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Proteolytic Cleavage of the Red Blood Cell Glycocalyx in a Genetic Form of Hypertension

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Abstract

Recent evidence suggests that the spontaneously hypertensive rat (SHR) has an elevated level of proteases, including matrix metalloproteinases (MMPs), involved in cell membrane receptor cleavage. We hypothesize that SHR red blood cells (RBCs) may be subject to an enhanced glycocalyx cleavage compared to the RBCs of the normotensive Wistar-Kyoto (WKY) rats. By direct observation of RBC rouleaux, we found no significant difference in RBC aggregation for unseparated SHR and WKY RBCs. However, lighter SHR RBCs have a greater tendency to aggregate than WKY RBCs when separated by centrifugation. When SHR plasma was mixed with WKY RBCs, SHR plasma proteases cleaved the glycocalyx of WKY RBCs, a process that can be blocked by MMP inhibition. When treated with MMPs, WKY RBCs showed strong aggregation in dextran but not in fibrinogen, indicating that RBC membrane glycoproteins from the inner core of the glycocalyx were cleaved and that dextran was able to bind to the lipid portion of the RBC membrane. In contrast, treatment with amylases produced fibrinogen-induced aggregation with fibrinogen binding to the protein core. MMP cleavage of RBC glycocalyx reduces RBC adhesion to macrophages as a mechanism to remove old RBCs from the circulation.

Keywords

Spontaneously hypertensive rat; matrix metalloproteinases; red blood cell aggregation; glycocalyx cleavage; dextran; fibrinogen

INTRODUCTION

Chronic arterial hypertension is accompanied by telltale cellular and molecular evidence for inflammation [24]. However, many aspects about the manifestation of this inflammatory process are not yet well defined. In the spontaneously hypertensive rat (SHR), besides an elevated blood pressure and oxygen free radicals [4], a multifaceted set of symptoms is present, including insulin resistance, capillary rarefaction, and immune suppression [5]. We recently obtained evidence that the SHR has enhanced MMP activity (including MMP-2, MMP-7, and MMP-9) within its microcirculation [5, 28], which causes cleavage of the extracellular domain of the receptors on several cell types, e.g. endothelial cells and leukocytes, with reduction of the associated cell functions. The plasma protease activity level is approximately 20-40% higher in the SHR compared to the WKY [5, 28]. However, no analysis of the impact of the unchecked protease activity on SHR red blood cells (RBCs) has been carried out.

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During their approximate three-month life span, human RBCs undergo several physiochemical changes, including an increased tendency to aggregate in plasma or in polymer solutions. In blood of patients, it has been shown that enhanced cell-cell attractive forces may exist [29]. Moreover, fibrinogen and other plasma proteins that stimulate rouleaux formation are increased in many clinical conditions, including hypertension [12]. The SHR shows without a significant increase in hematocrit [13] a higher plasma concentration of fibrinogen [9] and an increased level of RBC aggregation compared to its normotensive control, the Wistar Kyoto (WKY) rat [10]. We hypothesize that, in SHR plasma, elevated levels of MMP activity are responsible for RBC glycocalyx cleavage, thus leading to enhanced glycoprotein and glycocalyx cleavage.

Over the past decades, many studies have been devoted to RBC aggregation [19]. Yet, the causes for aggregation have not been fully identified. Here we explore whether MMPs are involved in the RBC membrane cleavage in the SHR. Specifically we determine the degree of surface glycoprotein cleavage on the SHR RBCs and their impact on RBC aggregation as well as on adhesion to macrophages as a mechanism for eventual removal of old RBCs from the circulation.

MATERIALS AND METHODS

Reagents

Active matrix metalloproteinases (MMP-7 and MMP-9) and pro-MMP-2 were purchased from EMD Biosciences, San Diego, CA. Pro-MMP-2 was activated with 1mM 2-aminophenylmercuric acetate (APMA) at a volume ratio of 1:10 (APMA: pro-MMP-2) in a 37°C water bath for 2.5 hours. Control groups were included such that APMA did not impose an effect on RBCs. The two representative subgroups of the MMP family studied in this report are described and classified below:

-Gelatinase: MMP-2 (gelatinase A), Mca-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH_2, 120 nM and MMP-9 (gelatinase B), N-(2,4-dinitrophenil)-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg, final concentration 120 nM.

-Matrilysin: MMP-7 (matrilysin-1), Dnp-Arg-Pro-Leu-Ala-Leu-Trp-Arg-Ser, final concentration 120 nM.

Other enzymes were also used to serve as control or as comparison groups against MMPs:

-Serine protease: Trypsin (from bovine pancreas, Sigma-Aldrich, St. Louis, MO, T1426, final concentration 0.1 $g.L^{-1}$) and α-chymotrypsin (from bovine pancreas, Sigma-Aldrich, St. Louis, MO, C3142, final concentration 0.1 $g.L^{-1}$).

-Amylase: Neuraminidase (from Clostridium perfringens, Sigma-Aldrich, N2876, final concentration 15 $mg.L^{-1}$), heparinase I (from Flavobacterium heparinum, Sigma-Aldrich, St. Louis, MO, H2519, final concentration 15 $mg.L^{-1}$).

For enzyme blockade, the following inhibitors were used:

-MMP inhibitor: GM6001 (Millipore, Billerica, MA, CC1100, final concentration 100μM).

-Serine protease inhibitor: ANGD (6-aminido-2-naphtyl p-guanidinobenzoate dimethanesulfate, Torii Pharmaceutical, Chiba, Japan, final concentration 370μM).

