THE SYNTHETIC HYDROXYPROLINE-CONTAINING COLLAGEN ANALOGUE (GLY-PRO-HYP)₁₀ PROMOTES ENZYMATIC ACTIVITY OF MATRIXMETALLOPROTEINASE-2 *IN VITRO*

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Diseases such as liver fibrosis and intestinal inflammation are characterized by accumulated components of the extracellular matrix (ECM). Given that fibrillar collagen structures were shown to serve as storage site for inactive proforms of matrixmetalloproteinases (MMPs), modulating this MMP–collagen interaction might offer a rational interventional (therapeutic) approach to enhance degradation of accumulated ECM. The synthetic triple helical collagen analogue (Gly–Pro–Hyp)₁₀ – (GPO)₁₀ – was shown to trigger release and enzymatic activation of collagen sequestered proMMP-2. In the presented study, we, for the first time, investigated how MMP–(GPO)₁₀ interaction impacts cellular responses *in vitro*. We found that recombinant proMMP-2 induced proliferation of hepatic stellate cells (HSC), which was enhanced after addition of (GPO)₁₀ reaching comparable levels following incubation with fully activated MMP-2. In addition, (GPO)₁₀ induced HSC migration similar to the platelet-derived growth factor subunit-B. Further, the MMP-2-dependent invasion of HT1080 fibrosarcoma cells through an ECM membrane was enhanced after addition of (GPO)₁₀. Since cellular proliferation and migration concomitant with matrix degradation is stimulated, we conclude that the MMP–(GPO)₁₀ interaction also functions in a physiological environment. Thus, a potential therapeutic effect of (GPO)₁₀ should be further tested in animal models for MMP-associated diseases such as colitis or fibrosis.

Keywords: (GPO)₁₀, extracellular matrix, *in vitro*, matrixmetalloproteinase, proMMP-2, gelatinases, enzymatic activation, HT1080, hepatic stellate cells

Abbreviations: DMEM, Dulbecco's Modified Eagle's Medium; DQ-gelatin, dye-quenched gelatin; DSS, dextran sulfate sodium; ECM, extracellular matrix; FCS, fetal calf serum; GAP, H–Gly–Ala–Cys–(Gly–Ala–Pro)₅–Gly–Phe–Hyp–Gly–Glu–Arg–(Gly–Ala–Pro)₅–NH₂; (GPO)₁₀, H–Gly–Cys–Hyp–(Gly–Pro–Hyp)₁₀–Gly–Cys–Hyp–Gly–NH₂; HSC, hepatic stellate cell; HT1080, human fibrosarcoma cell line; MMP, matrixmetalloproteinase; PBS, phosphate buffered saline; PDGF-BB, platelet-derived growth factor subunit B; PI3-K, phosphoinositide 3-kinase

Introduction

Matrixmetalloproteinases (MMPs) form a large family of zinc-dependent metalloendopeptidases that degrade extracellular matrix (ECM) molecules such as collagens, gelatin, elastin, or fibronectin [1]. MMPs are assumed to be sequestered into the ECM [2, 3] and are involved in the regulation of cellular differentiation, proliferation and migration, tumour growth and metastases, and fibrosis of organs such as liver [4–6]. Among the MMPs, the gelatinases MMP-2 and MMP-9 are considered to play vital roles in tumour invasion and liver fibrosis [7–11]. Both gelatinases can degrade virtually all structural components of the ECM and are therefore considered interesting targets for potential ECM-targeted therapeutic approaches.

MMP-2 and MMP-9 were shown to be involved in human inflammatory bowel diseases (IBD) such as Crohn's disease and ulcerative colitis [12, 13], as well as experimental acute small intestinal [14] and large intestinal inflammation [15].

