Anti-apoptotic Activity of Human Matrix Metalloproteinase-2 Attenuates Diabetes Mellitus

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Abstract

Background: Chronic progression of diabetes is associated with decreased pancreatic islet mass due to apoptosis of β-cells. Patients with diabetes have increased circulating matrix metalloproteinase-2 (MMP2); however, the physiological significance has remained elusive. This study tested the hypothesis that MMP2 inhibits cell apoptosis, including islet β-cells.

Methods: Samples from diabetic patients and newly developed transgenic mice overexpressing human MMP2 (hMMP2) were harnessed, and diabetes was induced with streptozotocin.

Results: Circulating hMMP2 was significantly increased in diabetic patients compared to controls and significantly correlated with the serum C-peptide levels. The diabetic hMMP2 transgenic mice showed significant improvements in glycemia, glucose tolerance and insulin secretion compared to diabetic wild type mice. Importantly, the increased hMMP2 levels in mice correlated with significant reduction in islet β-cell apoptosis compared to wild-type counterparts, and an inhibitor of hMMP2 reversed this mitigating activity against

diabetes. The increased activation of Akt and BAD induced by hMMP2 in β-cells compared to controls, links this signaling pathway to the anti-apoptotic activity of hMMP2, a property that was reversible by both an hMMP2 inhibitor and antibody against integrin-β3.

Conclusion: Overall, this study demonstrates that increased expression of hMMP2 may attenuate the severity of diabetes by protecting islet β-cells from apoptosis through an integrin-mediated activation of the Akt/BAD pathway.

Key words: Diabetes; metalloproteinases; islet β-cell; apoptosis; Akt signaling pathway; integrins,

Abbreviations

MMP2, matrix metalloproteinase-2; TG, transgenic; STZ, streptozotocin; DOX, doxycycline; NF, normal food; FCS, fetal calf serum; HUVECs, human umbilical vein endothelial cells; NHMCs, normal human mesangial cells; EDTA, ethylenediaminetetraacetic acid; WT, wild type, SAL, saline

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Introduction

Diabetes mellitus (DM) is a major global health problem with more than 300 million people living with DM worldwide [1, 2]. DM is the fourth highest cause of death and one of the most important risk factors for disabling diseases including retinopathy, peripheral ischemic angiopathy, ischemic heart disease and nephropathy with renal failure [1, 3]. Palliative, but no curative, therapy is currently available for type 1 and type 2 clinical forms of DM [4]. The etiology has been associated with genetic or autoimmune factors in type 1 DM, and with lifestyle-related insulin resistance and/or abnormal insulin secretion in type 2 DM. Histopathological studies have shown that apoptosis with reduced number of insulin-secreting pancreatic islet β-cells are common features in both types of DM [4, 5]. However, the underlying regulatory mechanism of islet β-cell apoptosis is not completely understood.

Matrix metalloproteinases (MMPs) are a large group of calcium-dependent zinc-containing endopeptidases that play critical roles in wound healing, tissue repair/remodeling and

morphogenesis [6]. The human MMP family comprises 24 members synthesized in a variety of cells and released as latent proenzymes (pro-MMPs). Plasmin, urokinase and other proteases can activate pro-MMPs. Apart from degrading connective tissue components, the active MMPs can also cleave and release active growth factors, membrane-anchored receptors, and cell-adhesion or apoptosis-related molecules, and by this mechanism, they can indirectly regulate cell proliferation, motility, survival and angiogenesis [6-8]. MMP2, a 72-kDa type IV collagenase, is a member of this MMP family that plays an important role in several physiological and pathological processes. A recent study suggested that pro-MMP2 can also directly affect cell function by stimulating the secretion of vascular endothelial growth factor to promote angiogenesis [9]. However, to date there is no study showing effects of MMP2 on cell apoptosis.

Increased level of the proenzyme and active forms of MMP2 has been previously reported in the peripheral blood from patients with diabetes and metabolic syndrome [10-15]. In addition, clinical correlation studies suggested that high circulating MMP2 level may indicate severity of microangiopathic complications in advanced stages, but its significance

in early stages of diabetes is unknown [12, 14]. Previous studies have demonstrated that

MMPs can interact with integrins, thereby regulating cell survival by activating Akt and

that MMP2 can also modulate the Akt signaling pathway [9, 16-21]. The aim of the present

study was, therefore, to test the hypothesis that human MMP2 (hMMP2) can inhibit

pancreatic β-cell apoptosis by interacting with integrin-β3 and thereby activating the

Akt/BAD pathway, and that overexpression of hMMP2 attenuates diabetes in mice.

Materials and Methods

Subjects

Blood samples were made available by 22 patients with type 2 DM (mean age 55.6 ± 10.2

year-old; females 9, males 13) with different durations of diabetes, and 34 healthy

volunteers (mean age 53.9 ± 7.6 year-old; females 25, males 9) to measure clinical

parameters. Table 1 shows the demography and laboratory data of the subjects. Written

informed consent was given by all patients and healthy subjects, and the study protocol was

approved by the Ethics Committee for Clinical Investigation of Mie University (approval

No 107 and 2194).

