Matrix Metalloproteinases-1 and -8 Improve the Distribution and Efficacy of an Oncolytic Virus

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Abstract
Oncolytic viral vectors show enormous potential for the treatment of many solid tumors. However, these vectors often suffer from insufficient delivery within tumors, which limits their efficacy in both preclinical and clinical settings. We have previously shown that tumor collagen can significantly hinder diffusion, and that its degradation can enhance the distribution and efficacy of an oncolytic herpes simplex virus (HSV) vector. Here, we identify two members of the matrix metalloproteinase (MMP) family of enzymes, MMP-1 and MMP-8, which can modulate the tumor matrix and enhance HSV delivery and efficacy. We show that overexpression of MMP-1 and MMP-8 in the human soft tissue sarcoma HSTS26T leads to a significant depletion of tumor-sulfated glycosaminoglycans. This increases the hydraulic conductivity of these tumors and enhances the flow of virus during injection. In control tumors, injected virus accumulates primarily in the periphery of the tumor. In contrast, we observed a more widespread distribution of virus around the injection site in MMP-1- and MMP-8–expressing tumors. Due to this enhanced vector delivery, MMPexpressing tumors respond significantly better to oncolytic HSV treatment than control tumors. Thus, these findings introduce a new approach to improve the delivery and efficacy of oncolytic viral vectors: modulation of tumor glycosaminoglycans to enhance convection. [Cancer Res 2007;67(22):10664–8]

Introduction
Oncolytic viruses have shown promise for the treatment of various tumors in both preclinical tumor models and clinical trials (1). However, the inability to distribute throughout the tumor mass is well documented and has limited clinical efficacy, even when multiple intratumor injections are used (2). Unlike traditional chemotherapeutics, most novel targeted therapeutics face tremendous transport barriers in the tumor interstitium due to their large size. We and others have documented the role of the extracellular matrix (ECM) in preventing efficient distribution of macromolecules in tumors (3–6). One method to overcome this therapeutic delivery barrier is to modify the tumor matrix. Matrix metalloproteinases (MMP) serve as ideal agents because they can degrade all components of the ECM (7). In particular, MMP-1 and MMP-8 have the ability to degrade fibrillar collagens, proteoglycans, and other structural matrix components (7). Here, we reveal for the first time the ability of MMP-1 and MMP-8 to improve convective transport in tumors and enhance the efficacy of oncolytic herpes simplex virus (HSV) therapy.

Materials and Methods

Animals and tumor models. Human soft tissue sarcoma HSTS26T cells and transfected clones were grown s.c. in the leg and in the dorsal skinfold chamber of severe combined immunodeficient (SCID) mice as described previously (5).

Plasmid construction and stable transfections. The cDNA coding for human MMP-1 (American Type Culture Collection) and MMP-8 (gift of David Tarin, University of California, San Diego, CA) were subcloned into the retroviral vector pBMN-I-GFP (gift of Garry Nolan, Stanford University, Stanford, CA). Packaging of retroviral vectors and stable transduction was carried out as described previously (8).

Western blot. AB806 and AB8115 (Millipore) were used to detect MMP-1 and MMP-8, respectively. β-Actin was detected with a polyclonal antibody (Santa Cruz Biotechnology).

Second harmonic generation imaging and quantification. Imaging of second harmonic generation (SHG) in dorsal chamber tumors was performed with a custom-built multiphoton laser scanning microscope (9). Excitation was at 880 nm, and SHG signal was detected via a 435DF30 emission filter with a high pass 475 dichroic. Three-dimensional image stacks were obtained of the top 130 μm from the surface of the tumor (27 images of 5 μm thickness). Six to ten image stacks were obtained to create a montage covering the entire tumor or a significant portion of it. The total SHG signal intensity in each stack was measured.

Histology. Tumor tissue was harvested, fixed in 4% paraformaldehyde, and embedded in paraffin. Sections 5 μm thick were cut and stained using a polyclonal anti–HSV-1 antibody (DAKO) and a biotinylated hyaluronic acid (HA)–binding protein (Calbiochem). For quantification of HA staining, eight random images were taken per section. The brown pseudocolor was selected and thresholded, and the mean brown pixel count for each section was measured. For HSV staining, individual images were taken to create a montage of the entire tumor. A square grid with 100-μm-length grid boxes was overlaid on the images, and grid boxes containing brown staining were counted. In both cases, the analysis was performed on single tissue sections from six to seven separate tumors for each experimental group.