In previous studies, we identified the $\alpha 2$ chain of collagen type VI as the main binding structure for sequestration of collagenases and stromelysin-1 proforms in fibrotic tissue [16], and showed that gelatinase binding sites are located on fibrillar collagen structures and the synthetic collagen analogue (Gly–Pro–Hyp)₁₀ ((GPO)₁₀) [17]. Using (GPO)₁₀ as a binding competitor for the interaction of the gelatinase proMMP-2 with collagen, a dissociation of the proMMP-2 prodomain accompanied by high enzymatic activation of MMP-2 could be achieved [17].

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In the present cell culture experiments, we investigated whether (GPO)₁₀ modulates the enzymatic activation/activity of proMMP-2/MMP-2. We demonstrate that (GPO)₁₀ acts as a mitogen for hepatic stellate cells and supports the MMP-dependent invasion of HT1080 fibrosarcoma cells via *in vitro* activation of proMMP-2, implicating (GPO)₁₀ or structural-related peptides as therapeutic options in MMP-associated diseases.

Materials and methods

Cell culture

Hepatic stellate cells (HSC) isolated from rats and treated with carbon tetrachloride as described earlier [18] were obtained from Prof. W. Dieterich (University of Erlangen, Germany). Human fibrosarcoma cells (HT1080) were purchased from ATCC (CCL-121). All cell lines were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, and 100 U/ml penicillin–streptomycin, and incubated at 5% CO₂ and 37 °C humidified atmosphere.

Preparations of collagen peptides

The triple helical collagen analogue (GPO)₁₀–H–Gly–Cys–Hyp–(Gly–Pro–Hyp)₁₀–Gly–Cys–Hyp–Gly–NH₂ – and the linear control peptide GAP – H–Gly–Ala–Cys–(Gly–Ala–Pro)₅–Gly–Phe–Hyp–Gly–Glu–Arg–(Gly–Ala–Pro)₅–NH₂ – were synthesized and characterized as described previously [17, 19]. The lyophilized peptides were dissolved and stored in stock solutions of 2 mg/ml in 150 mM acetic acid at –20 °C. In the cell culture experiments, the peptides were diluted in the supernatants of the cells.

In vitro activation of MMP-2

ProMMP-2 was activated by incubation with 4-aminophenylmercuric acetate (APMA) as described [17]. To exclude APMA-specific effects, the respective controls contained APMA alone.

Table 1. Concentrations of (pro)MMP-2, (GPO)₁₀, GAP, ilomastat and PDGF-BB used in the cell culture experiment

Treatment	Concentration [M]
MMP-2	1.7×10^{-9}
proMMP-2	1.7×10^{-9}
(GPO) ₁₀	1.7×10^{-8}
GAP [17]	1.7×10^{-8}
Ilomastat	6.4×10^{-6}
PDGF-BB	2.1×10^{-6}

Determination of hepatic stellate cell proliferation

As described in a previous study [20], effects of (GPO)₁₀, GAP with or without proMMP-2, MMP-2, or the MMP-inhibitor ilomastat on the proliferation of HSC were tested by [³H]-thymidine incorporation. All peptide analogue and chemical concentrations used in the proliferation assay and the following assays are given in Table 1.

Wound healing assay

For migration experiments, 5×10^4 HSC were seeded into 24-well plates in triplicate. After reaching confluence artificial wounds were created using a sterile yellow 100 µl pipette tip. After washing with PBS, the cells were treated with proMMP-2 and MMP-2 with or without (GPO)₁₀/GAP or platelet-derived growth factor subunit B (PDGF-BB, Biomol, Hamburg, Germany) in DMEM supplemented with 0.25% FCS. Treatment with DMEM supplemented with 5% FCS was used as positive control. After 8 h, migrated cells were photographed using a digital camera coupled to a phase contrast microscope. Five randomly chosen fields were analyzed for each well. Wound diameters were analyzed using ImageJ (version 1.41; National Institutes of Health, Bethesda, MA) software.