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Animals

Generation of hMMP2 overexpressing transgenic (hMMP2-TG) mice

The hMMP2-TG mouse with C57BL/6J genetic background was generated using a full-length hMMP2 cDNA subcloned into a vector containing the CAG-promoter [cytomegalovirus enhancer + chicken β-actin promoter] and rabbit β-globin polyadenylation sites (Riken Bioresources, Tsukuba, Japan). The hMMP2 cDNA of pENTR221 vector was subcloned into the pBS-CAG-DEST vector using the Gateway technology (Invitrogen), and the sequence of the hMMP2 cDNA was confirmed by direct sequencing and by restriction analysis **(Supplementary Fig. 1A,B,C)**. Expression of hMMP2 cDNA was driven by the chicken β-actin promoter linked to a human cytomegalovirus immediate-early enhancer, followed by the first exon and intron of chicken β-actin (**Fig 1.A**). The vector was digested, purified, and microinjected into fertilized eggs from C57BL/6J mice and then implanted (CLEA Japan Inc.). Transgenic founders were assessed by Southern blotting and genotyping by polymerase chain reaction (PCR).

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C57BL/6 wild-type (WT) mice were used as controls. Male WT and hMMP2-TG mice

15-to-17-weeks old and weighing 20-40g were used in the experiments and they were

maintained in a specific pathogen-free environment and subjected to a 12-h light:dark cycle

in the Mie University animal house. The Committee on Animal Investigation of Mie

University approved the experimental protocols (Approval No 24-50) and all procedures

were carried out following the institutional guidelines. All mice received humane care

according to the "Guide for the Care and Use of Laboratory Animals" prepared by the

National Academy of Sciences and published by the National Institutes of Health. The

research followed the ARRIVE Guidelines for animal investigation. Mice were randomized

and researchers that measured the parameters in the samples were blinded to the treatment

groups.

DM mouse model

Mice were randomized, all mice that started the experiments were accounted for, and

researchers that measured parameters were blinded to treatment groups. DM was induced in

WT (WT/STZ) and hMMP2-TG (hMMP2-TG/STZ) mice by intraperitoneal injection of

streptozotocin (STZ; Sigma-Aldrich, St. Louis, MO) at a dose of 40 mg/kg for 5 consecutive days. Control mice were injected with the same volume of saline (SAL) solution (WT/SAL, hMMP2-TG/SAL) for 5 consecutive days. We sacrificed all mice after 4 weeks of STZ administration.

DM evaluation

Fasting blood glucose levels were measured once a week for 4 weeks. On the 4th week after STZ or saline injection, an intraperitoneal glucose tolerance test (IPGTT) was performed after 16h fasting by intraperitoneal glucose injection (1 g/kg mouse body weight). One day later, a glucose stimulated insulin secretion test was performed after 16h fasting by intraperitoneal glucose injection (3 g/kg mouse body weight). Tail vein blood was drawn for measurement of glucose and insulin levels. After completion of the insulin secretion test, mice were sacrificed by pentobarbital overdose and pancreatic tissues collected for subsequent evaluation.

Histopathological evaluation

After sacrifice of mice by anesthesia overdose, the pancreas was resected, dehydrated,

embedded in paraffin, and then 3-μm-thick sections were prepared. Immunostaining of

insulin and glucagon was performed at Biopathology Institute Corporation by using

antibodies from Dako Corporation (Carpinteria, CA). All visible islets (WT/SAL [n=4],

hMMP2-TG/SAL [n=3], WT/STZ [n=6], hMMP2-TG/STZ [n=6]) in the pancreatic

sections were evaluated, photographed using a VS120 virtual slide microscopy and

transferred to Olyvia software (Olympus, Tokyo, Japan) and quantification was performed

using the WinROOF software (Mitani Corporation, Tokyo, Japan) as previously described

and the values were averaged for each individual mouse [22].

Evaluation of apoptosis in pancreatic islets

In vivo evaluation of apoptosis within pancreatic islets was carried out by the terminal

deoxynucleotidyl transferase dUTP nick and labeling (TUNEL) staining with a kit

(Chemicon International, Temecula, CA). Five days after the last injection of STZ as

described above, the mice were sacrificed and the pancreas was resected. After TUNEL

staining microphotographs of high-power field of islets were taken using a Olympus BX50,

DP 70 digital camera, and a DP Controller software (Olympus, Tokyo, Japan). The number

of TUNEL-positive cells was counted in all visible islets in individual mouse of each group (WT/SAL [n=5], hMMP2-TG/SAL [n=4]), WT/STZ [n=5], hMMP2-TG/STZ [n=7]) to calculate the number of apoptotic cells per islet in each individual mouse as previously described [22].

Cell culture

The murine pancreatic β-cell line MIN6, kindly provided by J. Miyazaki, Osaka University, Japan, the rat pancreatic β-cell line INS-1, purchased from AddexBio Technologies (San Diego, CA), were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St Louis, MO) or RPM1 (Sigma-Aldrich) containing 10% heat-inactivated fetal calf serum (FCS), respectively. Human umbilical vein endothelial cells (HUVECs) and normal human mesangial cells (NHMCs) were obtained from Lonza (Walkersville, MD) and cultured in DMEM with 15% heat-inactivated FCS. The human liver cancer cell line HepG2 was from RIKEN Bioresource Center (Tsukuba, Japan). All cell culture media contained 100 IU/ml of penicillin and 100 μg/ml of streptomycin.

Apoptosis assay

HepG2 cells, HUVECs and NHMCs were seeded in 12-well plates, cultured to sub-confluency, and then washed with FCS (-) medium and starved by FCS deprivation for 72h (HepG2 cells) or 24h (HUVECs, NHMCs). After an hour, the cells were treated with 0.5 μg/ml active hMMP2 (Calbiochemi Co, San Diego CA), and the culturing was continued for 72h followed by analysis of apoptosis by flow cytometry (FACScan, BD Biosciences, Oxford, UK) after staining with fluorescein-labelled annexin V and propidium iodide (FITC Annexin V Apoptosis Detection Kit with PI, Biolegend, San Diego, CA).