Collagenase activity assay. Collagenase activity in tumor lysates was determined using an in vitro assay modified from the method of Johnson-Wint (10).

Tumor growth delay. Tumors were implanted s.c. in the leg of SCID mice and allowed to reach 60 mm3 average volume (n = 7 for each group). Tumors were treated with 10–12 plaque-forming units (p.f.u.) of oncotyic HSV MGH2 (gift from E. Antonio Chiocca, Ohio State University, Columbus, OH) in PBS or PBS alone. A second injection was performed 2 days later. Tumor volume was measured every 3 to 4 days and calculated as volume = πAB2/6, where A and B are the maximum and minimum diameters, respectively.

Fluorescence recovery after photobleaching. A custom-built multiphoton microscope (9) was adapted for fluorescence recovery after photobleaching (FRAP) based on a previous design (11). One-half microliter...
of tetramethylrhodamine-labeled $2 \times 10^6$ molecular weight dextran (Invitrogen) was injected at a concentration of 2 mg/mL as described previously (12). The laser was set at 800 nm, and a 40\(/\)0.75 numerical aperture objective lens was used with a 525/100 filter. During each bleach/recovery cycle, the sample was bleached with a 160-mu s pulse train of light, followed by an 40 ms recovery monitored in 40-mu s time bins. Each bleach/recovery cycle was repeated 500 to 1,000 times for a given spot/measurement. Ten to twenty measurements were taken for each individual tumor.

**Hydraulic conductivity.** Measurements were performed using a tissue clamp apparatus as described previously (13).

**Cell proliferation assay.** Mock and MMP-transfected HSTS26T cells ($1 \times 10^4$) were seeded in 96-well plates. After a 48-h incubation, the media was replaced with 10 μL of WST-1 reagent (Roche) in fresh media. The cells were incubated for 2 h at 37°C and 5% CO₂, and the absorbance at 450 nm was measured.

**Viral titer.** Cells ($4 \times 10^5$) were seeded in 12-well plates and infected with MGH2 at a multiplicity of infection (MOI) of 0.1. After 48 h incubation, cells and supernatant were collected, freeze/thawed, and titered with HSTS26T cells by counting enhanced green fluorescent protein–expressing cells.

**Measurement of glycosaminoglycan content.** Sulfated glycosaminoglycan content was measured using the Blyscan assay (Biocolor) as described previously (4).

**Statistical analysis.** Data are expressed as mean ± SE. Statistical significance between groups was determined by unpaired Student’s t test. Differences were considered statistically significant at $P < 0.05$.

**Results and Discussion**

MMP-1 and MMP-8 expression enhances the efficacy of oncolytic HSV therapy. Human soft tissue sarcoma HSTS26T cells were stably transfected with full-length human MMP-1 and MMP-8, and tumors were grown s.c. in the flank of SCID mice. Western
blots were performed on tumor lysate to confirm the expression of each enzyme (Fig. 1A). Both MMP-1 and MMP-8 contain an NH₂-terminal pro-peptide domain, which must be cleaved to form the active enzyme. Thus, both MMPs can exist in two forms: the full-length zymogen or the smaller active form. The Western blots show expression of MMP-1 and MMP-8 in the tumors grown from their respective transfected cells, but not tumors grown from mock-transfected cells. MMPs are present in both the latent and active forms, although the majority is in the zymogen state.

To determine whether MMP-1 and MMP-8 expression could affect the efficacy of oncolytic HSV, we treated control and MMP-expressing s.c. tumors with the oncolytic HSV vector MGH2 (14). For all three groups, tumors were treated with two intratumor injections of either 2.5 × 10⁶ t.u. of the oncolytic HSV vector MGH2 or PBS control. A significant delay in growth between control and treated tumors was observed for both MMP-1– and MMP-8–expressing tumors, but not the mock transfected (Fig. 1B). Thus, MMP-1 and MMP-8 expression can enhance the efficacy of oncolytic HSV treatment.