-Amylase inhibitor: α-amylase inhibitor (from Triticum aestivum, Sigma-Aldrich, A1520, final concentration 50 $mg.L^{-1}$).

Centrifuged RBCs were suspended in buffer consisting of 1X Phosphate Buffer Saline (Sigma-Aldrich) with bovine serum albumin (Sigma-Aldrich, St. Louis, MO, A3059, final concentration 5 $g.L^{-1}$) and diluted in a volume ratio of 1:20 (RBCs : buffer). The absence of calcium chloride in buffer did not impact the ability of RBCs to form rouleaux.

Blood collection and pretreatment

The animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee at the University of California San Diego. Blood was collected from the great saphenous vein of male SHR and WKY rats (Harlan Laboratories Inc., Indianapolis, IN) of mature age (16 weeks old) and added into 1% heparin (APP Pharmaceuticals, Schaumburg, IL, final concentration 10 $U.mL^{-1}$).

In order to eliminate potential MMP inhibitors in the plasma, the blood samples were centrifuged at 250 G and 20°C for 5 minutes before each experiment. The plasma (supernatant) was removed and replaced with an equal volume of buffer. Treatment of red blood cells with MMPs (-2, -7, and -9) were performed for a duration of 20 minutes at the MMP concentration of 120nM.

Red blood cell density separations were performed by first centrifuging whole blood at 600 G for 10min at 20°C. An aliquot of blood from the top fraction of the cells was collected for analysis as was an aliquot from the bottom fraction for comparison among the following groups: WKY and SHR RBCs, WKY RBCs exposed to SHR plasma, and WKY RBCs exposed to SHR plasma inhibited with 100μM of GM6001.

Image analysis

Bright field micrographs of RBCs as well as fluorescent images (using 100-Watt mercury lamp) for the FITC-lectin and rhodamine dextran labeling of RBCs were captured in the upright microscope (Leitz Wetzlar SM-LUX) with an oil immersion objective (100X, numerical aperture 1.25) and digitized (VC500 One-Touch Video Capture USB 2.0, Diamond Multimedia, Chatsworth, CA). All image analysis was digitally performed (ImageJ, NIH).

We counted the average number of RBCs per rouleaux (in bright field) with the minimum size of one rouleaux being two RBCs. A value of one RBC per aggregate therefore refers to no aggregation. The average number of RBCs for multiple (n=30) randomly selected aggregates was used as the average aggregate size.

Fluorescent images for the PKH26 red blood cell membrane labeling experiment were captured using a 100-Watt mercury lamp in a Leitz Wetzlar intravital microscope with a 60X water immersion objective. We captured fluorescent images using a silicone-intensified target (SIT) camera (MTI66, DAGE, Michigan City, IN). Average grey value or image intensity of each fluorescent image was calculated by ImageJ.

Fluorescence labeling

Lectin binds to the sugar residues of the RBC glycoproteins (10). To trace it, we used a fluorescein isothiocyanate-labeled (FITC) BS-I lectin (from Bandeiraea simplicifolia, Sigma-Aldrich, St. Louis, MO, L9381, final concentration 80 $mg.L^{-1}$, excitation 490 nm, emission 525 nm). After suffusing different samples of centrifuged blood for 30 minutes, the supernatant (containing free-floating lectin and bound particles) was removed and cells monitored via fluorescence microscopy with a blue filter.

To detect dextran binding to WKY RBCs after treatment with MMP-9 and neuraminidase (Figure 6), WKY RBCs were labeled with a 70 kDa rhodamine B isothiocyanate dextran (Sigma-Aldrich, St. Louis, MO, R9379, final concentration 15 $g.L^{-1}$, excitation 540 nm, emission 625 nm), and centrifuged RBCs were suspended in a solution of buffer containing the fluorescent dextran at a 1:10 volume ratio. Excess rhodamine dextran in the supernatant was removed after centrifugation at 200G for 5min at 20°C. A green filter (494 nm

excitation, 520 nm emission wavelengths) was used to detect the presence of the fluorescent dextran under the microscope.

The red fluorescence cell membrane linker PKH26 (Sigma-Aldrich, #MINI26, final concentration 5μM) was used to label SHR RBCs and WKY RBCs exposed to SHR plasma (with or without the MMP inhibitor GM6001 at 100μM). The fluorescence excitation and emission spectra of PKH26 are centered at 551nm and 567nm, respectively. After 200μl of blood was drawn from WKY rat or SHR, the blood from each group was centrifuged at 400G for 5min at 20°C to separate plasma from cells. WKY rat plasma was removed from WKY cells, and SHR plasma was added to WKY rat cells and allowed to react for 20min at 20°C. In tandem, GM6001 (final concentration 100 μM) was added to one aliquot of SHR plasma and subsequently added to a separate tube of WKY cells. In addition, SHR RBCs in its native plasma were also included as another experimental group for PKH26 labeling.

Application of fluid shear stress with a cone-and-plate viscometer

The rotational viscometer (Sherline P/N 4345, 90V DC Motor) includes a 2-inch cone tapered at an angle of 1 degree. Sterile polystyrene petri dishes (Fisher Scientific, Pittsburgh, CA) served as containers for blood samples during fluid shear.

To quantify the strength of the RBC aggregates, selected shear rates (10, 20, 30, 35, and 40 s^{-1}) were applied for one minute to 50 μL of the blood sample (centrifuged blood diluted in the solution of buffer in a ratio of 1:20). At the end of the shear period images of the aggregates were recorded immediately after shear as basis for determination of the average number of RBCs per aggregate.