We found in a preliminary experiment that treatment with active hMMP2 for 30-min significantly inhibits apoptosis. Thus, to evaluate the effect of hMMP2 on MIN6 β-cell apoptosis, cells were pre-treated with 0.5 μg/ml of the proenzyme (R&D Systems, Minneapolis, MN) or active hMMP2 30min before adding 2 mM STZ to the cell media. The number of apoptotic cells was assessed by flow cytometry after 24h. To assess interaction of hMMP2 with cell surface integrins (β1, β2, β3), the cells were incubated with 5 μg/ml of anti-integrin-β1, -β2 or -β3 (BioLegend, San Diego, CA) antibody for 30min before treating with active hMMP2, and then 2 mM STZ was added to the culture media

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and apoptosis was assessed by flow cytometry after 24h. Experiments to assess the interaction of hMMP2 with cell surface integrin-β3 on INS-1 cells were performed following similar methods.

Primary mouse islet cell isolation

Pancreatic tissues were resected from diabetic WT and diabetic hMMP2-TG mice and islet cells were isolated as previously described [22]. Briefly, the pancreatic tissue was cut into 2-3 mm pieces, and incubated for 30 min at 37°C in 1 mg/mL collagenase, before centrifugation. After resuspension the cells were placed on a discontinuous Percoll gradient, centrifuged and the islet cell layer collected, washed and dispersed with trypsin/EDTA solution for assays.

Evaluation of apoptotic pathways in pancreatic islet β-cells

Apoptosis of primary murine islet cells and of MIN6 β-cell lines was analyzed by flow cytometry as described above. Activation of the Akt pathway in primary murine islet cells and MIN6 β-cells was evaluated by Western blotting using antibodies against Akt, p-Akt, Bcl-xL/Bcl-2 associated death promoter (BAD), p-BAD, cleaved form of caspase-3 and

β-actin (Cell Signaling Technology, Danvers, MA). Activation of the Akt pathway in MIN6

β-cells or INS-1 β-cells was evaluated in presence of 0.5 μg/ml of active hMMP2 (EMD

Millipore Corp, Billerica, MA), and 5 μg/ml of anti-integrin-β3 antibody (Biolegend, San

Diego, CA).

In vivo inhibition of hMMP2 by doxycycline

After induction of DM by STZ in mice following the protocol described above, one group

each of WT or hMMP2-TG mice was given food containing 0.3% doxycycline (LKT

Laboratory, St Paul, MN). There were 4 treatment groups: 1) WT/STZ/NF group: WT

treated with i.p. STZ and normal food, 2) WT/STZ/DOX group: WT treated with i.p. STZ

and doxycycline-containing food, 3) hMMP2-TG/STZ/NF group: hMMP2-TG treated with

i.p. STZ and normal food, 4) hMMP2-TG/STZ/DOX group: hMMP2-TG treated with i.p.

STZ and doxycycline-containing food. Intraperitoneal glucose tolerance test (IPGTT) was

performed on the 4th week after STZ injection and then mice were sacrificed to evaluate

apoptosis of primary islet cells from resected pancreas by flow cytometry.

In vitro inhibition of hMMP2 by doxycycline

MIN6 β-cells were pretreated with 10 μg/ml of doxycycline or vehicle for 30min before adding 0.5 μg/ml hMMP2. The cells were then treated with 5 mM STZ or saline, cultured for 24h and the number of apoptotic cells was evaluated by flow cytometry.

In vivo blockade of Akt-mediated inhibition of apoptosis

MK-2206 (AdooQ BioScience, Irvine, CA) was dissolved in sterile water containing 30% sulfobutylether-β-cyclodextrin (ChemScene, Monmouth Junction, NJ). One dose of MK-2206 or vehicle was delivered (100 mg/kg) by oral gavage after the last injection of STZ or saline administered as described above. Mice were allocated to the following five treatment groups: WT/SAL/VEH group treated with i.p. saline and oral vehicle, WT/STZ/VEH group treated with i.p. STZ and oral vehicle, WT/STZ/MK-2206 group treated with i.p. STZ and oral MK-2206, hMMP2-TG/STZ/VEH group treated with i.p. STZ and oral vehicle, and hMMP2-TG/STZ/MK-2206 group treated with i.p. STZ and oral MK-2206. Mice were sacrificed by pentobarbital overdose 48h after MK-2206 treatment and the pancreas was resected for evaluation of apoptosis of primary islet cells by flow cytometry.

***In vitro* blockade of Akt-mediated inhibition of apoptosis**

MIN6 β-cells were pre-treated with 0.5 μg/ml of active hMMP2 30min before adding

saline or 2 mM STZ with or without MK-2206 to the cell media. The number of apoptotic

cells was assessed by flow cytometry after 24h.

Zymography

Gelatinolytic activity of secreted MMP-2 was analysed by zymography on

gelatin-containing polyacrylamide gels as described [23].

Biochemical analysis

The peripheral blood level of glucose was measured by the glucose-oxidase assay and the

level of insulin using a commercial kit from ALPCO Diagnostics (Salem, NH).

Hemoglobin A1c was measured by high-performance liquid chromatography, and total

cholesterol, triglycerides and C-peptide by automated enzymatic methods at the clinical

laboratory of Mie University Hospital. The level of C-peptide was measured using a

commercial kit (Daiichi III, TBC Inc, Tokyo, Japan). The blood levels of hMMP2 in

healthy diabetic subjects were measured using a commercial enzyme immunoassay kit

(detection limit: 0.63 ng/ml) from R&D Systems (Minneapolis, MN). The levels of both

mouse MMP2 (mMMP2) and hMMP2 in mouse plasma were measured using a

commercial enzyme immunoassay kit (Total MMP-2 Quantikine ELISA kit, R&D Systems)

that cross reacts with both mMMP2 and hMMP2 proteins (detection limit: 0.082 ng/ml)

and that measures both active and pro forms of MMP2.