MMP expression does not affect cell proliferation or infectivity. To determine the cause of this enhanced efficacy, we first measured the effect of MMP-1 and MMP-8 expression on cell viability and proliferation. A WST assay showed no difference between the mock- and MMP-transfected tumor cells (Fig. 2A). Next, we measured the effect of MMP expression on viral replication. Tumor cells were infected with MGH2 at a MOI of 0.1.

![Figure 2](image1)

Figure 2. Effect of MMP-1 and MMP-8 expression on tumor cell proliferation and viral infection and replication. A, WST-1 cell proliferation assay performed on mock-, MMP-1–, and MMP-8–transfected cells. Cells were allowed to grow for 48 h following seeding, and the absorbance of 450 nm light was measured following the application of WST-1. There is no significant difference between cell lines. B, comparison of the viral titer 48 h following infection at a MOI of 0.1. Viral titer was assessed with wild-type HSTS26T cells. There is no significant difference between cell lines. Columns, mean values for each assay; bars, SEs.

The viral yield 48 h after infection was compared. There was no difference in viral yield between cell lines (Fig. 2B). These results show that the enhanced response of MMP-1– and MMP-8–expressing tumors to oncolytic HSV is not due to a modulation in cell viability or the infectivity of HSV.

Enhanced convection improves virus distribution. To gain insight into the differential response to MGH2 treatment, HSV immunostaining was performed on tumor sections 7 days following treatment with a single injection of MGH2. In control tumors, HSV particles were present only in the tumor periphery (Fig. 1C). In contrast, for MMP-1– and MMP-8–expressing tumors, HSV particles were located in both the tumor periphery and center (Fig. 1D and E). The fraction of tumor area containing HSV staining was quantified (Fig. 1F). As expected from the tumor growth delay results, MMP-expressing tumors had a significantly larger HSV staining fraction than mock. When the tissue HSV staining was subdivided into “periphery” and “center” subfractions, we found that the majority of the difference was due to the increase in HSV staining in the center of MMP-expressing tumors.

The lack of HSV staining in the center of the mock-transfected tumors is intriguing because MGH2 was injected directly into the center of the tumors. These results suggest that MMP-1 and MMP-8 expression has altered the tumor such that the delivery of viral particles, either by diffusive or convective interstitial transport, has been enhanced. We tested this hypothesis by probing these two
transport mechanisms. We found that whereas the diffusion coefficient of tracer particles was no different in each tumor type (Fig. 3A), the hydraulic conductivity of MMP-1– and MMP-8–expressing tumors was 2-fold greater than the control tumors (Fig. 3B). Thus, it seems that MMP expression enhances the therapeutic response to oncolytic HSV vectors by improving the flow of viral vectors into the tumor tissue during intratumor injection.

Our observation that injected virus distributes heterogeneously in the tumor and that this distribution can be made more widespread by decreasing the resistance to fluid flow is in agreement with previous studies with HSTS26T tumors. Netti et al. (4) measured the elastic modulus and hydraulic conductivity in a panel of tumors and found that HSTS26T was the hardest and had the greatest resistance to flow. The extreme stiffness of this tumor likely causes the unique distribution of injected agents. Boucher et al. (15) found that when they infused Evans blue-labeled albumin into the center of HSTS26T tumors, it distributed asymmetrically from the source and often in necrotic regions. Fluid accumulation was found away from the infusion site, including at the surface of tumors, with channels of fluid connecting regions of accumulation. The pattern of fluid flow through preferential pathways rather than bulk flow to the surrounding tissue in this study replicates the previous findings. Furthermore, when Boucher et al. (15) performed similar injections in the colon adenocarcinoma LS174T, which has a 5-fold greater hydraulic conductivity than HSTS26T, they found that dye distributed symmetrically around the infusion needle in the center of the tumor. Thus, our results suggest that the increase in hydraulic conductivity in these tumors can significantly enhance the delivery of injected viral vectors.

