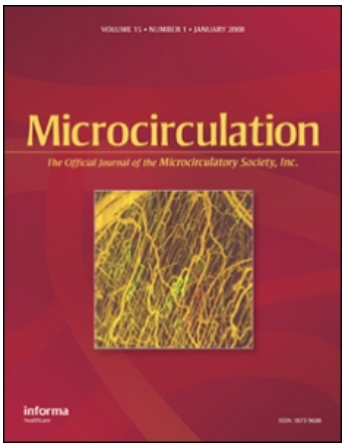

Microcirculation
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713723262

Inhibition of Glycan Shedding and Leukocyte-Endothelial Adhesion in Postcapillary Venules by Suppression of Matrixmetalloprotease Activity with Doxycycline

Aaron W. Mulivor [a]; Herbert H. Lipowsky [a]
[a] Department of Bioengineering, Pennsylvania State University, University Park, Pennsylvania, USA

First Published on: 04 August 2009

To cite this Article Mulivor, Aaron W. and Lipowsky, Herbert H.(2009)'Inhibition of Glycan Shedding and Leukocyte-Endothelial Adhesion in Postcapillary Venules by Suppression of Matrixmetalloprotease Activity with Doxycycline',Microcirculation,99999:1,

To link to this Article: DOI: 10.1080/10739680903133714

URL: http://dx.doi.org/10.1080/10739680903133714

Microcirculation. iFirst: 1–10. 2009

ISSN: 1073-9688 print / 1549-8719 online
DOI: 10.1080/10739680903133714

informa
healthcare

Inhibition of Glycan Shedding and Leukocyte-Endothelial Adhesion in Postcapillary Venules by Suppression of Matrixmetalloprotease Activity with Doxycycline

AARON W. MULIVOR AND HERBERT H. LIPOWSKY
Department of Bioengineering, Pennsylvania State University, University Park, Pennsylvania, USA

ABSTRACT

Objective: The aims of this study were to examine the role of matrixmetalloproteinases (MMPs) in causing shedding of glycan components of the endothelial glycocalyx and delineate the efficacy of doxycycline as an inhibitor of white blood cell–endothelial cell (WBC-EC) adhesion and glycan shedding in postcapillary venules. **Methods:** WBC-EC adhesion in postcapillary venules of mesentery (rat) was examined in response to superfusion with the chemoattractant, f-Met-Leu-Phe (fMLP). Glycan shedding was delineated by using fluorescently labeled microspheres (FLMs; 0.1 μm in diameter) coated with lectins and infused into the systemic circulation. The shedding of FLMs in response to fMLP was examined during superfusion with graded concentrations of doxycycline and the zinc chelator, ilomastat. **Results:** Superfusion of mesentery with 10^{-7} M of fMLP caused a reduction in FLM adhesion due to shedding of the glycocalyx and a rise in WBC-EC adhesion. WBC-EC adhesion and FLM shedding were reduced with subantimicrobial concentrations of doxycycline equal to or greater than 0.5 μM with an EC_{50} value of 0.15 μM. MMP activation was verified by inhibition of shedding and attenuation of circulating MMP substrate cleavage at the venular wall with the zinc chelator, ilomastat (GM6001, 2.6 μM; US Biological, Swampscott, Massachusetts, USA).**Conclusions:**MMPs play a significant role in glycan shedding and WBC-EC adhesion, and doxycycline may stabilize the endothelial glycocalyx by inhibition of MMP activation.
Microcirculation (2009) **iFirst,** 1–10. doi:10.1080/10739680903133714

KEY WORDS: glycocalyx, matrix metalloproteinases, MMP, doxycycline, leukocyte-endothelium adhesion

INTRODUCTION

The vascular endothelium is coated with a macromolecular layer of membrane-bound proteins and their associated glycosaminoglycans (GAGs; heparan sulfate, chondroitin sulfate, and hyaluronan), referred to as the glycocalyx [3], and a superficial layer of adsorbed proteins, both of which comprise the endothelial surface layer [32]. It has been demonstrated that the endothelial cell glycocalyx is a dynamic layer of polysaccharides whose composition reflects a balance of shear dependent removal [2] and continued biosynthesis of new glycans [29].

Address correspondence to Herbert H. Lipowsky, Department of Bioengineering, Pennsylvania State University, 205 Hallowell Building, University Park, PA 16802, USA. E-mail: HHLBIO@engr.psu.edu

Received 2 February 2009; accepted 22 June 2009.

The most common macromolecular components of the glycocalyx are carbohydrates and glycoproteins, such as GAGs, proteoglycans, and glycolipids [19,33]. Studies have suggested that heparan sulfate, one of the major GAG constituents of the glycocalyx, limits leukocyte-endothelial interactions by hindering the ability of leukocytes, such as white blood cells (WBCs), to bind to intracellular adhesion molecule-1 (ICAM-1) on endothelial cells (ECs) [6,28]. Further, it has been shown that components of the glycocalyx can be shed rapidly during an inflammatory response and ischemia to facilitate increased WBC-EC adhesion [29]. Modification of the glycocalyx is significant because it is indicative of endothelial dysfunction and has been linked to pathological disease states, such as atherosclerosis [22,39] and diabetes [30].

Considerable evidence suggests that matrix metalloproteinases (MMPs) have the ability to modify components of the endothelial glycocalyx and thus

facilitate the shedding of its constituents under pathological conditions. MMPs represent a family of over two dozen zinc-dependent proteases that play a role in normal tissue remodeling during bone growth, wound healing, reproduction, cancer, inflammation, and cardiovascular disease [35]. Recent studies suggest that MMPs (-1 and -9) serve to cleave the endothelial insulin receptor and CD18 on leukocytes in the spontaneously hypertensive rat [10]. Modification of the extracellular matrix by MMPs has been shown to be a critical step in angiogenesis [18] and atherosclerosis [24]. MMP-2, -7, and -9 were shown to be capable of directly cleaving chondroitin sulfate [17]. In addition, MMP-1 was shown to cleave the heparan sulfate proteoglycan, syndecan-1 [12]. MMPs can be stored within and released by the endothelium. It has been shown [38] that both the active and proactive forms of MMP-2 and -9 are stored in vesicles within the EC. Similarly, MMP-7, in both its constitutive and latent forms, has a high affinity for, and binds to, heparan sulfate [42]. This suggests that there exist mechanisms by which MMPs can be rapidly released by ECs without the need to wait for the upregulation of mRNA. Inhibition of MMPs occurs naturally by a class of tissue inhibitors of metalloproteinases (TIMPs). TIMPs are comprised of a family of four different molecules made unique by their expression, localization, and inhibitory activity. Much like the MMPs, TIMPs are capable of binding heparan sulfate and chondroitin sulfate in the glycocalyx [42].