Gene expression

Total RNA was extracted from mouse organs by Trizol Reagent (Invitrogen, Carlsbad, CA)

and then subjected to reverse-transcription (RT) using oligo-dT primers and DNA

amplification by polymerase chain reaction (PCR) using the Superscript Pre-amplification

system kit (Invitrogen). **Supplementary Table 1** describes the sequences of the primers.

All PCR studies were performed under pre-plateau condition for each amplification. PCR

products were run on a 2% agarose gel and the bands were visualized by ethidium bromide

staining and ultraviolet transillumination, and the gene expression was normalized by the

GAPDH transcription level. We used the restriction enzyme KpnI to evaluate the specificity

of the hMMP2 PCR product (**Supplementary Fig. 1D**).

Statistical analysis

Data were expressed as the mean ± standard deviation of the mean (S.D.). Statistical analyses were carried out by ANOVA with *post hoc* analysis using Fisher's least significant difference test to assess differences among 3 or more variables, and by unpaired Student t-test between 2 variables. Statistical analyses were carried out with the StatView software package version 5.0 for Windows (SAS Institute Inc., Cary, NC).

Results

Circulating levels of hMMP2 in diabetic patients are increased

The durations of type 2 DM among all patients and between genders were highly variable. The plasma concentration of hMMP2 was higher in type 2 DM patients than in healthy subjects in agreement with previous reports in both male and female patients (Table 1). Lipid parameters were elevated in both male and female diabetic patients compared to healthy subjects except for high-density lipoprotein, which was reduced (**Table 1**). Circulating hMMP2 was positively correlated with serum C-peptide levels in all diabetic patients (**Supplementary Table 2**).

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Increased expression of endogenous MMP2 in diabetic mice

To clarify whether the high expression of MMP2 under diabetic conditions can be reproduced in mice we induced diabetes in mice by STZ injection and evaluated the expression of endogenous mMMP2. Mice had significantly high levels of glycemia after STZ injection. Relative mMMP2 mRNA expression and the activity of endogenous mMMP2 in pancreatic tissue and the plasma activity of endogenous mMMP2 were increased in mice with DM (WT/STZ) compared to their non-diabetic counterparts (WT/SAL) (**Supplementary Fig 2.A**). RT-PCR analysis showed mMMP2 expression in all organs tested from WT diabetic mice (**Supplementary Fig 2.B**) in agreement with a previous report [24].

hMMP2 inhibits apoptosis of β-cell lines

We then investigated whether hMMP2 can prevent β-cell apoptosis. Apoptosis of murine MIN6 β-cells induced in the presence of STZ was significantly inhibited by the proenzyme or active form of hMMP2; however, suppression of apoptosis by active hMMP2 was significantly stronger than that observed for human proMMP2 (**Supplementary Fig. 3**).

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hMMP2 inhibits apoptosis of cells from different organs

The effect of hMMP2 on apoptosis of cells from different organs was evaluated to clarify

whether the antiapoptotic activity of hMMP2 is restricted to β-cell lines. Apoptosis of liver

cells (HepG2 cells), vascular endothelial cells (HUVECs) and glomerular mesangial cells

(NHMCs) induced by serum starvation was significantly reduced by the presence of active

hMMP2 (**Supplementary Fig. 4, Supplementary Fig. 5, and Supplementary Fig. 6**).

Increased circulating and tissue levels of hMMP2 in hMMP2-TG mice

To further study the biological significance of the anti-apoptotic activity of hMMP2 *in vivo*,

we created a transgenic (TG) mouse line that overexpresses hMMP2 in several tissues.

Evaluation of transgenic founders and germ line transmission by Southern blotting of tail

DNA showed that two of 68 progeny contained the hMMP2 transgene (**Fig. 1A,B**). The

mouse line with 3 hMMP2 gene copies in the founder was used in all subsequent

experiments (**Fig. 1B**). Peripheral organs expressed hMMP2 in the transgenic mice as

demonstrated by RT-PCR (**Fig. 1C**). Restriction analysis confirmed that the PCR product is

hMMP2 (**Supplemental Fig. 1D**). The plasma concentration of total (mouse and human)

MMP2 was enhanced 1.8-fold in hMMP2-TG mice compared to WT mice using an ELISA

that detects both mouse and human MMP2 (**Fig. 1D**).

DM is attenuated in hMMP2-TG mice

DM was induced with STZ in WT and hMMP2-TG mice and the status of their diabetes

was compared. Fasting blood glucose levels in the hMMP2-TG/STZ group were decreased

compared to the WT/STZ group on weeks 2, 3 and 4 after STZ injection (**Fig. 2A**).

Furthermore, during the intraperitoneal glucose tolerance test (IPGTT), blood glucose

levels were reduced in the hMMP2-TG/STZ mice compared to WT/STZ mice (**Fig. 2B**);

comparative analysis of the areas under the curve among groups also revealed similar

results (**Supplementary Fig. 7**). One day after IPGTT, a glucose-stimulated insulin

secretion test was performed, and the results showed an increase in plasma concentration of

insulin in hMMP2-TG/STZ mice compared to their WT counterparts; the increase was

statistically significant 10min after glucose infusion (**Fig. 2C**). The plasma levels of

glucose and insulin were not different between the control groups (WT/SAL and

hMMP2-TG/SAL). The plasma concentration of total (mouse and human) MMP2 was

increased in diabetic TG mice (hMMP2-TG/STZ) compared to their non-diabetic

counterparts (hMMP2-TG/SAL) (**Supplementary Fig. 8**).