Macrophage culture

To study attachment of normal and treated RBCs to the scavenger receptor of the macrophage, mouse macrophages were used (American Type Culture Collection (ATCC), Manassas, VA, TIB-71). The macrophages were a generous gift from Professor Christopher K. Glass of the Department of Cellular and Molecular Medicine, University of California, San Diego.

We cultured the mouse macrophages with Dulbecco's Modified Eagle Medium (DMEM; ATCC, 30-2002) supplemented with 10% Fetal Bovine Serum (FBS; Thermo Scientific, SH30071.03). Cells were passaged every 3 days (1:5) and used in our studies prior to passage 12. Cells were also cryopreserved at passage 8 with DMEM containing 10% FBS with 5% dimethyl sulfoxide (Sigma Aldrich, D2650).

Macrophage-RBC attachment

WKY whole blood was centrifuged at 200G in room temperature for 5min. Macrophages were first detached from their culture dish using a standard cell scraper and then suspended in dextran solution ($15 g.L^{-1}$ in PBS-albumin buffer). WKY RBCs (treated or non-treated by enzymes) were added to the macrophage solution at 0.5% hematocrit or 1:80 volume dilution (RBC volume: macrophage-dextran solution volume). The final number ratio between macrophage and RBC was kept consistently at 1:150. The red blood cells and macrophages were mixed for 20min. An aliquot of the RBC-macrophage mixture was then placed on a glass slide where the cells were allowed to sediment and excess RBCs were rinsed away with buffer.

Statistics

All measurements are presented as mean ± standard deviation. Each experiment was carried out on three different samples coming from three rats in each group. Student's t-test was

used to determine differences between pair-wise comparison groups. A probability $p < 0.05$ was considered statistically significant.

RESULTS

In the following we present evidence that the red blood cell glycocalyx is subject to enzymatic cleavage in the SHR, a hypertensive model which has elevated levels of plasma MMPs. Proteolytic cleavage of the RBC glycocalyx leads to swelling in SHR RBCs, reduction in RBC mass density, enhanced RBC aggregation, increased dextran-binding capability to RBC surface, and decreased attachment of RBC to macrophages.

RBC density in SHR

The mass density of cells is reduced when swollen. Compared to WKY RBCs, a subpopulation of SHR RBCs displays a partially swollen morphology with reduced biconcavity (Figure 1A). Similar to SHR RBCs, WKY RBCs exposed to SHR plasma for 20min also tend to become swollen (Figure 1A). When the MMP inhibitor GM6001 (100μM) is added to the SHR plasma the majority of WKY cells retain normal biconcave RBC morphology (Figure 1A). Swelling of a subpopulation of SHR RBCs and WKY RBCs exposed to SHR plasma is in line with a lower density of such cells after centrifugation. After labeling with the PKH26 lipophilic membrane linker, SHR RBCs and WKY RBCs exposed to SHR plasma were mixed with equal volumes of non-labeled WKY RBCs. After centrifugation of this RBC mixture, many labeled SHR RBCs and WKY RBCs exposed to SHR plasma deposited in the top fraction of WKY RBCs (Figure 1B and 1C). Average PKH26 label intensity of SHR RBCs show that the top RBC fraction in a mixture of unlabeled control WKY RBCs contains a higher number of SHR cells than the bottom fraction (Figure 1B and 1C). Similarly, the majority of the labeled WKY RBCs exposed to SHR plasma deposited at the top fraction of the mixture of non-labeled and labeled RBCs significantly more than the bottom fraction (Figure 1B and 1C). In contrast, labeled WKY RBCs and labeled WKY RBCs exposed to SHR plasma and the MMP inhibitor GM6001 are found evenly distributed in the top and bottom fractions of centrifuged WKY RBCs (Figure 1B and 1C). These results indicate the SHR RBCs and also control WKY RBCs exposed for less than an hour to SHR plasma have a fraction of RBCs that accumulate among light control WKY RBCs. Evidence of an increased ability of SHR RBCs or WKY RBCs exposed to SHR plasma to aggregate is also critical in evaluation of the pathophysiological importance of RBC glycocalyx cleavage.

RBC aggregation in the SHR

When unseparated WKY and SHR RBCs are resuspended in an aggregating agent after centrifugation, there is a mild fibrinogen-induced aggregation in the SHR RBCs (but not in the WKY RBCs) and a notable dextran-induced aggregation (Figure 2A). After separation of lighter and heavier RBCs by centrifugation at the top (below the buffy coat) and at the bottom of the RBCs, respectively, we found light RBCs to exhibit a higher degree of aggregation than heavy RBCs, in line with previous results [18]. Whereas heavy RBCs from SHR and WKY rats showed the same low level of *dextran*-induced aggregation as whole blood, light RBCs of the SHR tended to aggregate significantly more than light WKY RBCs (Figure 2B). However, a smaller *fibrinogen*-induced aggregation was observed in SHR RBCs compared to that in dextran (Figure 2A).

Aggregation of WKY RBCs after exposure to SHR plasma

To further study RBC aggregation and the effect of SHR plasma on WKY RBCs, we applied SHR plasma with and without enzyme inhibitors to WKY RBCs and measured the RBC aggregation size.

After centrifugation of both WKY and SHR blood, SHR plasma and WKY RBCs were mixed in a volume ratio of ~70% RBC and 30% plasma for 30min. The RBCs were resuspended in dextran for another 20min incubation. Blockade of the SHR plasma protease activity with a serine protease inhibitor (ANGD) still yields dextran-induced aggregation, while blockade with a MMP inhibitor (GM6001) dramatically decreases this aggregation (Figure 3A and 3B). No effect on fibrinogen-induced aggregation by the protease inhibitors themselves was observed (not shown).

