expression persisting for three weeks after infection. Our data have implications for future gene therapy in glaucoma.

Key words— matrix metalloproteinase-2, adenovirus vector, trabecular meshwork, primary open-angle glaucoma, zymography.

INTRODUCTION

Glaucoma is one of the leading causes of irreversible blindness. A major pathogenic factor in primary open-angle glaucoma (POAG) is increased resistance to aqueous humor flow in the trabecular meshwork (TM).[1] An excessive accumulation of extracellular matrices (ECMs) in the juxtacanalicular tissue of the TM has been postulated to cause increased outflow resistance in eyes with POAG. ECM turnover is regulated by balancing degradation by matrix metalloproteinases (MMPs)/tissue inhibitors of metalloproteinases (TIMPs) with construction by a variety of structural proteins.

MMPs are a family of zinc-dependent enzymes secreted from many cell types, including cells in the TM.[2] Changes in MMP levels can affect outflow in specific cases such as after laser trabeculoplasty[3,4] or upon artificial manipulation of ECM turnover in the human anterior chamber perfusion system.[5,6] MMPs are secreted as zymogens and proteolytically cleaved to their active forms. In the TM, several MMPs and TIMPs — MMP-1, -2, -3, and -9, and TIMP-1, -2 — are secreted and help maintain homeostasis of the conventional outflow architecture.[7]

MMP-2 (72 kDa gelatinase, type IV collagenase), which is secreted from TM cells and stably expressed in the normal aqueous humor, degrades gelatin, types IV, V, VII, XI collagen, laminin, fibronectin, elastin, and proteoglycan.[8-11] Bradley et al. demonstrated that adding purified MMPs, including MMP-2, increased outflow facility,[5] and that doubling the flow rate in perfused human organ cultures increased MMP-2 levels in the perfusate.[6] WuDunn showed that TM cells subjected to mechanical strain had increased MMP-2 activity.[12] Moreover, fibronectin, a MMP-2 substrate, is located in sheath-derived plaques that are increased in the TM of POAG eyes. These reports suggest that MMP-2 regulate the outflow facility of the aqueous humor through ECM turnover, and thus control intraocular pressure (IOP). In

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Abbreviations – ECM, extracellular matrix; FBS, fetal bovine serum; IOP, intraocular pressure; MMP, matrix metalloproteinase; POAG, primary open-angle glaucoma; RT-PCR, polymerase chain reaction; TIMP, tissue inhibitors of metalloproteinase; TM, trabecular meshwork.

this study, we devised a method to overexpress MMP-2 as a potential gene therapy and investigated the efficiency and duration of MMP-2 adenoviral gene transfer into cultured porcine TM cells.

MATERIALS AND METHODS

Cloning of full-length MMP-2 cDNA

Mouse MMP-2 cDNA was synthesized from total RNA extracted from newborn C57BL/6 mouse lungs employing the following strategy. Three segments of the MMP-2 cDNA (segment 1-3) were amplified by reverse transcription–polymerase chain reaction (RT-PCR) with three sets of primers designed according to the reported mouse MMP-2 cDNA sequence (GenBank accession No. M84324). The primers used were as follows: segment 1, 5'-GCTCTAGAATGGAGGCACGAGTGGCCTG (F1, forward primer) and 5'-CGGGATCCCCAGAGG AAGCCATCACTGC (R1, reverse primer); segment 2, 5'-GCTCTAGAATACCCGTTTGATGGCAAGG (F2) and 5'-GGTCCCAATGTCGGTGTGGG (R2); segment 3, 5'-GCTGGAACACTCTCAGGACC (F3) and 5' -GGGGTACCTCAGCAGCCCAGCCAGTCTG (R3). F1 and F2 primers contained six extra nucleotides at the 5' end corresponding to *Xba*I restriction sites. Similarly, R1 contained a *Bam*HI site and R3 a *Kpn*I site. R2 and F3 primers did not contain restriction sites because they had a *Bam*HI site in their targeted segment of MMP-2 cDNA (segments 2 and 3) at the 5' end. Individual cDNA segments were subcloned into pBSK and then excised by restriction enzyme digestion (segment 1, XbaI and ScaI; segment 2, ScaI and BamHI; segment 3, BamHI and KpnI). The 3 MMP-2 segments were ligated to obtain full-length MMP-2 cDNA. The MMP-2 sequence was confirmed using a genetic analyzer (ABI PRISM 310; Applied biosystems, USA).