The aim of the current study was to examine the rapid shedding of glycans from the venular endothelium in intestinal mesentery (rat) by using a well-defined model of inflammation and test the hypothesis that fMLP-induced shedding of the glycocalyx involves MMP activity. To this end, changes in glucose and galactose moieties of the glycocalyx were delineated with lectin-coated, fluorescently labeled microspheres (FLMs; 0.1 μm in diameter). Adhesion of microspheres was quantified during normal steady-state conditions, as well as following the topical application of fMLP, which resulted in a decrease in the adhesion of the microspheres. Pretreating tissues with the topical application of the broad-spectrum MMP inhibitor, doxycycline, or the specific MMP hydroxamic acid inhibitor, ilomastat, was found to suppress glycan shedding as well as leukocyte adhesion.

MATERIALS AND METHODS

Animal Preparation

Male Sprague-Dawley rats, weighing 300–450 g, were anesthetized with Inactin® (Sigma, St. Louis, Missouri, USA; 125 mg/kg, intraperitoneally; i.p.), tracheostomized, and allowed to breathe spontaneously. The right internal jugular vein was cannulated with polyethylene (PE-50) tubing to enable the administration of supplemental doses of anesthetic, as required, to maintain a surgical plane of anesthesia. The left carotid artery was cannulated with PE-90 tubing and connected to a strain gage-type pressure transducer to monitor arterial blood pressure. Systemic arterial pressure averaged 125.0 ± 12.7 (standard deviation; SD) mmHg and did not change with any of the treatments. To facilitate the systemic infusion of fluorescently labeled microspheres (FLMs), the right femoral vein was cannulated with PE-10 tubing and connected to an infusion syringe pump (PHD-2000; Harvard Apparatus, Holliston, Massachusetts, USA).

Intravital Microscopy and Experimental Protocol

The intestinal mesentery was exteriorized through a midline abdominal incision and placed on a glass pedestal to facilitate viewing under either bright-field microscopy by either trans- or incident-illumination. Fluorescence microscopy was performed under incident Hg-lamp illumination with a dichroic mirror and filters appropriate for fluorescein excitation and emission spectra. The tissue was superfused with HEPES-buffered Ringer's solution (pH, 7.4) at a temperature of 37.0°C. Solutions of the synthetic peptide, f-Met-Leu-Phe (fMLP, 10^{-7} M; Sigma, St. Louis, Missouri, USA), were prepared in HEPES-buffered Ringer's solution (pH, 7.4) for irrigation of the tissue. The protocol used for these experiments was as follows. A bolus of labeled FLMs was infused at 2×10^{12} spheres/mL/kg to obtain a circulating concentration of 10^7 FLMs/mL and maintained at that level by an intravenous (i.v.) infusion of 2×10^9 spheres/kg/min via the femoral vein. The circulating concentration of FLMs was measured by using a hemocytometer under fluorescence microscopy for blood samples withdrawn from the carotid artery. To model the inflammatory process, following a control period of tissue stabilization, the mesentery was superfused with fMLP (10^{-7} M) in Ringer's solution or Ringer's solution with either doxycycline (Sigma) or ilomastat (GM6001;

US Biological, Swampscott, Massachusetts, USA) for the duration of the experiment. Studies of the effects of doxycycline and ilomastat were done by irrigation of the mesentery with these agents solubilized in Ringer's solution for 30 minutes prior to superfusion with fMLP, followed by their continued application with the addition of fMLP.

Mesenteric postcapillary venules, ranging in width from 25 to 50 μm, were viewed with a Zeiss water-immersion 40X/0.75 NA objective (Carl Zeiss MicroImaging, Thornwood, NY, USA) for measurement of red-cell velocity and counting of microspheres adhered to the ECs. The image was projected onto a low-light-level silicon-intensified target camera (model 66; Dage-MTI, Michigan City, Indiana, USA) for an effective width of the video field of 100 μm. By focusing above and below the microvessel diametral plane during video recording, all of the intense FLMs adhered in a 100-μm length of venule could be counted during off-line video analysis. *In vitro* calibration studies [25] of the adhesion of FLMs to glass surfaces, to which various concentrations of chondroitin sulfate were covalently linked, revealed that the number of bound FLMs was proportional to GAG concentration and was invariant with wall shear stresses over the physiological range of 1–50 dynes/cm^2, with or without the presence of 10^{-7} M of fMLP.

Centerline red-cell velocity (V_{RBC}) was measured in arterioles and venules by using the two-slit photometric technique (self-tracking correlator; IPM, San Diego, California, USA), as described previously [28]. The mean velocity of blood (V_{MEAN}) was calculated from the following relationship: $V_{MEAN} = V_{RBC}/1.6$ [26]. Microvessel diameter (D) was measured by the video-image–shearing technique, using an image-shearing monitor (IPM).

Fluorescent Microsphere Preparation and Lectin Labeling

Fluorescent (yellow-green) carboxylate-modified polystyrene microspheres, 0.1 μm in diameter (FLMs, Fluospheres; Molecular Probes, Eugene, Oregon, USA), were labeled by covalent linkage (carbodiimide reaction) of lectins with preferential binding to either galactose or glucose moities on the EC surface, as described previously [29]. The lectin, *Bandeiraea simplificifolia* (BS1; Sigma), was used to probe for galactose, and *Lycopersicon esculentum* (LEA; Sigma) was used to probe for glucose. These two lectins were selected because of the abundance of these sugars on the GAGs, condroitin sulfate and heparan sulfate, respectively, which are major components of the endothelial glycocalyx.

Measurements

WBC and FLM Adhesion. The numbers of WBCs and FLMs firmly adhered were measured by frame-by-frame analysis of video recordings and quantified as the number adhering per 100 μm of venule length. WBCs and FLMs were judged to be firmly adhered if they remained stationary for at least five seconds. All FLMs and WBCs were counted by frame-by-frame playback of video recordings obtained while focusing the microscope objective above and below the diametral plane.