WKY RBC aggregation after enzyme treatment

WKY blood was subject to treatment with three kinds of enzymes: the matrix metalloproteinases MMP-2, MMP-7, and MMP-9 (applied for 20 minutes), the serine proteases trypsin and chymotrypsin (20 minutes), and the amylases neuraminidase and heparinase (applied for 60 minutes). Each shear experiment was repeated with corresponding inhibitors to these enzymes (see reagents) present during the treatment period. The enzymes and their corresponding inhibitors were removed after the treatment by centrifugation and before determination of the aggregation level. Dextran-induced aggregation was greatly enhanced after treatment with each of the MMPs (Figure 4A, upper images and histogram) and the serine proteases (Figure 4A histogram; Figure 4B, upper images) but remained at low non-aggregating control levels when protease inhibitors were added (not shown). In contrast, only the amylase treatment without inhibitor induces strong aggregation with fibrinogen (Figure 4C, lower images), while the MMPs or serine proteases did not lead to fibrinogen-induced RBC aggregation (Figure 4A, B lower images). In order to quantitatively measure the strength of RBC aggregation, we applied fluid shear stress to RBC rouleaux using a cone-and-plate device.

Aggregation of MMP-treated WKY RBCs

The shear rates required to break up dextran-induced rouleaux (Figure 5A) is higher with MMP treatment ($> 40\ s^{-1}$) than without ($< 20\ s^{-1}$). The average number of RBCs per aggregate increased with MMP concentration (Figure 5B; 1.2, 12, and 120 nM) and MMP treatment time (Figure 5C; 10, 20, 40, and 60 minutes). This response is the same for MMP-2, -7, and -9.

Glycocalyx cleavage of MMP-treated WKY RBCs

To confirm cleavage of RBC glycocalyx, we labeled the sugar residues of the glycocalyx with a fluorescent lectin. The fluorescent lectin intensity is significantly higher for non-treatment controls, i.e. WKY RBC, (44 average digital units; Figure 6B) than for WKY RBC subjected to MMP treatment or SHR RBC (11.3, 9.4, 12.7, and 14.3 average digital units for SHR, MMP-9, MMP-7, and MMP-2, respectively; Figure 6B).

Neuraminidase and MMP-9 increase dextran binding in WKY RBC membrane

To assess the effect of proteolytic cleavage of the RBC glycocalyx on dextran binding, we used a fluorescent dextran to label the RBCs. Rhodamine-dextran binding to the RBC membrane was determined in four different groups, each with WKY RBCs (Figure 7): without any digestive enzyme or inhibitor addition (control); with neuraminidase (final concentration 15 $mg.L^{-1}$); with MMP-9 (final concentration 120 nM); and with MMP-9 (final concentration 120 nM) as well as GM6001 (final concentration 100 μM).

The fluorescence intensity, as measured by light intensity (digital units), was significantly higher for the RBCs treated with neuraminidase and MMP-9 (63.1 and 69.5 average digital units, respectively; Figure 7B) than for the control cells and the blood cells treated with combined MMP-9 and GM6001 at the same time (22.7 and 29.4 average digital units,

respectively; Figure 7B). Evidence of RBC glycocalyx cleavage may also impede their ability to bind to macrophages.

Attachment to macrophages

Membrane attachment of RBCs to macrophages facilitates removal of old RBCs from the circulation. It can be mediated by the macrophage scavenger receptors, which bind to a glycoprotein of the RBC membrane. Heavy WKY and SHR RBCs and WKY RBCs treated with MMP-9 showed no attachments to macrophages (Figure 8). In contrast, membranes of light WKY RBCs attached to the macrophages more than light SHR RBCs (Figure 8). In our experimental setup, the macrophages are attached to the glass substrate and each has on average one light WKY RBC attached. When treated with amylases, the macrophages have on average even more than one WKY RBCs attached. Light SHR RBCs had an intermediate level of attachment with an average of about one attached RBC for every other macrophage (Figure 8).

DISCUSSION

The present results bring to light that the SHR has enhanced cleavage of its RBC glycocalyx in line with its enhanced protease activity. Proteolytic cleavage of the SHR RBC glycocalyx can lead to a number of abnormal cellular properties, such as swelling of RBCs, increase in RBC aggregation, and impairment of RBC attachment to macrophages. Cleavage of core glycoproteins on SHR RBCs by MMPs causes an increase in dextran- but not fibrinogen-induced aggregation (Figure 4A). There is a limited degree of RBC aggregation in the microcirculation of the SHR, in spite of an elevated concentration of fibrinogen [11]. The proteases responsible for the cleavage are present in the SHR plasma and can be inactivated by MMP inhibitors. Cleavage of control RBCs with SHR plasma or with several MMPs reveals a dextran-mediated, but not a fibrinogen-mediated form of aggregation, suggesting that fibrinogen binds to a residue of a core glycoprotein in the glycocalyx of lighter RBC membranes, which is cleaved from light SHR RBCs.

Membrane Receptor Cleavage in the SHR

The combined evidence from this and our previous studies suggests that RBC glycocalyx protein cleavage in the SHR may occur together with several other forms of receptor cleavage, each of which is associated with specific functional cell defects. For example, cleavage of the insulin receptor-α is associated with insulin resistance[5], cleavage of the β_2-adrenergic receptor can lead to arteriolar constriction[22], cleavage of the vascular endothelial growth factor receptor (VEGFR-2) may result in endothelial apoptosis and capillary rarefaction [28], and cleavage of the formyl peptide receptor (FPR) has been shown to impair neutrophil fluid shear response and the response to chemotactic formyl peptides[1].