Adenoviral vector construction and propagation

We prepared replication-deficient recombinant adenovirus vectors using the following strategy. The E1 and E3 regions were deleted, and the vectors were engineered to express MMP-2 under the control of the CAG [cytomegalovirus IE enhancer, chicken β-actin promoter, and rabbit β–globin poly (A) signal] promoter. The adenovirus vectors carrying the MMP-2 (AdMMP-2) or LacZ (AdLacZ) genes were constructed using an adenovirus expression vector kit (TaKaRa,Tokyo) according to the manufacturer's instructions. Briefly, cosmid vectors carrying the MMP-2 or LacZ genes were constructed and cotransfected with the *Eco*T221-digested DNA-terminal protein complex of Ad5-dIX into HEK293 cells (American Type Culture Collection, Manassas, VA) to generate the recombinant viruses by homologous recombination.[13] The recombinant viruses, designated AdMMP-2 and AdLacZ, were propagated in HEK293 cells. After the fourth propagation, the virus was recovered from HEK293 cells by sonication, purified with a double cesium step-gradient, dialyzed against a vehicle solution, and stored at −80°C. The titers of the recombinant viruses were determined by a modified endpoint cytopathic effect assay on HEK293 cells to calculate plaque-forming units (pfu). We obtained recombinant adenoviruses with titers higher than $1x10^8$ pfu/ml.

Porcine trabecular meshwork cell culture and adenovirus infection

TM cells were prepared from porcine eyes (the Niigata Internal Organ Ltd., Niigata) as previously reported.[14] Briefly, TM explants were isolated by blunt dissection from eyes enucleated within 12 h postmortem, and cells were plated on dishes containing Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, California, USA) supplemented with 20% fetal bovine serum (FBS; Gibco-BRL, California, USA). Once confluent, the cells were trypsinized, split at a ratio of 1:4, subcultured, and used for further experiments within four passages.

TM cells were infected with AdMMP-2 or AdLacZ as a negative control at final concentrations of $1x10^5$, 10^6, and 10^7 pfu/ml. One day prior to harvest, the cells were washed thrice with phosphate buffered saline (PBS) to remove the old medium and cultured in a serum-free medium as FBS has MMP activities (data not shown).

Western blotting

To confirm MMP-2 expression, culture media samples were electrophoresed on 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels under reducing conditions and transferred to a nitrocellulose membrane (Schuleicher & Schuell, Dassell, Germany). The membrane was blocked for one h at room temperature, followed by incubation with a rabbit anti-human MMP-2 polyclonal antibody (Chemicon, Temecula, CA) diluted 1:1000 in Tris-buffered saline containing 0.1% Tween-20 (TBS-T, pH7.6) for one h at room temperature. This MMP-2 anti-human antibody cross-reacts with mouse MMP-2. An alkaline phosphatase conjugated goat anti-human IgG-antibody (Chemicon, Temecula, CA, 1:4000 dilution) was used for band identification in combination with Enhanced ChemiLuminescence(ECL) Chemiluminescence's Detection System (Amersham, Buckes,UK).

Zymography

Culture medium were analyzed by gelatin zymography to determine gelatinolytic activities. The media samples were mixed with an equal volume of a nonreducing sample buffer without boiling and loaded onto 8.15% polyacrylamide gels containing 0.15% gelatin. After electrophoresis, the gels were incubated twice with 2.5% Triton X-100 to remove SDS for renaturing and then with a proteinase reaction buffer (50 mM Tris-HCl (pH7.6) and 10mM $CaCl_2$) at 37°C for 12 h. The gels were stained with Coomassie blue, destained, and photographed.

RESULTS

Adenovirus-mediated MMP-2 protein expression in porcine trabecular meshwork cells

Adenovirus-mediated MMP-2 gene expression in porcine TM cells was determined by Western blot analysis using an antibody against MMP-2. Five days after viral infection at a concentration of $1x10^7$ pfu/ml, MMP-2 was detected in the supernatant of AdMMP-2 infected cells but not in AdLacZ infected cells (Fig.1).