Statistical analysis of trends in the data were performed by using SigmaStat (SPSS Inc., Chicago, Illinois, USA) and employed either the Student's *t*-test for paired tests or the Holm-Sidak method for the analysis of variance (ANOVA) of multiple comparisons.

In Situ Microzymography

To verify that MMPs were activated by superfusion of the mesenteric tissue with fMLP, techniques of microzymography were employed. The fluorescent FRET (fluorescence resonance energy transfer) substrate, 520 MMP FRET Substrate XIV (Anaspec Inc., San Jose, California, USA), was solubilized to 17.4 μM in normal saline and administered as an initial i.v. bolus of 0.5 mL, followed by a continuous infusion (syringe pump) at a rate of 0.033 mL/min. This broad-spectrum substrate consists of fluorescence donor and quencher molecules on either end of a peptide sequence that is cleaved by activated MMP-1, -2, -3, -7, -8, -9, -10, -12, -13, or -14. Cleavage of the substrate results in loss of the quenching molecule and increased fluorescence. Fluorescence was observed by using a spinning disk confocal microscope [Yokogawa CSU-10 with a Stanford Photonics intensified charge-coupled device (CCD) camera; Solamere Technology, Salt Lake City, Utah, USA] with a Zeiss 20X/0.50-NA water-immersion objective, using laser excitation at 488 nm and capturing fluorescence at 520 nm. Fluorescence images were digitized at 1,024 × 1,024 pixels × 10 bits depth and corrected by background subtraction and field flattening against a uniform

field standard. Corrected images were analyzed by using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). *In vitro* calibration studies of fluorophores of known concentrations revealed a linearity and accuracy within 5% in the determination of unknown concentrations.

RESULTS

Leukocyte Adhesion

Presented in Figure 1 are representative images of WBC adhesion prior to (panels A and B) and 5 minutes following (panels C and D) the onset of superfusion of the mesenteric tissue with 10^{-7} M fMLP. Systematic sampling of the number of WBCs adhered to the ECs, by focusing up and down along the optical axis, revealed a significant rise in the number of WBCs adhered per 100 μm of venule length, as shown in the subsequent figures. Also shown is the adhesion of BS-1–coated FLMs used to indicate the amount of glycans present on the surface of the endothelium. As shown in Figure 1B and 1D, with the topical application of fMLP, the number of bound FLMs fell significantly, from 6 to 2 per 100 μm of venule length.

Shown in Figure 2 is the number of WBCs adhered along the length of single unbranched venules (expressed as number of WBCs/100 μm of venule length) for groups of postcapillary venules ($n=6$) superfused with either Ringer's solution alone, doxycycline, or doxycycline plus 10^{-7} M of fMLP for five concentrations of doxycycline, ranging from 5 nM to 250 μM. The well-established, fMLP-induced increase in WBC adhesion was significantly inhibited by doxycycline superfusion at concentrations equal to or greater than 0.5 μM (0.25 mg/L; $P<0.05$). Fitting the data with a dose-response

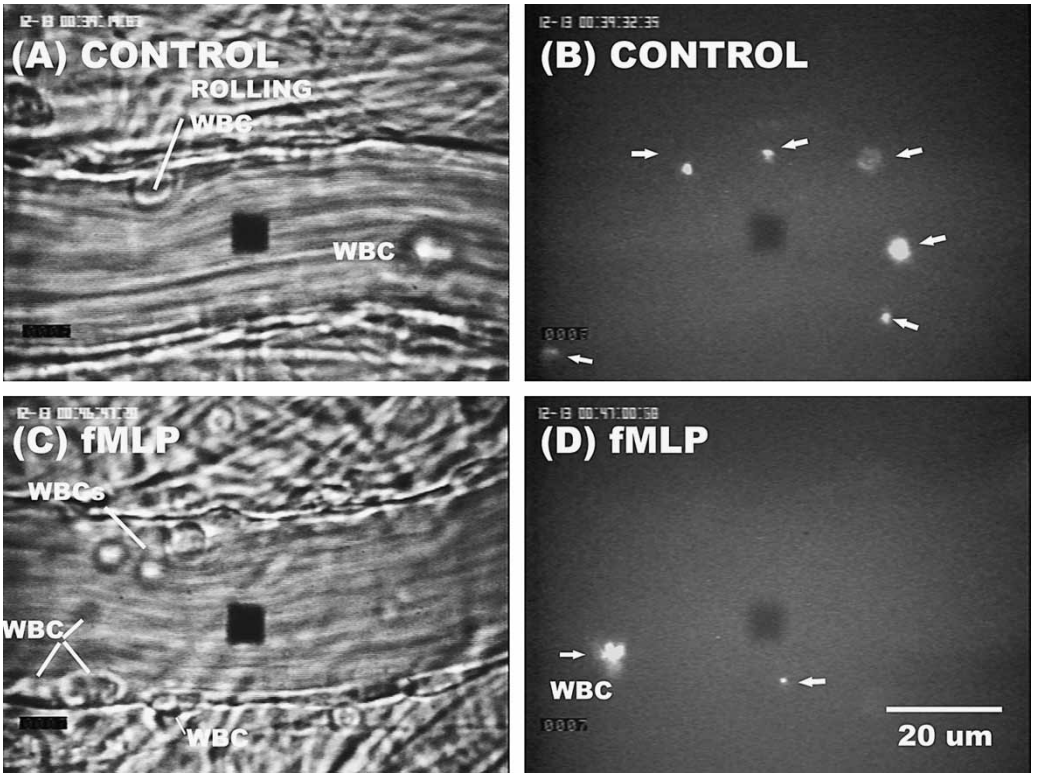

Figure 1. Representative video scenes of WBC adhesion (**A, C**) to the endothelium (EC) under bright-field illumination and BS-1–coated fluorescently labeled microspheres (FLMs) (**B, D**) under fluorescence illumination, prior to (control), and five minutes following, the onset of superfusion of the mesentery with 10^{-7} M of fMLP. Under control conditions, on the order of one WBC can be observed firmly adhered to the EC per 100 μm of venule length, which increases to six WBCs/100 μm in response to fMLP (panel C). In contrast, the number of BS-1–coated FLMs (arrows in **B** and **D**) fell from six to two FLMs/100 μm, as glycans were shed from the EC surface. Panel **D** also shows a WBC that was coated with multiple FLMs, which were not counted in the analysis of glycan shedding. These snapshots focused on the diametral plane, thus resulting in some objects being out of focus or not visible.