Despite a loss of membrane charge with loss of the glycocalyx, the impact on RBC aggregation in the SHR is relatively limited [10]. Heavy SHR RBCs, which already show elevated RBC aggregation, do not have a major impact on the elevation of blood pressure. Visual observations on the SHR microcirculation, as carried out repeatedly in the past [25, 30], do not suggest a significant level of RBC aggregation, even in a low flow state and in venules. The enhanced ability of the light SHR RBCs to aggregate is revealed when dextran is used as an aggregating agent, but less so in fibrinogen (Figure 2). Therefore, the full in-vivo consequences of membrane glycocalyx cleavage in SHR RBCs may have an impact on RBC functions other than just aggregation and they remain to be determined.

Prolonged circulation and aging of the SHR RBCs may not only involve cleavage of the sugar residues on glycoproteins, as is the case for amylase-mediated cleavage (Figure 4C),

but appears to involve the core proteins in the glycocalyx. Residues of RBC membrane core proteins after cleavage of the glycocalyx with amylases serve as binding-site for fibrinogen (Figure 4C) as well as the macrophage scavenger receptors (Figure 8) as part of the removal of RBCs from the circulation. These core protein residues may be cleaved in the aging SHR RBCs due to the plasma proteases (Figure 3, 4A).

It has been suggested that SHRs overexpress a RBC membrane glycoprotein of 110 kDa [17]. This protein may be a type of integrin [16] and a modification in its expression could cause a change in the RBC surface charge.

SHR Plasma Proteases

The current evidence is in line with the fact that during their life span in the circulation the RBC sare exposed to higher protease activity in the SHRs than in the WKY rats [5, 28]. The MMP activities in the SHR are enhanced in the plasma and on the endothelium. Several MMPs with enhanced activity in the SHR [28] have the ability to cleave the RBC fibrinogen binding sites (Figure 4) and a broad-based MMP inhibition (GM6001) blocks the ability of SHR plasma to cleave the glycocalyx in control RBCs (Figure 3).

Besides MMP activity, the SHR plasma has also some serine protease activity [5, 6]. Trypsin and chymotrypsin are serine proteases, i.e. enzymes that cleave with high likelihood peptide bonds in proteins in which one of the amino acids at the active site is serine. They are synthesized in the pancreas as zymogens and released into the digestive system. Trypsin can interact with positively charged residues such as arginine and lysine on the substrate peptide to be cleaved; chymotrypsin prefers phenylalanine, tryptophan and tyrosine, which fit into its hydrophobic pocket. Unlike amylases, they have a radical effect on RBC glycocalyx, since they remove almost the whole set of glycoproteins that are attached to the membrane. Our results show that, after serine protease treatment, RBC aggregation with dextran is still present (Figure 4B upper images) but has disappeared with fibrinogen (Figure 4B lower images).

Serine proteases can also activate MMPs [23] so they may play a role in the cascade leading to glycocalyx cleavage [14]. A serine protease inhibitor in fresh SHR plasma did not prevent the dextran-mediated aggregation (Figure 3) suggesting that the MMPs present in SHR plasma are already activated before blood was collected.

MMP treatment and serine protease treatment appear to have a similar impact on RBC aggregation by cleaving a RBC glycoprotein required for fibrinogen binding. Removal of a protein of any kind from the inner core of the endothelial glycocalyx leads to the deletion of the glycocalyx, whereas removal of a sugar only damages a part of its coating [11]. Since neuraminidase and heparinase only cleave carbohydrates, the results support the hypothesis that fibrinogen binds to a glycoprotein core after sugar cleavage (Figure 4C lower images), whereas dextran binds to an element exposed after cleavage of RBC membrane proteins (Figure 4C upper images). Future experiments with specific RBC glycocalyx labels need to determine which proteins are cleaved by the MMPs.

MMP-2, -7, and -9 are capable of cleaving myelin associated glycoproteins in humans (J Neuroimmunol (2008) 193(1-2): 140-8). This was demonstrated tby treating cells (that have myelin associated glycoprotein expression on the cell surface) with MMPs, and using cell lysates to detect glycoprotein cleavage products by Western blot. The specificity of MMPs with respect to potential cleavage sites on glycocalyx glycoproteins should be further investigated (Nature Biotechnology (2001) 19:661-667).

Amylase Cleavage

Amylases degrade starch into sugar. Neuraminidase and heparinase are both able to remove specific carbohydrates from the RBC glycocalyx coating. The negative surface charge of normal RBCs leads to a surface potential which in turn creates an electrostatic repulsive force inversely related to the distance, which separates two RBCs. Neuraminidase removes the sialic acid from the RBC glycocalyx, thus significantly reduces the surface charge and enhances fibrinogen and dextran-induced aggregation, which were demonstrated by several techniques [21]. Heparinase belongs to the family of carbon-oxygen lyases acting on polysaccharide. Therefore, heparinase and neuraminidase actions on RBC glycocalyx are similar.