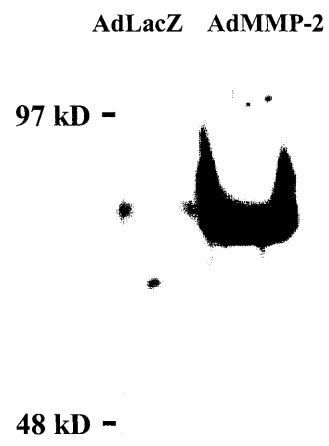

Fig. 1. The MMP-2 adenovirus yields highly expressed MMP-2 in cultured porcine TM cells. TM cells were infected with AdMMP-2 at a final concentration of $1x10^7$ pfu/ml. MMP-2 expression was assayed five days post infection by Western blot using a rabbit anti-human MMP-2 antibody. A major band of 62 kDa corresponds to the active form of MMP-2 detected in the conditioned medium from TM cells infected with the MMP-2 adenoviral vector (AdMMP-2) but not the LacZ adenovirus control (AdLacZ).

Adenovirus-delivered MMP-2 has gelatinolytic activity in porcine trabecular meshwork cells

Adenovirus-mediated MMP-2 gene expression was further confirmed by gelatin zymography, which detects both activated forms and latent pro forms of MMP-2. SDS in the gel and sample buffer activated the pro enzymes without changing the molecular weight. Cultured porcine TM cells infected with three different doses of AdMMP-2 were assessed five days after infection. Although endogenous gelatinase activities were observed in the supernatant from cells infected with AdLacZ, a dose-dependent increase in MMP-2 activity was observed only in the AdMMP-2-infected culture, confirming MMP-2 overexpression (Fig.2). We further investigated whether the MMP-2 adenovirus could induce MMP-2 activity for longer periods. MMP-2 gelatinolytic activity was detectable two days post infection, peaked at four to seven days post infection (Fig.3A), and persisted for at least three weeks (Fig.3B). Baseline gelatinase activities in AdLacZ-infected TM cells (negative control) were due to endogenous MMP-2 in TM cells.

DISCUSSION

There are several reports regarding the duration of adenoviral gene expression. Borras et al. reported that adenovirus-delivered X-gal appeared in cells two and seven days post infection and lasted at least seven days.[15] Budenz et al. reported that LacZ expression in TM cells was retained at least 14 days after adenoviral injection into the anterior chambers *in vivo*.[16] Borras et al. showed the fluorescence could be observed as soon as 72 h after adenoviral GFP injection and persisted for at least one month. However, the expression of these proteins was analyzed histologically using X-gal staining or GFP fluorescence *in vivo*. These techniques examine only a portion of the eye, and this technique is not sufficient to visualize overall changes in expression after adenoviral gene transfer.

We biochemically and functionally examined the duration of MMP-2 gene expression with adenovirus vectors in cultured TM cells using gelatin zymography, and obtained new data that adenoviral gene delivery is successful in cultured TM cells. The transgenic MMP-2 protein was expressed within 48 h after infection, peaked at four to seven days (Fig.3A), and then decreased gradually over three weeks (Fig.3B), eventually consistent with previous reports.

The problems associated with adenovirus vectors

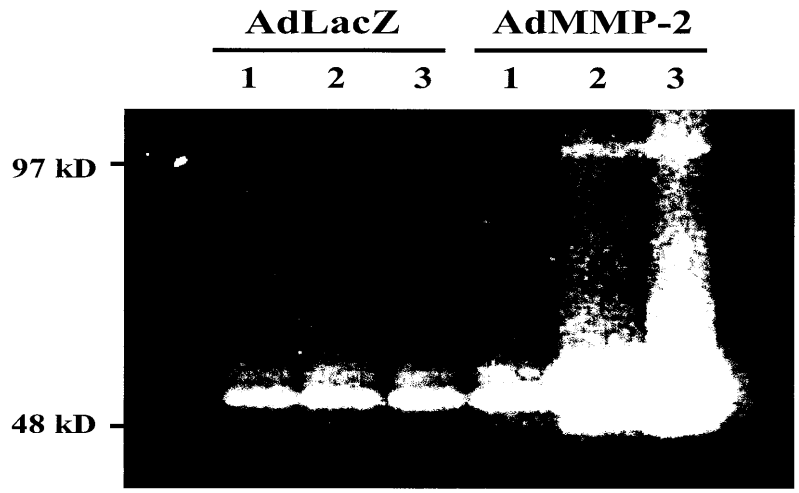

Fig. 2. MMP-2 gelatinase activities increase in a dose dependent manner with adenovirus titers in cultured porcine TM cells. TM cells plated onto a 24-well plate were infected with AdMMP-2 or AdLacZ at final concentrations of 1×10^5 pfu/ml (*lane 1*), 1×10^6 pfu/ml (*lane 2*), or 1×10^7 pfu/ml (*lane 3*). MMP-2 expression was assayed five days post infection by gelatin zymography.