In vitro invasion assay

Fifty microliters of 3 mg/ml Matrigel™ (BD Biosciences, Heidelberg, Germany) diluted in ice cold, serum-free DMEM were used to coat the upper compartments of 24-well FluoroBlok transwell inserts (BD Biosciences; pore size 8 µm) for 16 h at 37 °C. 2×10^5 HT1080 cells diluted in 300 µl serum-free medium were seeded into the upper compartments followed by addition of ilomastat, (GPO)₁₀, GAP or MMP-2. The lower compartment was also filled with serum-free medium. As a positive control, an FCS gradient was created by adding DMEM supplemented with 10% FCS as stimulating agent into the lower compartment. The plates were incubated for up to 24 h at 37 °C in a humidified atmosphere with 5% CO₂. Cells that remained in the upper compartment were gently removed with a cotton swab. The inserts were then washed with PBS and invaded cells on the lower surface were quantified after calcein AM (Life Technologies, Carlsbad, CA, USA) labeling using a fluorescence microplate reader (Molecular Devices, Sunnyvale, CA).

Determination of MMP-activity in cellular supernatants

Enzymatic activity of cellular supernatants was studied spectrofluorometrically by cleavage of the fluorogenic MMP-substrate DQ-gelatin as described [21]. HSC and HT1080 were treated for 24 h as indicated in the respective figures. As control, MMP-activity was blocked by addition of the MMP-inhibitor ilomastat. Supernatants were collected, 40 µl was mixed with 150 µl dye-quenched

(DQ)-gelatin solution, and substrate conversion was measured for 1.5 h. Background subtraction was applied to all measurements. The experiments were performed with a fluorescence microplate reader (Molecular Devices, Sunnyvale, CA) and black 96-well microtiter plates with clear bottoms (Greiner Bio One, Frickenhausen, Germany).

Statistical analysis

One-way ANOVA/Tukey tests were performed using SigmaStat for Windows (version 2.03; Systat, San Jose, CA). $p < 0.05$ was considered significantly different.

Results

$(GPO)_{10}$ and (pro)MMP-2 enhance the proliferation of HSC

First, effects of $(GPO)_{10}$ and (pro)MMP-2 on the proliferation of HSC were tested serum-starved cells. Recombinant proMMP-2 treated HSC reached 60% of maximum proliferation obtained by treatment with 10% FCS (Fig. 1). Simultaneous treatment of HSC with proMMP-2 and $(GPO)_{10}$ enhanced the stimulating effect of proMMP-2 alone, reaching 75% of maximum stimulation. This corresponded with the effects obtained by treatment of HSC with fully (pre)activated MMP-2, which induced an HSC proliferation of ~77% of the maximum level. HSC treated with $(GPO)_{10}$ showed a proliferation of ~66% of the maximum (Fig. 1) whereas treatment with the linear control-peptide GAP [17] showed no stimulatory effects on HSC proliferation as compared to control (not shown).

Fig. 1. Proliferation of hepatic stellate cells treated with $(GPO)_{10}$. Cell cycle-synchronized hepatic stellate cells were treated with proMMP-2 or MMP-2 with or without a tenfold molar excess of $(GPO)_{10}$ or GAP for 24 h as described in the Materials and methods section. Proliferation was determined by [3H]-thymidine incorporation. The proliferation of cells treated with DMEM supplemented with 10% fetal calf serum (FCS) was set to 100%. Shown are mean values and SD of five parallel measurements. ns=non-significant, *** $p < 0.001$

Fig. 2. Effects of $(GPO)_{10}$ on the migration of hepatic stellate cells. Confluent layers of hepatic stellate cells were scratched with a pipette tip and were treated with or without $(GPO)_{10}$, proMMP-2 or platelet-derived growth factor subunit B (PDGF-BB) and documented as described in the Materials and methods section. Indicated are the data of one representative experiment out of three individual experiments