We explored here a possible role of amylase activity and cleavage of the RBC glycocalyx in SHR plasma by use of an α-amylase inhibitor (results not shown). Treatment of WKY RBCs by the SHR plasma showed no signs of fibrinogen-induced aggregation, supporting the hypothesis that RBC alteration in the SHR involved no significant action by amylase. If amylases were involved, the inhibitor treatment would have led to some measurable level of dextran-induced aggregation, which was not the case (i.e. the RBCs remained uniformly dispersed). Direct measurements of amylase activity in the SHR are required in the future.

Mechanisms for RBC Aggregation

Two major mechanisms for rouleaux formation have been proposed, due to macromolecular cross-bridging [2] and formation of a depletion layer [7]. Neither of these two mechanisms has received universal acceptance and both mechanisms may be involved in this phenomenon to different degrees depending on the nature of the aggregant molecules and the receptor sites on the RBC membrane.

The macromolecular cross-bridge hypothesis is based on the assumption that large molecules inducing aggregation adhere to the membranes of adjacent cells over a distance sufficient to bridge the repulsion distance generated by negatively charged membrane glycoproteins [20]. The current results indicate that different molecules, known to facilitate rouleaux formation, adhere to surface structures on the RBC membrane depending on whether proteolytic or amylytic cleavage occurred. The molecular aggregation mechanism becomes specific; dextran adheres to the phospolipids after removal of the core glycoproteins while fibrinogen adheres to the glycoproteins that remain after amylase cleavage.

The principle of the depletion layer hypothesis is based on molecular exclusion by steric repulsion of the large aggregating molecules from the glycocalyx on the cell surface, thus giving rise to a depletion layer and to osmotic forces pushing the cells membranes together [7]. This model has the distinct advantage, over the cross-bridging hypothesis, that it requires a less specific molecular species and is therefore more compatible with the different properties of macromolecules that mediate RBC aggregation.

Cell-specific Factors for Aggregation

The first observations pointing towards more cell-specific factors responsible for RBC aggregation were presented by Rampling et al [20]. They showed that cells of different subjects aggregate to different levels under identical suspension conditions. Cells of a single subject, if separated by age, i.e. the time they were in the circulation, aggregate to different degrees.

It was proposed that there is a direct relation between cell aging and increased cell density [18]. One can then separate young and old RBCs by high-speed centrifugation [15].

Taking this approach the less dense cells tend to aggregate more than heavier cells [20]. Not only does the average cell population differ among various individuals, but also within one individual, cells have different aggregating potential. It does not seem to come from age-dependent differences in the deformability or size of the cells.

The aggregation in light RBCs may rather be due to an evolution of its ability to absorb aggregant molecules and possibly a reduction of the glycocalyx thickness as a result of non-specific enzymatic digestion. Specifically, we propose here that digestion of the RBC membrane by degrading enzymes may be involved in aggregation. Neuraminidase as an amylase removes sialic acid, and thus most of the negative membrane charge and consequently the repulsion between membranes. A large body of evidence has brought to light evidence that both fibrinogen and dextran-induced aggregation is enhanced by this treatment [3, 8]. The current results suggest further that digesting the RBC membrane proteins by MMPs, serine proteases, and amylases removes different parts of the glycocalyx core proteins and in the process no longer supports a fibrinogen-mediated aggregation (Figure 4A, B, and C lower images) and neither does it allow attachment of macrophage receptors (Figure 8).

Swelling of SHR RBCs

The current results in the SHR bring to light the phenomenon of the swelling of red blood cells with a reduction of their average cell (mass) density. RBC swelling may be due to an enzymatic attack on their membrane proteins. The SHR has many red blood cells that have a relatively low density and tend to accumulate after density separation in the top or light fraction of cells when mixed with WKY RBCs (Figure 1B and 1C). While currently unexplored, there is a possibility that SHR ion channels and other membrane proteins may also be subject to enzymatic degradation in the presence of uncontrolled plasma proteins. It remains to be determined whether aging of RBCs is associated with an enzymatic degradation of their membrane proteins and to what degree such an event will influence the cell density.

Previous experiments indicate that older cells are less dense than younger cells, one can separate young and old RBCs by high-speed centrifugation (Nash JB, Wenby RB, Sowemimo-Coker SO, and Meiselman HJ. Influence of cellular properties on red cell aggregation. *Clinical Hemorheology* 7: 93-108, 1987.). The less dense cells tend to aggregate more than heavier cells (Rampling MW, Meiselman HJ, Neu B, and Baskurt OK. Influence of cell-specific factors on red blood cell aggregation. *Biorheology* 41: 91-112, 2004). The current observations with SHR RBCs are not in line with such behavior in non-pathophysiological situations, such as the SHR with an elevated and unchecked protease activity. The SHR RBCs tend to swell and assume lower density and therefore mix with light (i.e. young) WKY RBCs.

MMP Upregulation in the SHR

The mechanism for MMP upregulation in the SHR has not been fully delineated. MMP levels are kept in check by tissue inhibitors of metalloproteinases (TIMPs), We have preliminary results suggesting that TIPMSs may be downregulated in the SHR compared to the WKY (unpublished observations). The transcription factor NF-κB is upregulated in the SHR (Hypertension (2011) 57(2):261-8) and is involved in MMP upregulation in the SHR.

Macrophage membrane attachment

After about three months, old cells are removed from the circulation in the spleen and the liver [26]. Our experiments with macrophages show that MMPs cleave certain proteins from the RBC membrane so that it cannot be recognized by scavenger receptors on macrophages.

This feature may influence the life-time of RBCs in the SHR circulation which remains to be determined.