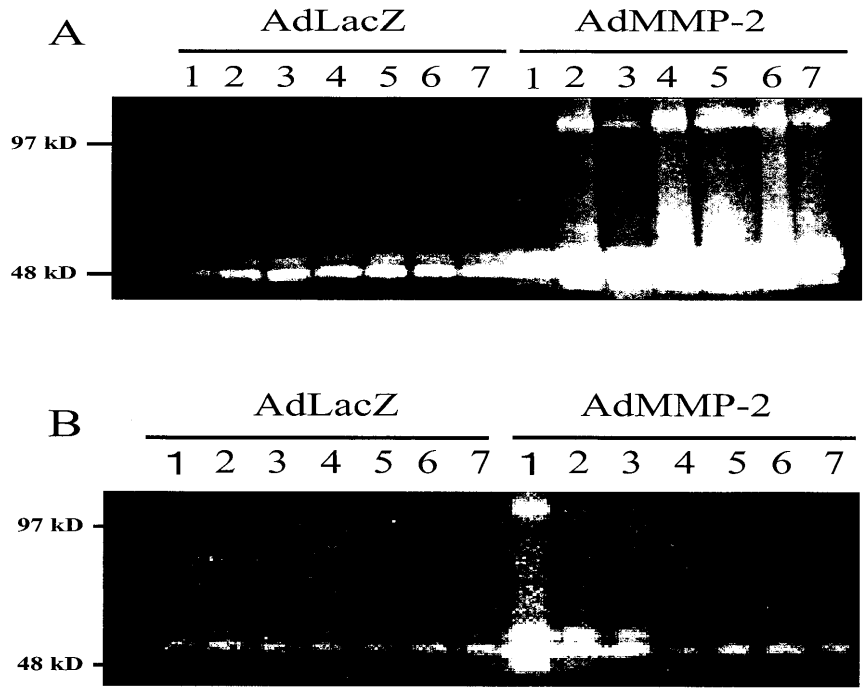

Fig. 3. Time course of MMP-2 expression and enzymatic activity assayed by gelatin zymography one to seven days. **A**. or one to seven weeks. **B**. after AdMMP-2 or AdLacZ infection.

are that they are immunogenic[17] and transferred gene is expressed for a relatively short duration.[18] Borras et al. showed that three of eight rabbits injected with adenoviral vectors developed severe inflammation at high viral titers,[19] but no signs of clinical inflammation were observed in the eyes and IOP did not also increase under certain adenovirus titers.[20, 21] Gene expression can be prolonged in immunodeficient mice[22] or mice treated with immunosuppressive agents.[22, 23] These studies have demonstrated that the host innate immune response can be manipulated to favor adenovirus vector survival and extend transgene expression. In this study, we investigated the relationship between the adenovirus titer and transgene expression. Transgene activity increased in a dose-response manner, indicating that the gelatinolytic activity of the transgene can be controlled by adjusting the titer or dose of adenoviral vectors. Future studies should determine the appropriate dose of viral vectors for gene expression that yield minimal side effects in animal models.

One potential therapy for POAG is lowering the IOP by manipulating tissues of the anterior segment. MMP-2 was reported to affect outflow facility.[5, 6, 12] To determine whether MMP-2 is a candidate gene for gene therapy, future studies should investigate whether gene transferred MMP-2 can morphologically change the TM and alter outflow facility using a well-established anterior segment organ culture.[5, 15, 20]

In summary, we used a MMP-2 adenovirus to examine the relationship between adenoviral titers and transgene expression and to determine the fate of the transgene in cultured porcine TM cells using zymography. Our study is the first report regarding the fate of MMP-2 transgene expression mediated by an adenovirus vector in cultured TM cells. We belive this report provides useful information for gene transfer in TM cells or for future gene therapy.

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