Insulin-secreting cells are protected in hMMP2-TG mice after DM induction

The insulin- and glucagon-secreting cells were evaluated by immunostaining and their islet

areas were calculated after DM induction. The insulin-positive (β-cell) area per total islet

area was larger in hMMP2-TG/STZ mice than in WT/STZ mice (**Fig. 3A**). Inversely, the

glucagon-positive (α-cell) area per total islet area was smaller in hMMP2-TG/STZ mice

than in WT/STZ mice. In the negative control (hMMP2-TG/SAL and WT/SAL) groups that

received injection of saline alone, no differences in insulin- or glucagon-positive area were

observed.

hMMP2-TG mice have fewer apoptotic islet cells after DM induction

The number of apoptotic cells in pancreatic islets was decreased in hMMP2-TG/STZ mice

compared to WT/STZ mice; apoptosis was not observed in mice treated with saline alone

when apoptosis was evaluated by TUNEL (**Fig. 3B**). This *in vivo* observation was

confirmed by *in vitro* experiments in which MIN6 β-cells were treated with STZ in the

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presence or absence of the active form of hMMP2. Cleaved caspase-3, a marker of cell apoptosis, was evaluated by Western blotting and found to be less abundant in MIN6 β-cells treated with active hMMP2 (hMMP2/STZ) than in untreated cells (SAL/STZ) (**Supplementary Fig. 9**).

Inhibition of MMP2 abolishes its protective activity against DM *in vivo* and *in vitro*

We administered doxycycline, an inhibitor of the activity and expression of MMP2 [25-27], to evaluate if it renders the mice more susceptible to STZ-induced DM. hMMP2-TG or WT mice received normal food (hMMP2-TG/STZ/NF, WT/STZ/NF) or food containing doxycycline (hMMP2-TG/STZ/DOX, WT/STZ/DOX). The plasma level of total (mouse and human) MMP2 was decreased in hMMP2-TG/STZ/DOX mice compared to non-treated (hMMP2-TG/STZ/NF) TG mice (**Fig. 4A**). The IPGTT revealed increased blood glucose levels in hMMP2-TG/STZ/DOX mice compared to hMMP2-TG/STZ/NF mice but a decreased blood glucose level in WT/STZ/DOX mice compared to WT/STZ/NF mice (**Fig. 4B,C**).

Evaluation of primary islet β-cell apoptosis *in vivo* showed increased numbers of apoptotic

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β-cells in the hMMP2-TG/STZ/DOX group compared to hMMP2-TG/STZ/NF group (**Fig. 4D**). The blood glucose levels were followed for 4 weeks and the results showed that TG mice treated with doxycycline (hMMP2-TG/STZ/DOX) have significantly elevated levels of blood glucose compared to counterparts receiving normal food (hMMP2-TG/STZ/NF) (**Supplementary Fig. 10**). Inversely, blood glucose levels were lower in the WT/STZ/DOX group than in the WT/STZ/NF group (**Supplementary Fig. 10**).

To confirm these *in vivo* findings, MIN6 β-cells were treated *in vitro* with active hMMP2 in the presence of STZ with or without doxycycline. Active hMMP2 inhibited STZ-induced cell apoptosis but this effect was abolished by doxycycline (**Fig. 5**).

hMMP2 activates the Akt pathway in MIN6 β-cells and in primary islet cells

The Akt pathway was evaluated in murine MIN6 β-cells to decipher the mechanism of hMMP2-mediated apoptosis inhibition by examining phosphorylation of Akt and BAD with Western blots. The results showed increased phosphorylation of both Akt and BAD in MIN6 β-cells cultured in the presence of active hMMP2 compared to cells cultured in its absence (**Fig. 6A**). Interestingly, hMMP2 induced Akt phosphorylation even in MIN6

β-cells that were not treated with STZ (**Fig. 6A**).

The effect of hMMP2 on the Akt and BAD pathway was also assessed by Western blotting in both WT and hMMP2-TG mice treated with STZ. The results showed enhancement of Akt and BAD phosphorylation in pancreatic islets from hMMP2-TG/STZ mice compared to WT/STZ mice (**Fig. 6B**).

Akt orchestrates the hMMP2-mediated attenuation of islet cells apoptosis *in vivo*

WT and hMMP2-TG mice were treated with a daily dose of STZ for 5 consecutive days and then once with MK-2206, an Akt pathway inhibitor, after the last STZ injection. Cell apoptosis was evaluated by the surface expression of annexin V. Apoptosis was reduced in hMMP2-TG treated with STZ and vehicle (hMMP2/STZ/VEH) compared to their WT counterpart (WT/STZ/VEH) (**Fig. 7A,B**). However, the number of apoptotic cells was increased in TG mice treated with MK-2206 (hMMP2/STZ/MK-2206) compared to untreated mice (hMMP2/STZ/VEH) (**Fig. 7A,B**).

To confirm this finding, MIN6 β-cells were treated *in vitro* with active hMMP2 in the presence of STZ with or without MK-2206. Active hMMP2 inhibited STZ-induced cell

apoptosis but this inhibition was abolished by MK-2206 (**Supplementary Fig. 11**).