In contrast, amylase seems to enhance RBC aging but at the same time doesn't have an impact on the protein residues that facilitate macrophages to attach to the RBCs. The average number of light SHR RBCs attached to macrophages is lower than that of light WKY RBCs (Figure 8); this may be due to a greater plasma concentration of MMPs in the SHR. However, some lighter SHR RBCs remain untouched by MMPs, which can explain that the number of SHR RBCs attached to macrophages in these experiments is also significantly higher than with WKY RBCs treated with MMPs (Figure 8). This evidence is therefore in line with the hypothesis that besides a normal amylase glycocalyx cleavage as seen on the WKY RBCs, the SHR RBCs are subject to further cleavage by proteases, e.g. MMPs.

To maintain a homeostatic level of RBCs, the rate of production and removal of red blood cells in the circulation needs to balanced. When a reduced number of old red blood cells attach to macrophages in the SHR, the removal of old or defective RBCs from the circulation is impaired. Functionally the renewal of red blood cells is not as efficient, and activities such as scavenging of superoxide anions with superoxide dismutase (produced by RBCs) or nitric oxide synthase (Blood (2006) 107(7):2943-51) to produce nitric oxide and regulate vascular tone regulation and smooth muscle relaxation may be compromised. In addition, the ability of red blood cells to carry oxygen to peripheral tissues may be impeded. An excess of red blood cells can cause polycythemia, and low oxygen transport ability of RBCs may lead to anemia.

It is interesting to note that the spleen of the SHR, with an enhanced accumulation of RBCs and the animal model in general having a near normal hematocrit [13], is not enlarged compared to the spleen of WKY rats (unpublished observation). In contrast, the Dahl salt-dependent hypertensive model has an enlarged spleen with a significant reduction of the hematocrit [27]. The degree to which old SHR RBCs may be removed from the circulation by other types of cells (e.g. in the liver) remains to be examined.

Conclusion

An unchecked proteolytic activity in the plasma of the SHR causes cleavage of fibrinogen binding sites on the SHR RBC membrane, together with a reduction of macrophage membrane attachment sites. The cleavage of various membrane receptors by MMP activity in the SHR affects therefore not only receptors on the endothelium and leukocytes but also on red cells. The situation leads to an attenuation of various cell functions, which may lead to increased cell aging and turnover rates, and may contribute to an enhanced risk for end-organ failure in hypertension.

Acknowledgments

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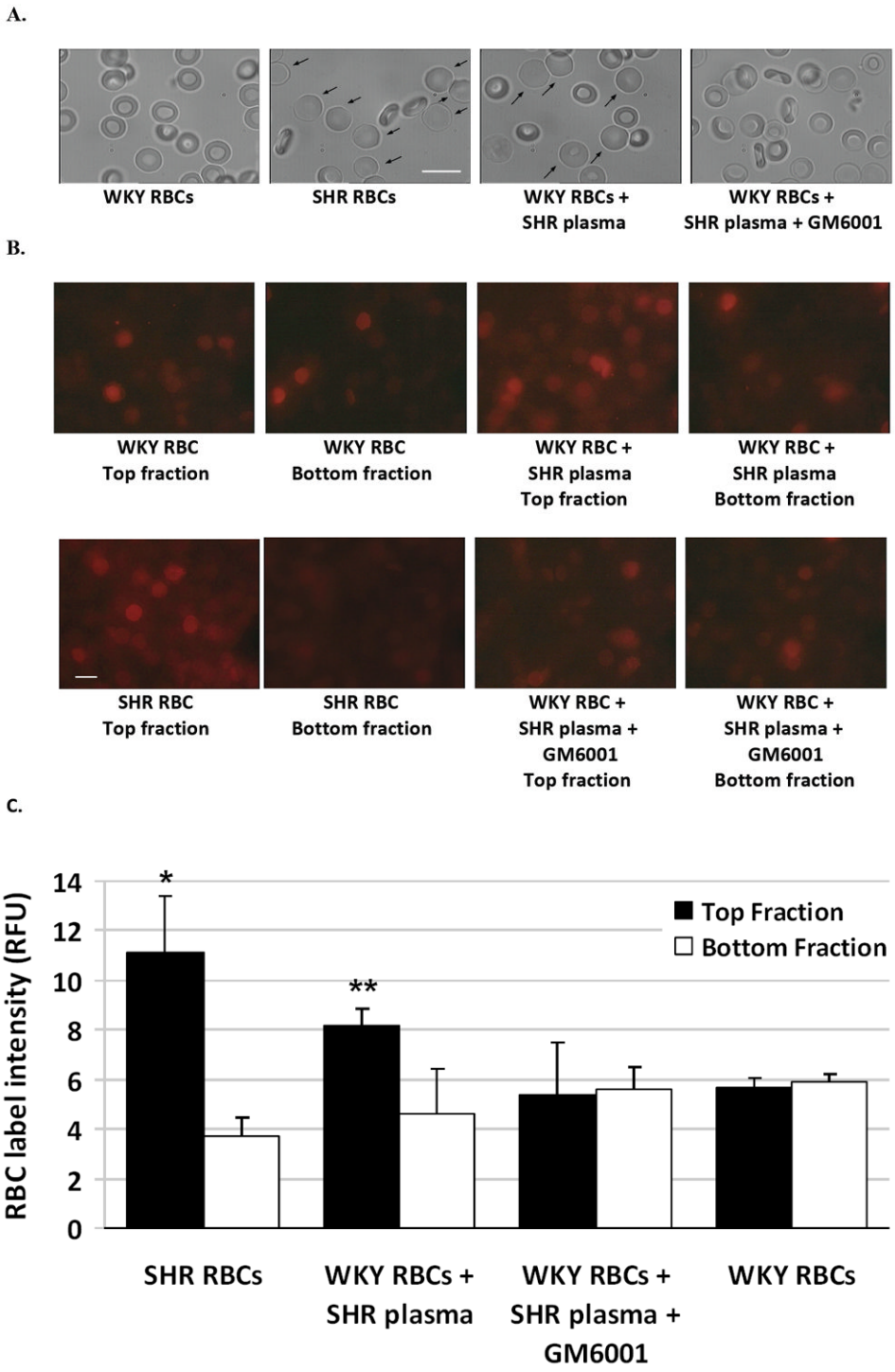

Figure 1.