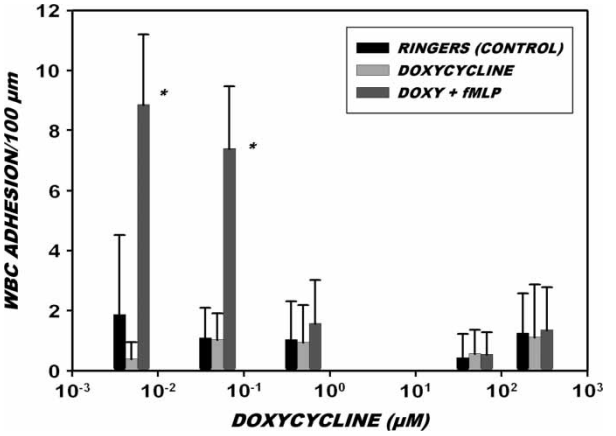

Figure 2. Adhesion of WBCs along the length of postcapillary venules in response to superfusion of mesentery with 10^{-7} M fMLP with increasing doses of doxycycline. Shown are mean (with SD) of WBC adhesion for six venules in each treatment with superfusion under control conditions (Ringer's solution alone) and doxycycline of the indicated concentration with and without fMLP (in Ringer's). Each group of bars is centered on the concentration of doxycycline used. A significant rise in WBC-EC adhesion* in response to fMLP ($P<0.05$) occurred for concentrations of doxycycline <0.5 μM, whereas for concentrations ≥ 0.5 μM, WBC adhesion did not rise significantly.

logistics curve revealed an EC_{50} value of 0.15 μM for 50% suppression of fMLP-stimulated adhesion. Red-cell velocity averaged 3.4 ± 0.9 (SD) mm/sec under control (Ringer's solution) and changed insignificantly during superfusion with fMLP or doxycycline at all concentrations ($P=0.25$). Hence, increases in shearing forces that may act to remove WBCs adhered to the endothelium played only a little role in the significant suppression of WBC-EC adhesion following increased concentration of doxycycline during fMLP superfusion.

The time course of fMLP stimulated adhesion is illustrated in Figure 3 during control conditions (Ringer's solution) and during superfusion with 0.5 μM of doxycycline or 2.6 μM of the hydroxamic acid zinc chelator, ilomastat (GM6001; US Biological), each in Ringer's solution. Initial adhesion of WBCs ranged from one to three cells per 100 μm, and were not significantly different for the three treatments ($P=0.285$). Both doxycycline and ilomastat significantly suppressed the adhesion response to fMLP ($P<0.001$). With doxycycline, WBC-EC adhesion increased slightly, but insignificantly, during the 40-minute duration of the experiment ($=0.362$). However, with ilomastat,

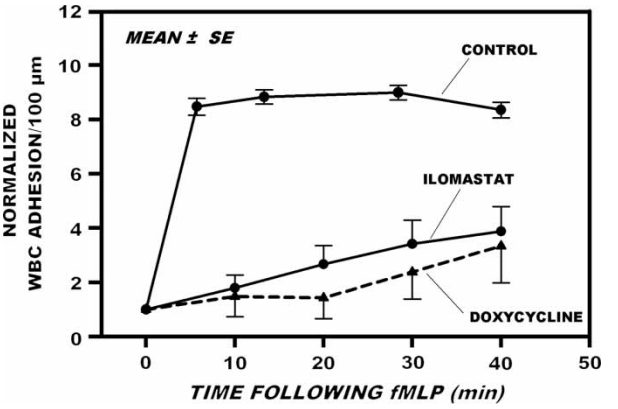

Figure 3. Normalized leukocyte-endothelial adhesion (WBCs/100 μm of venule length) following the onset of superfusion of the mesentery with 10^{-7} M of fMLP ($n=17$ venules). Also shown is the time course of adhesion with the addition of 0.5 μM of doxycycline ($n=17$) and 2.6 μM of ilomastat (GM6001; US Biological, Swampscott, Massachusetts, USA) ($n=9$). During the first 30 minutes of superfusion, there was no significant rise of WBC adhesion with either doxycycline ($P>0.10$) or ilomastat ($P>0.36$).

WBC adhesion rose significantly after 30 minutes ($p<0.03$). During the entire period of the study, WBC-EC adhesion was not significantly different between doxycycline and Ilomastat treatments ($p=0.447$). Average red-cell velocity changed insignificantly from control with ilomastat ($P=0.17$).

Quantitation of Glycan Shedding Using Lectin Microspheres

To elucidate the shedding of glycans from the endothelial surface layer, lectin-coated microspheres were infused systemically to attain a steady-state circulating concentration 10^7 FLMs/mL. This exposure led to a steady accumulation on the venular wall of 7.03 ± 2.27 (SD) FLMs/100 μm for the BS-1 FLMs and 12.5 ± 4.23 (SD) FLMs/100 μm for the LEA adhesion. As shown in Figure 4, following superfusion with the mixture of fMLP and 0.5 μM of doxycycline, these microsphere counts remained relatively constant and averaged 12.6 ± 4.74 and 10.6 ± 4.56 (SD) FLM/100 μm, for BS-1– and LEA-coated FLMs, respectively, which were not significantly different from their initial values ($P=0.818$). In the absence of doxycycline, microsphere counts fell to 20 and 50% of their initial values with a 10-minute exposure to fMLP, for BS-1– and LEA-coated spheres, respectively, as shown previously [29]. During the duration of the exposure to fMLP