**Sulfated glycosaminoglycan depletion is responsible for improved transport.** To determine the mechanism of enhanced fluid flow in the MMP-expressing tumors, we turned to the ECM. Diffusion and convection in normal and tumor tissue are primarily regulated by the ECM constituents. Our previous findings show that interstitial collagen type I is primarily responsible for diffusive hindrance in tumors (4, 6, 12). Addition of agents that degrade interstitial collagen can improve diffusion in tumors (4, 5). Although an increase in tumor collagen has been linked to a reduction in hydraulic conductivity, fluid flow is also regulated by the glycosaminoglycan content of the tissue (16). A strong negative correlation between tissue glycosaminoglycan concentration and hydraulic conductivity has been established (13). In particular, HA, a nonsulfated glycosaminoglycan, has been found to be a significant source of hindrance to fluid flow (17), and its degradation has been shown to significantly increase tissue hydraulic conductivity (16, 17). Sulfated glycosaminoglycans may also have a significant effect on hydraulic conductivity. Application of trypsin, a protease that can act on proteoglycan core proteins and glycoproteins, to corneal stroma caused a significant depletion of sulfated glycosaminoglycans and a greater increase in hydraulic conductivity than hyaluronidase (17). Collectively, these studies suggest that collagen I, HA, and sulfated glycosaminoglycans, and perhaps even glycoproteins, all play an important role in regulating interstitial transport.

To determine which components of the ECM were modified by MMP-1 and MMP-8 and contributed to enhanced fluid flow, we measured the level of collagen type I, HA, and sulfated glycosaminoglycans in these tumors. We found that there was no difference in collagen I or HA content between mock- and MMP-expressing tumors (Fig. 4A and C; Supplementary Figs. S1 and S2). However, MMP-1 and MMP-8 expression caused a significant reduction in sulfated glycosaminoglycan content (Fig. 4B). Because MMP-1 and MMP-8 are primarily known as collagenases, we also confirmed the lack of collagenase activity with a fibril degradation assay and by Western blot (Supplementary Fig. S3). Thus, it seems that MMP-1 and MMP-8 expression has improved the convective delivery and efficacy of
viral vectors by decreasing the tumor-sulfated glycosaminoglycan content.

The likely method of sulfated glycosaminoglycan depletion is the degradation of chondroitin sulfate proteoglycans. Although both MMP-1 and MMP-8 have traditionally been characterized as type I collagenases, they have multiple other ECM substrates (7). Both MMP-1 and MMP-8 can degrade the chondroitin sulfate proteoglycans aggrecan and brevican (18). Although aggrecan is found mainly in cartilage, tumors have been shown to have elevated levels of other chondroitin sulfate proteoglycans (19). Proteoglycans besides aggrecan have yet to be studied as substrates for either MMP-1 or MMP-8, but other aggrecanases are able to cleave multiple other proteoglycans in a similar manner (20). Furthermore, MMP-1 can degrade link protein, which stabilizes the interaction of various chondroitin sulfate proteoglycans with HA. These studies suggest that proteoglycans are a possible target of MMP cleavage. However, there are a host of other candidate ECM proteins whose degradation may lead to the alteration of tumor hydraulic conductivity because these MMPs have been found to cleave various other fibrillar collagens and glycoproteins (7).

In summary, we find that MMP-1 and MMP-8 expression can enhance the efficacy of an oncolytic HSV vector by improving its distribution within tumors. Our previous work has shown that collagen degradation can improve interstitial diffusion and therapeutic delivery (4–6). Here, we develop a novel approach to enhance treatment efficacy: depletion of tumor-sulfated glycosaminoglycans via matrix proteases to enhance the convection of a therapeutic agent into the surrounding tissue during intratumor injection. Much research has gone into improving the delivery of injected therapeutics by optimizing infusion parameters (21). Our work establishes a technique to enhance this process by modifying the tumor itself and making it more amenable to the injection of large, transport-limited agents such as viral vectors. Two MMPs are identified, which can be used to enhance therapeutic distribution during injection. Although MMPs in general have been found to promote various processes in cancer cell invasion and metastasis (7), the specific roles of MMP-1 and MMP-8 are still unclear. In fact, there is emerging evidence that MMP-8 is antimetastatic (22). Thus, this work suggests a clinically relevant method to enhance the effectiveness of many cancer therapeutics.

References


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