Integrins modulate inhibition of β-cell apoptosis by hMMP2

To clarify whether interaction of hMMP2 with integrins are associated with hMMP2-induced inhibition of β-cell apoptosis, MIN6 β-cells were cultured in the presence of activated hMMP2 and antibodies for integrin-β1, -β2 or -β3. The reduction in apoptosis of MIN6 β-cells treated with both STZ and hMMP2 was reversed when the cells were pre-treated with integrin-β3 antibody before the addition of hMMP2 (**Fig. 8A,B,C**). Similar results were obtained in experiments performed using INS-1 β-cells (**Supplementary Fig. 12**). Activation of the Akt pathway by active hMMP2 in the presence of anti-integrin-β3 was also assessed by Western blotting in both MIN6 and INS-1 cell lines and showed inhibition of hMMP2-mediated Akt activation in the presence of anti-integrin-β3 (**Supplementary Fig. 13, Supplementary Fig. 14**).

Discussion

MMP2 and diabetes

Increased circulating levels of hMMP2 occur in patients with type 1 and type 2 DM

compared to a healthy population [6, 15, 28]. The source of hMMP2 can be a variety of cells including vascular endothelial cells, smooth muscle cells and macrophages that appear to be stimulated by hyperglycemia and cytokines released during chronic inflammation [7, 29, 30]. The significant correlation with hyperglycemia suggests the clinical significance of the enhanced circulating hMMP2 under diabetic conditions [31]. Results have been contradictory on if this response is beneficial or harmful [11, 15, 29, 32-34]. Here we confirmed that circulating hMMP2 is increased in DM subjects and that it may have clinical relevance as it is positively correlated with the levels of C-peptide, a marker of insulin secretory activity [35]. We also showed that the expression and plasma activity of MMP2 are increased in STZ-induced DM in mice. To get more insights into the clinical implications we developed an hMMP2-TG mouse that has elevated circulating hMMP2.

MMPs and apoptosis

Previous investigations suggested that metalloproteinases can indirectly regulate cell proliferation and apoptosis by activating the latent forms of growth factors or signal proteins [6, 8, 36]. Here, we evaluated if hMMP2 acts directly on cells inhibiting cell

apoptosis. In preliminary *in vitro* experiments we found that pretreatment of cells with

active hMMP2 reduces apoptosis of MIN6 β-cells and other tissue-derived cells suggesting

that hMMP2 possesses direct anti-apoptotic properties. Apoptosis of islet β-cells leads to

reduced islet β-cell mass and consequently deficient insulin secretion in both type 1 and

type 2 DM [4, 5, 37].

To validate the potential regulatory function of MMP2 on apoptosis *in vivo*, we induced

DM in hMMP2-TG mice with STZ and found that they have reduced blood glucose levels

and an improved insulin secretory response that is associated with a reduction in islet cell

apoptosis and increased number of insulin-secreting cells compared to diabetic WT mice.

To demonstrate that these findings are related to MMP2, the experimental DM model was

carried out in mice fed with doxycycline, an inhibitor of activity and expression of MMP2

[25-27]. TG mice fed with doxycycline had DM decompensation and more apoptosis of

islet cells compared to mice receiving normal food. Interestingly, unlike diabetic

hMMP2-TG mice, diabetic WT receiving doxycycline had lower glucose levels compared

to counterpart mice fed with normal food; amelioration of insulin sensitivity by

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doxycycline may explain this difference in blood glucose levels between WT mice treated with and without doxycycline [38]. In an *in vitro* experiment, doxycycline also inhibited the anti-apoptotic activity of MMP2 in MIN6 β-cells. Overall, these observations suggest that hMMP2 protects islet β-cells from apoptosis under diabetic conditions. However, interpretation of these data needs caution because doxycycline is not a specific MMP2 inhibitor and the MMP2-TG mouse is an artificial model.

In the present study, we focused on the cellular effects of extracellular MMP2. Our hMMP2-TG mice overexpress the inactive form of hMMP2 (pro-MMP2) that is then activated extracellularly under pathological conditions, and we used an *in vitro* cell system where cells were treated with active hMMP2 added to the culture media. Distinct substrates and functions have been attributed to intracellular MMP2 [39], which may explain the discrepant result in a previous *in vitro* study where experiments were performed using the β-cell line INS-1 overexpressing intracellular MMP2 [40].

MMP2 and the integrin-β3/Akt/BAD axis

Akt is a serine/threonine protein kinase B that plays a critical role in the protection of islet

β-cells from apoptosis [41, 42]. The Akt pathway regulates cell death by multiple

mechanisms that include phosphorylation of BAD, a pro-apoptotic protein [41, 43]. BAD

promotes cell death by directly binding to and inactivating prosurvival Bcl-2 family

members such as Bcl-XL [41, 43]. Phosphorylation of BAD by Akt disrupts its ability to

bind and inactivate Bcl-2 proteins leading to increased availability of anti-apoptotic factors

and thus promoting cell survival [44]. It was unknown if hMMP2 regulation of the

activation of the Akt pathway affects cell apoptosis [9]. In the present study, we found that

MIN6 β-cells pre-treated with active hMMP2 and islet cells from hMMP2-TG mice show

increased phosphorylation of both Akt and BAD compared to controls, pointing to a role

for hMMP2 in Akt pathway activation. Interestingly, phosphorylation of BAD remains

unchanged in cells not treated with STZ even in the presence of high levels of hMMP2.

Apart from its role in stimulating β-cell apoptosis, BAD phosphorylation may also promote

glucose-stimulated insulin secretion in β-cells by interacting with glucokinase [45, 46];

therefore, different regulatory mechanisms at a physiological level may explain why

baseline BAD phosphorylation remained unchanged. Increases in apoptosis induced by

MK-2206, a specific Akt inhibitor, in hMMP2-treated MIN6 β-cells and islet cells from

hMMP2 TG mice treated with STZ further support the involvement of the Akt pathway in

the suppression of apoptosis by hMMP2.