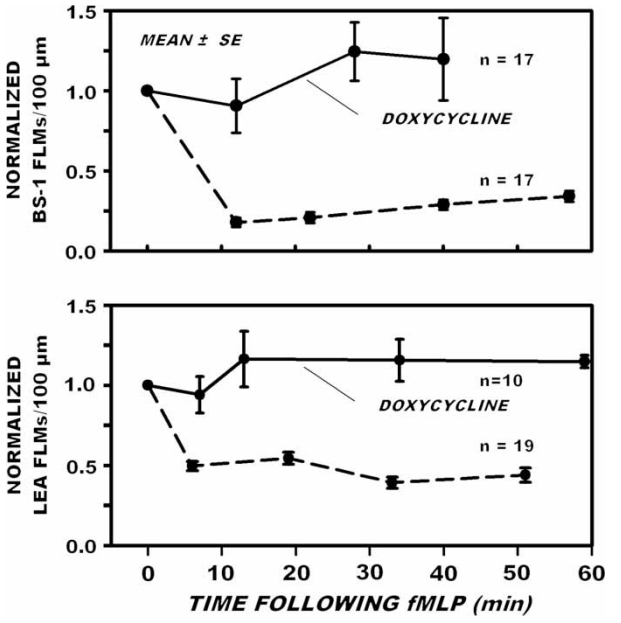

Figure 4. The adhesion of fluorescently labeled microspheres (FLMs) coated with lectins: LEA, with preferential binding to glucose moieties on the EC surface, and BS-1, with preferential binding to galactose. Doxycycline (0.5 μM in Ringer's solution) was superfused onto the tissue from $t=-30$ to $t=0$ minutes, and fMLP (10^{-7} M) was superfused from $t=0$ to $t=60$ minutes. In the absence of doxycycline, a rapid shedding of FLMs occurred in response to fMLP, as shown previously [29]. All data are shown as the mean ± SE.

without doxycycline, BS1 and LEA FLMs averaged 25 ± 13 and $47\pm16\%$ (SD) of initial values, respectively, which remained significantly reduced ($P<0.05$).

MMP Inhibition of Glycan Shedding

The effect of specific inhibition of MMP activity on glycan shedding is illustrated by the response of BS-1–coated FLMs to fMLP superfusion during superfusion with 2.6 μM of ilomastat (Figure 5). As in the case with doxycycline, initial FLM adhesion did not fall significantly during 40-minute exposure to fMLP ($P=0.693$). The response to fMLP was not significantly different from that of doxycycline ($P=0.651$). Chelation of divalent cations by superfusion with 5 mM of ethylene diamine tetraacetic acid (EDTA) resulted in the complete inhibition of WBC adhesion and shedding of BS-1 FLMs at 10 minutes following the onset of fMLP. However, the effects of EDTA were found to be time dependent and caused

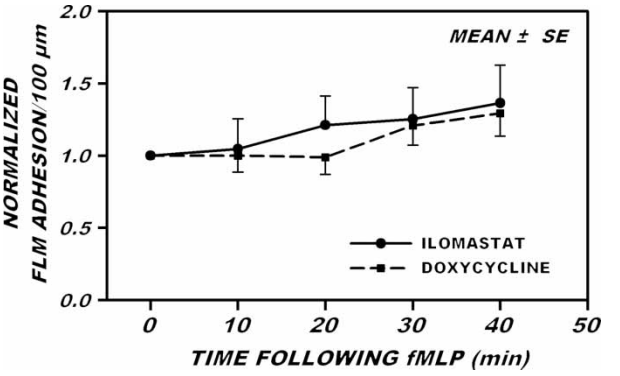

Figure 5. Normalized adhesion of BS-1–coated fluorescent microspheres (FLMs) following superfusion of the mesentery with 10^{-7} M of fMLP. The tissue was superfused with either 0.5 μM of doxycycline ($n=10$ venules) or 2.6 μM of ilomastat (GM6001; US Biological, Swampscott, Massachusetts, USA) ($n=9$ venules) for 30 minutes prior to and during superfusion with fMLP. Initial adhesion levels were 8.2 ± 2.5 (SE) FLMs/100 μm for doxycycline and 7.9 ± 1.2 (SE) for ilomastat. There was no significant change in FLM adhesion during superfusion with ilomastat ($P=0.693$) and no significant difference between ilomastat and doxycycline ($P=0.651$). Venule diameters ranged from 20 to 40 μm.

significant damage to the permeability barrier, as noted below.

To further support the hypothesis that MMPs are activated by stimulation with fMLP, studies of *in situ* microzymography are presented in Figure 6 for postcapillary venules during superfusion with fMLP ($n=14$), fMLP + ilomastat ($n=14$), and fMLP + doxycycline ($n=14$). As illustrated by the representative radial fluorescence intensity in Figure 6A, the fluorescence intensity of the FRET substrate attained a peak value at the microvessel wall (EC). Following five-minute superfusion with fMLP, the peak intensity increased to 1.71 ± 0.13 (standard error; SE) times the control intensity immediately preceding the onset of fMLP (Figure 6B). With the addition of 2.6 μM of ilomastat to the fMLP superperfusate, the fluorescence intensity was significantly reduced to a post- to presuperfusion ratio of 1.29 ± 0.03 (SE) ($P<0.001$), and with 0.5 μM of doxycycline, this ratio was significantly reduced to 1.33 ± 0.05 (SE) ($P<0.002$). There was no significant difference between ilomastat and doxycycline ($P=0.76$; Holm-Sidak multiple comparison test). Hence, these results confirm that MMPs on the EC surface are activated by fMLP and are significantly inhibited by either ilomastat or doxycycline.