$(GPO)_{10}$ is a mitogen for HSC

To determine $(GPO)_{10}$ effects on the motility of HSC, we next performed a wound healing migration assay. As expected, treatment of HSC in DMEM containing 0.25% FCS with the strong mitogenic homodimer of platelet-derived growth factor subunit B (PDGF-BB) provoked a migratory stimulus reaching ~70% of maximum HSC migration obtained by treatment with 5% FCS (Fig. 2). In this experimental setting, proMMP-2-treated cells displayed no differences to untreated cells. The addition of $(GPO)_{10}$ to the cells, however, induced a migratory stimulus of up to 80% of maximum migration. Interestingly, $(GPO)_{10}$ treatment alone also induced a strong HSC migration reaching ~57% of the maximum (Fig. 2). This on one hand demonstrates a proMMP-2-specific effect of $(GPO)_{10}$ but on the other hand also points to yet another mode of $(GPO)_{10}$ action. Of note, HSC treatment with GAP had no mitogenic activity (not shown).

$(GPO)_{10}$ enhances the invasive properties of HT1080 tumour cells

Given that HT1080 invasion through an ECM barrier depends on the proteolytic activity exerted by MMPs, we next applied $(GPO)_{10}$ to HT1080 cells in matrigel invasion assays. Addition of preactivated MMP-2 to the medium in the upper chamber induced an enhanced invasion of the tumour cells into the lower compartment reaching ~70% of maximum invasion capacity (Fig. 3). By adding inactive proMMP-2 to the media, however, only ~39% of maximum invasion could be observed. Furthermore, similar as in proliferation and migration experiments, the relatively low pro-invasive effects of proMMP-2 could be increased in the presence of $(GPO)_{10}$ to the level obtained

Fig. 3. Invasion of HT1080 cells following (GPO)₁₀ treatment. HT1080 cells were allowed to invade membranes coated with basement collagen in the absence or presence of (GPO)₁₀/GAP and (pro)MMP-2 as described in the Materials and methods section. After 24 h, transmigrated cells were detected by calcein AM labeling. Shown are the mean values $\pm$ SD of three independent experiments with four parallel measurements.

** $p < 0.01$, *** $p < 0.001$

by treatment with activated MMP-2 pointing towards an increased activation/activity of (pro)MMP-2 following (GPO)₁₀ treatment (Fig. 3).

The mitogenic effects of (GPO)₁₀ are due to enhanced activation of proMMP-2

The hypothesis that the stimulatory effects of (GPO)₁₀ on HSC proliferation and HT1080 invasion could be referred to enhanced enzymatic activity of MMP-2 in the supernatants was next investigated applying fluorescent based gelatinase-activity assays.

Supernatants from HSC and HT1080 containing exogenous (pre)activated MMP-2 showed the highest gelatinolytic activity (Fig. 4). In contrast, the gelatinolytic activity in supernatants containing exogenous proMMP-2 was lower, but was markedly enhanced in the presence of (GPO)₁₀ reaching the level of fully active MMP-2. This clearly demonstrates activating effects of (GPO)₁₀ on proMMP-2 *in vitro*. Taken together, we here show for the first time that the triple helical peptide (GPO)₁₀ modulates cellular responses *in vitro* by activation of proMMP-2 in the supernatants.

Discussion

Fibrillar collagen structures were shown to serve as storage site for inactive proforms of matrixmetalloproteinases (MMPs) and accumulate in extracellular matrices under pathological conditions such as liver fibrosis or intestinal inflammation. Thus, modulating this MMP–collagen interaction might offer a rational approach to enhance deg-

radation of accumulated ECM. In a previous study, the potential of the synthetic collagen analogue (GPO)₁₀ to act as a binding partner for the gelatinases proMMP-2 and proMMP-9 could be clearly demonstrated. Furthermore, (GPO)₁₀ induced enzymatic activation and activity of (pro)MMP-2, but not (pro)MMP-9 [17]. However, effects of (GPO)₁₀ on MMPs under physiological *in vitro* conditions such as cell culture experiments have not been tested so far. As a preceding step prior to the testing of (GPO)₁₀ *in vivo*, the present study provides evidence that (GPO)₁₀ promotes the activation and activity of (pro)MMP-2 *in vitro*.