Previous studies have demonstrated that MMPs can interact with integrins, which regulate

cell survival by activating Akt, and that MMP2 can also affect the Akt signaling pathway [6,

9, 16, 17, 21, 36, 47]. Here, our working hypothesis was that MMP2 triggers Akt activation

by directly interacting with integrins. To clarify the role of integrins in MMP2-mediated

Akt activation, we stimulated MIN6 β-cells with active hMMP2 in the presence of

anti-integrin antibodies and found that anti-integrin-β3 antibody blocks the effect of

hMMP2, implying that binding of hMMP2 to integrin-β3 is the initial stimulus for Akt

pathway activation in β-cells. Previous studies have shown that Akt pathway activation

increases expression of MMP2, therefore taken together, the results presented here suggest

that MMP2 may also stimulate its own expression by an autocrine mechanism [48].

Limitations of this study

Inclusion in our study of type 2 DM patients at different clinical stages as shown by the

high variability in the duration of disease limits the ability to generalize our conclusions. The ethnicity of our patients and volunteers also may limit generalizations because East Asian diabetic patients tend to have impaired insulin secretion from early disease stages compared to Caucasian and African patients [49]. Another weakness is that inhibition of apoptosis of pancreatic cells by hMMP2 was demonstrated using both *in vivo* and *in vitro* experiments but that of other organ cells only using *in vitro* experiments. In addition, the study showed the anti-apoptotic activity of hMMP in mice with systemic overexpression of hMMP2 but this experiment cannot be carried out ethically in humans. However, the significantly higher plasma level of MMP2 in our diabetic patients compared to controls suggests that overexpression of MMP2 occurs under diabetic conditions, while the high reproducibility of the anti-apoptotic activity of hMMP2 *in vitro* and *in vivo* experiments strongly supports the beneficial effects of hMMP2 in DM.

Conclusion and clinical implications

This study demonstrates that increased expression of hMMP2 attenuates the severity of DM by protecting islet β-cells from apoptosis through activation of the integrinβ3/Akt/BAD

pathway. Reduction of the number of insulin-secreting pancreatic islet β-cells is a major

and unresolved therapeutic challenge in diabetes. Development of novel drugs capable of

harnessing this novel anti-apoptotic function of MMP2 may provide a new and effective

therapeutic option for diabetic patients.

Conflict of Interest

The authors have declared that no conflict of interest exists regarding data reported in this

manuscript.

Author contributions

KN, TY and CND-G performed and prepared the DM mouse models. TK, RM-M and MI

measured and analyzed clinical parameters. MT, JAH, PBT, TT and AT measured

parameters in mouse model samples. ECG and YY conceived and designed the experiments

and analyzed the data. JM, IC, TK and YT contributed with critical revisions and

interpretations of the data. KN, ECG, JM and IC contributed to manuscript preparation. All

authors approved the final version of the manuscript.

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Figures legends

Figure 1. Generation of hMMP2 transgenic mice (hMMP2-TG). For the preparation of

the hMMP2 transgene plasmid, full-length hMMP2 cDNA was cloned into the pCAGGS

plasmid containing the CAG-promoter [Cytomegalovirus enhancer + chicken β-actin

promoter] and rabbit β-globin polyadenylation sites (A). The hMMP2 transcript, with its

own stop codon and poly(A) signal, was followed by a rabbit β-globin poly(A) sequence.

After confirmation by nucleotide sequencing, the plasmid was digested, purified, and

microinjected into fertilized eggs from C57BL/6J mice before implantation. Transgenic

founders and germ line transmission of transgenic construct were assessed by Southern

blotting (B) of EcoRI-digested DNA probed with [32P]-labeled hMMP2 cDNA fragment

generated by PCR. The copy number of the integrated transgene in the two lines was compared with the control signal intensity. Peripheral organs express the hMMP2 transgene (C). An immunoassay kit that measures both mouse and human MMP2 was used to determine the circulating level of total MMP2 protein (d). WT n=7, MMP2-TG n=5. Data are expressed as mean ± S.D. *p<0.05 vs WT group.

Figure 2. hMMP2-TG mice are resistant to STZ-induced diabetes. STZ or saline was injected intraperitoneally for five consecutive days. Blood glucose levels were measured weekly for four weeks after STZ or saline injection (A; WT/SAL n=4; hMMP2-TG/SAL n=3; WT/STZ n=6; hMMP2-TG/STZ n=6) and during the IPGTT (B; WT/SAL n=4; hMMP2-TG/SAL n=3; WT/STZ n=6; hMMP2-TG/STZ n=6) performed on the 4th week of STZ injection after 16h of food deprivation. After 16h of food deprivation an intraperitoneal injection of glucose was given and the level of plasma insulin was measured (C; WT/SAL n=4; hMMP2-TG/SAL n=3; WT/STZ n=6; hMMP2-TG/STZ n=6). The age of the WT and MMP2-TG mice was 15 to 17 weeks. Data are expressed as mean ± S.D. WT, wild-type; STZ, streptozotocin; hMMP2, human matrix metalloproteinase-2; SAL,

saline. *p<0.05 vs WT/STZ.

Figure 3. Diabetic hMMP2-TG mice have fewer apoptotic islet β-cells. Mice were sacrificed 32 days after intraperitoneal STZ or saline injection for immunostaining and 5 days after STZ injection for TUNEL evaluation. The pancreas was resected and processed for immune-staining of insulin (green) and glucagon (red). The positive areas for insulin or glucagon staining were evaluated in all visible islets in each mouse of each group (WT/SAL [n=4]; hMMP2-TG/SAL [n=3]; WT/STZ [n=6]; hMMP2-TG/STZ [n=6]) using an image processing software (WinROOF) (A). The number of TUNEL-positive cells was counted in all visible islets each group (WT/SAL [n=5]; hMMP2-TG/SAL [n=4]; WT/STZ [n=5]; hMMP2-TG/STZ [n=7]) to calculate the number of apoptotic cells per islet in each individual mouse (B). The age of the WT and MMP2-TG mice was 13 to 14 weeks. Data are expressed as mean ± S.D. Scale bars indicate 50 μm. WT, wild-type; STZ, streptozotocin; hMMP2, human matrix metalloproteinase-2; SAL, saline. *<0.05 vs WT/STZ group.