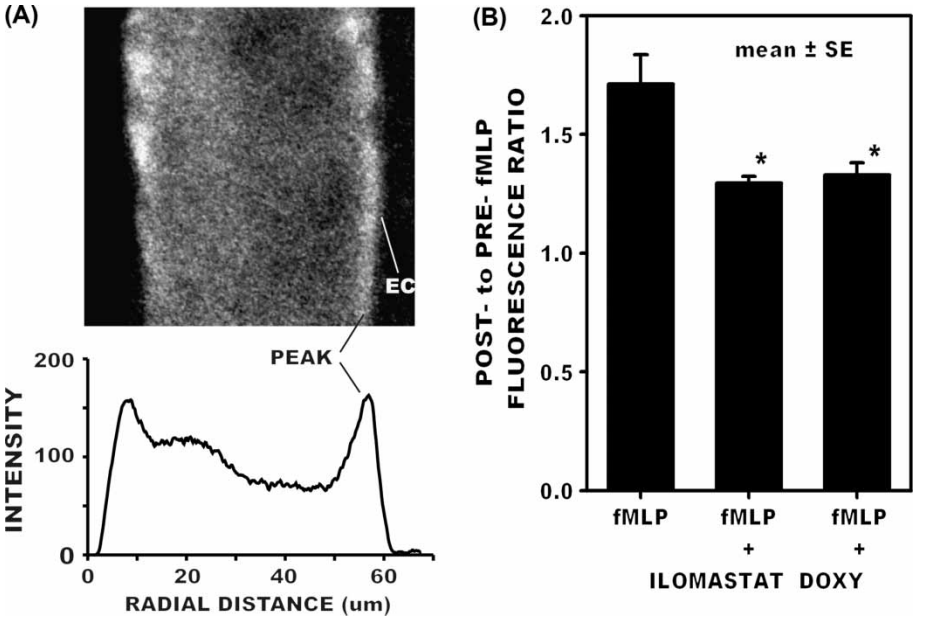

Figure 6. Fluorescence of FRET 520 MMP substrate infused into the systemic circulation and observed in postcapillary venules. (**A**) Representative 50-μm diameter venule following superfusion of the mesentery with fMLP for five minutes shows increased fluorescence at the EC surface. The radial distribution of average (along length of venule) fluorescence intensity is shown to scale below. The peak value of fluorescence at the venular wall was measured prior to, and five minutes following, superfusion of the tissue with fMLP. (**B**) Ratio of post- to pretreatment peak wall-fluorescence intensities for fMLP alone ($n = 14$) and with either 2.6 μM of ilomastat (GM6001; US Biological, Swampscott, Massachusetts, USA) ($n = 13$) or 0.5 μM of doxycycline ($n = 14$). *The fluorescence increase due to fMLP was significantly attenuated by the MMP inhibitor, ilomastat ($P < 0.001$) and doxycycline ($P < 0.002$).

DISCUSSION

The role of the glycocalyx as a barrier to cell adhesion has been examined in numerous studies in light of its structure and function on leukocytes and endothelium [40]. Analyses of the lengths of integrin and selectin receptors on the endothelium have shown a range from 20 nm for β2 integrins to 30–40 nm for E- and P-selectins [36]. These dimensions are well within the 300–500-nm thickness of the *in vivo* endothelial glycocalyx assessed by red-cell and macromolecule exclusion [39]. Modulation of specific components of the glycocalyx on phagocytic (monocytic THP-1) cells were shown to regulate their binding capacity [34]. The present studies have demonstrated that receptor-mediated leukocyte-endothelial adhesion in response to topical application of fMLP may be significantly reduced by inhibition of MMP activity, using the broad-spectrum inhibitor, doxycycline, and that the previously observed shedding of glycans from the endothelial surface in response to fMLP [29] is also significantly suppressed. These findings support the hypothesis that enzymatically induced shedding of the glycocalyx serves to expose cell-adhesion molecules and thus enhance the adhesion of WBCs to the ECs.

Doxycycline, a member of the tetracycline family of antibiotics, has raised considerable interest because of its ability to inhibit MMP activity at subantimicrobial doses, as well as in the case of other modified tetracyclines, scavenge divalent cations and reactive oxygen species, indirectly inhibit serine proteinases, inhibit the secretion of inflammatory cytokines, and block nitric-oxide synthase activity [16]. Until now, there has not been a direct *in vivo* study on the effects of doxycycline treatment on leukocyte-endothelium adhesion in the microcirculation, although there have been attempts to study its effects *in vitro* [9,14]. As shown therein, doxycycline inhibited binding of neutrophils to surfaces coated with albumin [9,14], fibronectin, or gelatin [14], which was attributed to the chelation of Mg^{2+}. In relation to MMP synthesis, doxycycline has been shown to inhibit the synthesis of MMP-8 and -9 from ECs following phorbol-12-myristate-13-acetate (PMA) activation [20].

The rapid shedding of glycans from the endothelial surface in response to fMLP has been shown to act through a G-protein–sensitive pathway, which can be inhibited by the pretreatment of tissue with pertussis toxin [29]. It has also been shown that fMLP can induce the release of MMP-9 from eosinophils in a p38 MAP-kinase–dependent pathway [41] and from neutrophils in a G-protein–dependent pathway [37]. Thus, it is conceivable that MMP degradation of the glycocalyx may arise from both leukocyte and EC activation. In the present studies, shedding of glycans were found to occur in areas along the length of postcapillary venules that were not paved with adherent WBCs, as evidenced by the reduction of lectin-coated microspheres bound to the ECs. Hence, it is likely that the present results reflect direct suppression of MMP activation on endothelium.

The reported interactions between MMPs and intracellular-signaling mechanisms support the hypothesis that MMPs can be released from cells by G-protein–sensitive pathways. In endothelial cells, the regulation of MMP-9 has been shown to be dependent on the phosphorylation of ERK. ECs stimulated with TNF-α and PMA could only have their MMP-9 production inhibited by blocking the MAPK cascade at the ERK phosphorylation stage [15]. Similar studies found that MMP-9 is activated by MMP-2 through the Ras-MAPK pathway [27] and by MMP-1 through a PTX-sensitive pathway [5]. In Con-A stimulated monocytes, it was shown that PTX can inhibit the production of MMP-1 [7]. These results are consistent with prior observations, that PTX inhibits glycan shedding in response to fMLP [29].

It is well recognized that chelation of zinc at the active site of MMPs inhibits their proteinase activity. The finding that both WBC-EC adhesion and glycan shedding were also inhibited with topical application of the hydroxamic acid zinc chelator, ilomastat, suggests that the effects of doxycycline arise from its MMP inhibition and not its ability to chelate divalent cations that may be necessary for WBC-EC adhesion. Inasmuch as doxycycline is known to chelate a broad spectrum of divalent cations, attempts were made to examine the fMLP-induced shedding of glycans during exposure to the broad-spectrum chelator, EDTA. Bathing the mesentery with 5 mM of EDTA resulted in a complete inhibition of WBC adhesion and shedding of glycans within 10 minutes of superfusion with fMLP. Similar inhibition of MMP activity in the microvessel wall with this concentration of EDTA has been demonstrated previously [10]. However, prolonged superfusion with EDTA (20–30 minutes) resulted in degradation of the mesenteric tissue and transudation of microspheres into the mesenteric tissue that precluded drawing conclusions on the extent of glycan shedding in response to fMLP.