Initially, we examined the effects of (GPO)₁₀ on the proliferation of HSC. These cells are known to produce proMMP-2, and recent studies have suggested that MMP-2 itself acts as a mitogen for HSC [22]. The latter was confirmed by our experiments, in that the addition of pre-activated MMP-2, in contrast to enzymatically inactive proMMP-2, induced a marked increase of HSC proliferation. In the presence of (GPO)₁₀, treatment of HSC with proMMP-2 showed a stimulatory effect similar to activated MMP-2 alone. This points towards an activating effect of (GPO)₁₀ on proMMP-2 even in cell supernatants.

In another set of experiments, we investigated the potential of (GPO)₁₀ to act as a mitogen in a 'wound healing' model system. Given that PDGF signaling is the most effective mitogenic pathway in stellate cells [23], PDGF-BB was used as positive control. Similar to the proliferation experiments, a concomitant treatment of (GPO)₁₀ with proMMP-2 provoked a stronger mitogenic stimulus than proMMP-2 alone. Interestingly, even treatment with (GPO)₁₀ alone showed a comparable stimulatory effect like PDGF-BB. Thus, besides acting via the activation of endogenous proMMP-2, (GPO)₁₀ might also impact cellular processes directly, as it does via activation of immune receptors such as the platelet glycoprotein VI (GPVI) or the leukocyte-associated immunoglobulin-like receptor-1

Fig. 4. Effects of (GPO)₁₀ on MMP activity in cellular supernatants. Supernatants from the preceding proliferation or invasion measurements were mixed with dye-quenched (DQ)-gelatin substrate, and gelatinolytic activity was measured as described in the Materials and methods section. Results are given as mean values $\pm$ SD from three independent experiments with five parallel measurements. *** $p < 0.001$

(LAIR1) [19]. An intracellular target of (GPO)₁₀ might be phosphatidylinositol 3-kinase (PI3-K) signaling, which is involved in PDGF-induced migration [23]. The latter needs to be addressed in ongoing studies.

In a third approach, we tested the influence of (GPO)₁₀ on the invasion of tumour cells through an ECM barrier which requires the release and proteolytic activity of matrix degrading enzymes such as MMPs [24]. The human fibrosarcoma cell line HT1080 is known to secrete relatively high amounts of proMMP-2 [25]. Treatment of the cells with (GPO)₁₀ almost doubled the invasive capacity of the tumour cells, indicating an enhanced activation of endogenous proMMP-2 by (GPO)₁₀ in the supernatant.

We finally confirmed our hypothesis that (GPO)₁₀ activates proMMP-2 *in vitro* by performing fluorescent-based MMP-activity assays with aliquots of the respective supernatants from the preceding assays. As expected, the presence of (GPO)₁₀ strongly increased the gelatinolytic activity in the supernatants compared to untreated cells. In line with this, adding exogenous proMMP-2 concomitant with (GPO)₁₀ resulted in a comparable strong increase in gelatinolytic MMP-activity as obtained by adding exogenous pre-activated MMP-2. This is in accordance with substrate gel zymography experiments in previous studies, which clearly showed the generation of activated, pro-domain free MMP-2 due to the treatment with (GPO)₁₀ [17].

In all experiments, the usage of the linear control-peptide GAP showed no effects on investigated cellular processes (not shown in the figures). We conclude that the (GPO)₁₀-mediated effects result from its unique triple helical structure.

In summary, we here demonstrate that (GPO)₁₀ is capable to induce MMP-2 activity *in vitro* which could impact MMP-associated processes like proliferation and invasion.

In conjunction with our previous study, which focused on the mechanisms of proMMP-2 activation by (GPO)₁₀ in binding studies including *in situ* experiments, the present study adds a solid background of *in vitro* data, which provides a rationale for the appliance of (GPO)₁₀ or structure related peptides in animal models such as inflammatory bowel disease where MMPs play a central role for the inflammatory response [12, 13].

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