Figure 4. Doxycycline blocks the beneficial effect of hMMP2 overexpression. Plasma

total MMP2 in plasma was measured by an enzyme immunoassay kit that detects both human and mouse MMP2 (A). hMMP2-TG mice made diabetic with STZ were fed with or without doxycycline and the intraperitoneal glucose tolerance test was performed (B). To evaluate apoptosis of islet cells *in vivo,* mice were sacrificed on day 33 after STZ injection, the pancreas was resected, and islet cells isolated. The age of the WT and MMP2-TG mice was 12 to 17 weeks. Data are expressed as mean ± S.D. For (A), (B) and (C) experiments: WT/STZ/NF n=11, WT/STZ/NF n=8, hMMP2-TG/STZ/NF n=10, hMMP2-TG/STZ/DOX n=8. For (D) experiment: WT/STZ/DOX n=5, WT/STZ/DOX n=4, hMMP2-TG/STZ/NF n=5, hMMP2-TG/STZ/DOX n=4. WT, wild type; STZ, streptozotocin; hMMP2, human matrix metalloproteinases-2; DOX, doxycycline; NF, normal food. *p<0.05 WT/STZ/DOX vs WT/STZ/NF; †p<0.05 hMMP2-TG/STZ/DOX vs hMMP2-TG/STZ/NF; ‡p<0.05 hMMP2-TG/STZ/NF vs WT/STZ/NF.

Figure 5. Doxycycline blocks the anti-apoptotic activity of hMMP2 on MIN6 β-cells.

MIN6 β-cells were pretreated with 10 μg/ml of doxycycline or vehicle for 30min before adding 0.5 μg/ml hMMP2. The cells were then treated with 5 mM STZ or saline, cultured

for 24h and the number of apoptotic cells was evaluated by flow cytometry after Annexin

V-FITC/propidium iodide (PI) double staining (A). The percentage of apoptotic cells was

measured (B). Each bar represents the mean ± S.D. Experiment was done in triplicates.

STZ, streptozotocin; hMMP2, human matrix metalloproteinases-2; SAL, saline. *p<0.05 vs

STZ(+) hMMP2(-)Doxycycline (-) group. †p<0.05 vs STZ(+)/hMMP2(+)/Doxycycline (-)

group..

Figure 6. hMMP2 activates the Akt pathway *in vitro* and *in vivo*. Murine MIN6 β-cells

were treated with STZ in the presence of hMMP2 (0.5 μg/ml) or vehicle and

phosphorylation of Akt and BAD was assessed by Western blotting. Experiment was done

in triplicates (A). To assess the effect of hMMP2 on Akt activation *in vivo*, hMMP2-TG

mice were made diabetic with STZ. The mice were then sacrificed 48h after the last STZ

injection, pancreatic tissue was resected, islet cells isolated and phosphorylation of Akt and

BAD was assessed by Western blotting; n=3 in hMMP2-TG/SAL group and n=4 in

WT/SAL, WT/STZ and hMMP2-TG/STZ groups (B). Each bar represents the mean ± S.D.

WT, wild type; SAL, saline; STZ, streptozotocin; hMMP2, human matrix

metalloproteinases-2. *p<0.05 vs. STZ alone and WT/STZ group.

Figure 7. The Akt inhibitor MK-2206 increases apoptosis of pancreatic islet cells in hMMP2-TG mice. hMMP2-TG mice made diabetic with STZ were treated with one dose of MK-2206 (100 mg/kg) by oral gavage after the last injection of STZ or saline and sacrificed to assess apoptosis of islet β-cells by flow cytometry (A,B). The age of the WT and MMP2-TG mice was 8 to 10 weeks. Data are expressed as mean ± S.D. WT/SAL/VEH n=3, WT/STZ/VEH n=5, WT/STZ/MK-2206 n=5, hMMP2-TG/STZ/VEH n=5, hMMP2-TG/STZ/MK-2206 n=5. WT, wild type mice; STZ, streptozotocin; hMMP2, human matrix metalloproteinases-2; SAL, saline; VEH, vehicle. *p<0.05 vs WT/STZ/VEH, †p<0.05 vs hMMP2-TG/STZ/VEH.

Figure 8. Anti-integrin antibodies block the anti-apoptotic activity of hMMP2 on MIN6 β-cells. MIN6 β-cells were pretreated with 5 μg/ml of anti-integrin-β1, -β2, and -β3 antibody or vehicle for 30 min before adding 0.5 μg/ml hMMP2. The cells were then treated with 2 mM STZ or saline, cultured for 24h and the number of apoptotic cells was evaluated by flow cytometry after Annexin V-FITC/propidium iodide (PI) double staining

(A, B, C). Each bar represents the mean ± S.D. Experiment was done in triplicates. STZ,

streptozotocin; hMMP2, human matrix metalloproteinases-2. *p<0.05 vs

antibody(-)/STZ(-)/hMMP2(-) group; †p<0.05 vs antibody(-)/STZ(+)/hMMP2(-) group;

‡p<0.05 vs antibody(-)/STZ(+)/hMMP2(+)group.