Superfusion of the mesentery with lower concentrations of EDTA to find the minimum amount of EDTA that affected WBC interactions with the EC, presumably by chelation of divalent cations, revealed that in the absence of fMLP, 1 mM of EDTA significantly reduced venular WBC rolling flux. This reduction occurred in the presence of insignificant changes in red-cell velocity ($P = 0.20$). However, at this concentration of EDTA, WBC-EC adhesion and shedding of lectin-coated microspheres in response to fMLP was not significantly attenuated (unpublished results). These studies suggested that the effects of EDTA are time dependent, and further studies are warranted to understand the effects of differences in the relative binding capacities to specific cations (i.e., Zn^{2+}, Ca^{2+}, Mg^{2+}, etc.) and their relative influence on WBC adhesion and glycan shedding. In this context, the ability of doxycycline to chelate cations and affect WBC adhesion to protein substrates *in vitro* was shown to have no effect for doxycycline concentrations up to 52 μM [14], which was 100 times the concentration found here (0.5 μM) for the onset of inhibition of WBC-EC adhesion.

The putative role of MMPs in cleaving glycans from the EC surface is supported by the studies of *in situ* microzymography (Figure 6). Inhibition of the activation of MMPs on the surface of the endothelium by the hydroxamic acid inhibitor, ilomastat (GM6001; Sigma) and doxycycline supports the hypothesis that MMPs on the EC surface are responsible for the shedding of glycans during EC activation with fMLP. Hence, it is likely that cleavage of GAG-bearing proteoglycans by either membrane-bound or cytosolic MMPs in the EC may be responsible for shedding of the glycocalyx. This hypothesis is also supported by studies of syndecan-1 shedding from human embryonic kidney cells caused by membrane-type matrix metalloproteinase-1 (MT1-MMP) [12], shedding of syndecan-1 and -4 from HeLa tumor cells by MMP-9 [4], shedding of syndecan-1 from pancreatic carcinoma cells by MMP-7 [11], and shedding

of syndecan-1 by MMP-7 during transmigration of neutrophils from the interstitium to alveoli in the lung [23]. Thus, MMPs may, indeed, be the source of shedding of glycans in response to endothelial activation with fMLP, and further studies are needed to elucidate the specific proteases and their inhibitors involved.

CONCLUSIONS

In summary, given that MMPs may be stored within the glycocalyx or within close proximity, and rapidly activated through a cascade of events, makes them ideal candidates for modification of the glycocalyx in response to physiological stimuli. The inhibition of the MMP-induced shedding of components of the glycocalyx may attenuate the exposure of ligands that capture leukocytes during the inflammatory process [28] or affect the barrier to the transvascular exchange of water and solutes [1,8,21]. Given that the structure of the glycocalyx represents a balance between its removal and biosynthesis of new glycans, which are governed by either shear-dependent [2,13,29] or metabolic [31] mechanisms, it appears that stabilization of the endothelial glycocalyx with subantimicrobial levels of doxycycline may provide a valuable clinical tool for the treatment of many vascular disorders.

ACKNOWLEDGEMENTS

The authors express their appreciation to Anne Lescanic and Rachna Sah for their technical assistance. This work was supported by the National Heart, Lung, and Blood Institute (grant no. R01HL39286; Bethesda, Maryland, USA).

Declaration of interest: The authors report no financial conflicts of interest. The authors alone are responsible for the content and writing of this paper.

REFERENCES

1. Adamson RH. (1990). Permeability of frog mesenteric capillaries after partial pronase digestion of the endothelial glycocalyx. *J Physiol* 428:1–13.
2. Arisaka T, Mitsumata M, Kawasumi M, Tohjima T, Hirose S, Yoshida Y. (1995). Effects of shear stress on glycosaminoglycan synthesis in vascular endothelial cells. *Ann N Y Acad Sci* 748:543–554.
3. Bennett HS. (1963). Morphological aspects of extracellular polysaccharides. *J Histochem Cytochem* 11:14–23.
4. Brule S, Charnaux N, Sutton A, et al. (2006). The shedding of syndecan-4 and syndecan-1 from HeLa cells and human primary macrophages is accelerated by SDF-1/CXCL12 and mediated by the matrix metalloproteinase-9. *Glycobiology* 16:488–501.
5. Conant K, St Hillaire C, Nagase H, et al. (2004). Matrix metalloproteinase 1 interacts with neuronal integrins and stimulates dephosphorylation of Akt. *J Biol Chem* 279:8056–8062.
6. Constantinescu AA, Vink H, Spaan JA. (2003). Endothelial cell glycocalyx modulates immobilization of leukocytes at the endothelial surface. *Arterioscler Thromb Vasc Biol* 23:1541–1547.
7. Corcoran ML, Stetler-Stevenson WG, DeWitt DL, Wahl LM. (1994). Effect of cholera toxin and pertussis toxin on prostaglandin H synthase-2, prostaglandin E2, and matrix metalloproteinase production by human monocytes. *Arch Biochem Biophys* 310:481–488.
8. Curry FE, Michel CC. (1980). A fiber matrix model of capillary permeability. *Microvasc Res* 20:96–9.
9. Dahlgren C, Norberg B, Eriksson S. (1987). Doxycycline effects on the adherence of polymorphonuclear leukocytes to an albumin-coated glass surface. *Scand J Infect Dis* 19:545–549.
10. DeLano FA, Schmid-Schonbein GW. (2008). Proteinase activity and receptor cleavage: mechanism for insulin resistance in the spontaneously hypertensive rat. *Hypertension* 52:415–423.
11. Ding K, Lopez-Burks M, Sanchez-Duran JA, Korc M, Lander AD. (2005). Growth factor–induced shedding of syndecan-1 confers glypican-1 dependence on mitogenic responses of cancer cells. *J Cell Biol* 171:729–738.
12. Endo K, Takino T, Miyamori H, et al. (2003). Cleavage of syndecan-1 by membrane-type matrix metalloproteinase-1 stimulates cell migration. *J Biol Chem* 278:40764–40770.
13. Florian JA, Kosky JR, Ainslie K, Pang Z, Dull RO, Tarbell JM. (2003). Heparan sulfate proteoglycan is a mechanosensor on endothelial cells. *Circ Res* 93:136–142.
14. Gabler WL, Tsukuda N. (1991). The influence of divalent cations and doxycycline on iodoacetamide-inhibitable leukocyte adherence. *Res Commun Chem Pathol Pharmacol* 74:131–140.
15. Genersch E, Hayess K, Neuenfeld Y, Haller H. (2000). Sustained ERK phosphorylation is necessary, but not sufficient, for MMP-9 regulation in endothelial cells: involvement of Ras-dependent and -independent pathways. *J Cell Sci* 113(Pt 23):4319–4330.
16. Golub LM, Lee HM, Ryan ME, Giannobile WV, Payne J, Sorsa T. (1998). Tetracyclines inhibit

connective tissue breakdown by multiple nonantimicrobial mechanisms. *Adv Dent Res* 12:12–26.
17. Gronski TJ, Jr, Martin RL, Kobayashi DK, et al. (1997). Hydrolysis of a broad spectrum of extracellular matrix proteins by human macrophage elastase. *J Biol Chem* 272:12189–12194.
18. Haas TL, Milkiewicz M, Davis SJ, et al. (2000). Matrix metalloproteinase activity is required for activity-induced angiogenesis in rat skeletal muscle. *Am J Physiol Heart Circ Physiol* 279:1540–1547.
19. Haldenby KA, Chappell DC, Winlove CP, Parker KH, Firth JA. (1994). Focal and regional variations in the composition of the glycocalyx of large-vessel endothelium. *J Vasc Res* 31:2–9.
20. Hanemaaijer R, Visser H, Koolwijk P, et al. (1998). Inhibition of MMP synthesis by doxycycline and chemically modified tetracyclines (CMTs) in human endothelial cells. *Adv Dent Res* 12:114–118.
21. Huxley VH, Williams DA. (2000). Role of a glycocalyx on coronary arteriole permeability to proteins: evidence from enzyme treatments. *Am J Physiol Heart Circ Physiol* 278:H1177–H1185.
22. Lewis JC, Taylor RG, Jones ND, St Clair RW, Cornhill JF. (1982). Endothelial surface characteristics in pigeon coronary artery atherosclerosis. I. Cellular alterations during the initial stages of dietary cholesterol challenge. *Lab Invest* 46:123–138.
23. Li Q, Park PW, Wilson CL, Parks WC. (2002). Matrilysin shedding of syndecan-1 regulates chemokine mobilization and transepithelial efflux of neutrophils in acute lung injury. *Cell* 111:635–646.
24. Li Z, Li L, Zielke HR, et al. (1996). Increased expression of 72-kd type IV collagenase (MMP-2) in human aortic atherosclerotic lesions. *Am J Pathol* 148:121–128.
25. Lipowsky HH, Haynes CA. (2005). Synthesis of an artificial glycocalyx for studies of leukocyte adhesion. Proceedings of the 2005 Summer Bioengineering Conference, Vail, Colorado, USA, June 22–26, abstract 247851.
26. Lipowsky HH, Zweifach BW. (1978). Application of the "two-slit" photometric technique to the measurement of microvascular volumetric flow rates. *Microvasc Res* 15:93–101.
27. Liu E, Thant AA, Kikkawa F, et al. (2000). The Ras-mitogen–activated protein kinase pathway is critical for the activation of matrix metalloproteinase secretion and the invasiveness in v-crk–transformed 3Y1. *Cancer Res* 60:2361–2364.
28. Mulivor AW, Lipowsky HH. (2002). Role of glycocalyx in leukocyte-endothelial cell adhesion. *Am J Physiol Heart Circ Physiol* 283:1282–1291.
29. Mulivor AW, Lipowsky HH. (2004). Inflammation- and ischemia-induced shedding of venular glycocalyx. *Am J Physiol Heart Circ Physiol* 286:1672–1680.
30. Nieuwdorp M, van Haeften TW, Gouverneur MC, et al. (2006). Loss of endothelial glycocalyx during acute hyperglycemia coincides with endothelial dysfunction and coagulation activation *in vivo*. *Diabetes* 55:480–486.
31. Noble MI, Drake-Holland AJ, Vink H. (2008). Hypothesis: arterial glycocalyx dysfunction is the first step in the atherothrombotic process. *QJM* 101:513–518.
32. Pries AR, Secomb TW, Gaehtgens P. (2000). The endothelial surface layer. *Pflugers Arch* 440:653–666.
33. Rambourg A, Leblond CP. (1967). Electron microscope observations on the carbohydrate-rich cell coat present at the surface of cells in the rat. *J Cell Biol* 32:27–53.
34. Sabri S, Soler M, Foa C, Pierres A, Benoliel AM, Bongrand P. (2000). Glycocalyx modulation is a physiological means of regulating cell adhesion. *J Cell Sci* 113:1589–1600.
35. Spinale FG. (2007). Myocardial matrix remodeling and the matrix metalloproteinases: influence on cardiac form and function. *Physiol Rev* 87:1285–1342.
36. Springer TA. (1990). Adhesion receptors of the immune system. *Nature* 346:425–434.
37. Takafuji S, Ishida A, Miyakuni Y, Nakagawa T. (2003). Matrix metalloproteinase-9 release from human leukocytes. *J Investig Allergol Clin Immunol* 13:50–55.
38. Taraboletti G, D'Ascenzo S, Borsotti P, Giavazzi R, Pavan A, Dolo V. (2002). Shedding of the matrix metalloproteinases MMP-2, MMP-9, and MT1-MMP as membrane vesicle-associated components by endothelial cells. *Am J Pathol* 160:673–680.
39. Vink H, Duling BR. (1996). Identification of distinct luminal domains for macromolecules, erythrocytes, and leukocytes within mammalian capillaries. *Circ Res* 79:581–589.
40. Vitte J, Benoliel AM, Pierres A, Bongrand P. (2005). Regulation of cell adhesion. *Clin Hemorheol Microcirc* 33:167–188.
41. Wiehler S, Cuvelier SL, Chakrabarti S, Patel KD. (2004). p38 MAP kinase regulates rapid matrix metalloproteinase-9 release from eosinophils. *Biochem Biophys Res Commun* 315:463–470.
42. Yu WH, Woessner JF, Jr. (2000). Heparan sulfate proteoglycans as extracellular docking molecules for matrilysin (matrix metalloproteinase-7). *J Biol Chem* 275:4183–4191.