A. Bright field micrographs of WKY RBCs, SHR RBCs, WKY RBCs exposed to SHR plasma, and WKY RBCs exposed to SHR plasma with 100μM of GM6001. Black arrows point towards cells that have reduced biconcavity or swollen morphology. Length bar represents 10μm. **B.** Fluorescent micrographs of red blood cells labeled with PKH26 lipophilic cell membrane linker. Labeled RBCs from WKY, SHR, WKY cells exposed to

SHR plasma (20min treatment), WKY cells exposed to SHR plasma and 100μM of GM6001 (20min treatment), were added to equal volumes of non-labeled WKY RBCs and then centrifuged at 600G for 10min at room temperature. An aliquot of each of the top and bottom layer of RBCs were visualized. The hematocrit for each case, including unlabeled WKY RBCs and the labeled cells, is that of packed RBCs and is the same in each image (~97%). **C.** Histogram of average RBC label intensity (relative fluorescence units; RFU) for each experimental group described in part **B.** The intensity values are given after background subtraction of the mean intensity of non-labeled WKY RBCs. *$p<0.05$ for labeled SHR cells mixed in non-labeled WKY cells for top vs. bottom fraction. **$p<0.05$ for WKY RBCs exposed to SHR plasma (labeled) and then mixed in non-labeled WKY RBCs for top vs. bottom fraction. n=3 rats per group and 10 images per top or bottom fraction per rat. Length bar represents 10μm.

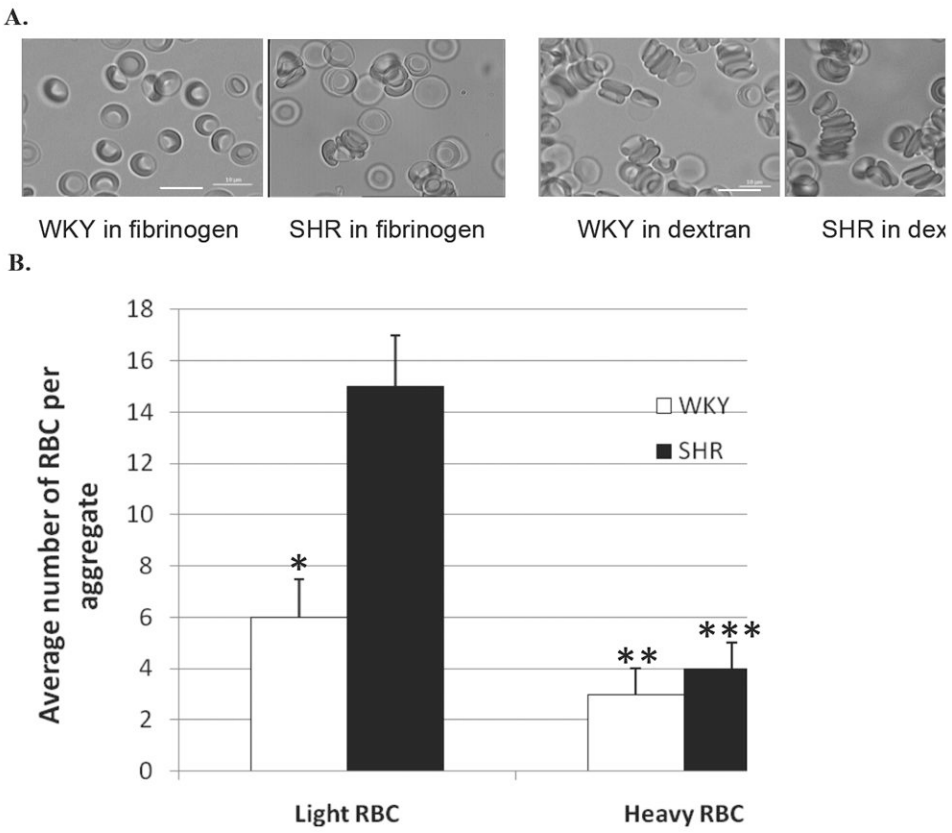

Figure 2.

A. WKY and SHR RBC-induced aggregation after centrifugation and plasma removal (250 G, 5 minutes, 20°C) followed by 20 minutes suspension in fibrinogen (6 $g.L^{-1}$) or dextran 70 kDa (15 $g.L^{-1}$), 2% hematocrit or 1:20 dilution by volume of blood in buffer containing fibrinogen or dextran. Length bar represents 10μm. **B.** WKY and SHR aggregate size for light and heavy RBC after centrifugation and plasma removal (250 G, 5 minutes, 20°C) followed by 20 minutes suspension in dextran 70 kDa (final concentration of 15 $g.L^{-1}$). *$p < 0.05$: light WKY RBC vs. light SHR RBC: **$p < 0.01$: heavy WKY RBC vs. light SHR RBC. ***$p < 0.01$: heavy SHR RBC vs. light SHR RBC.

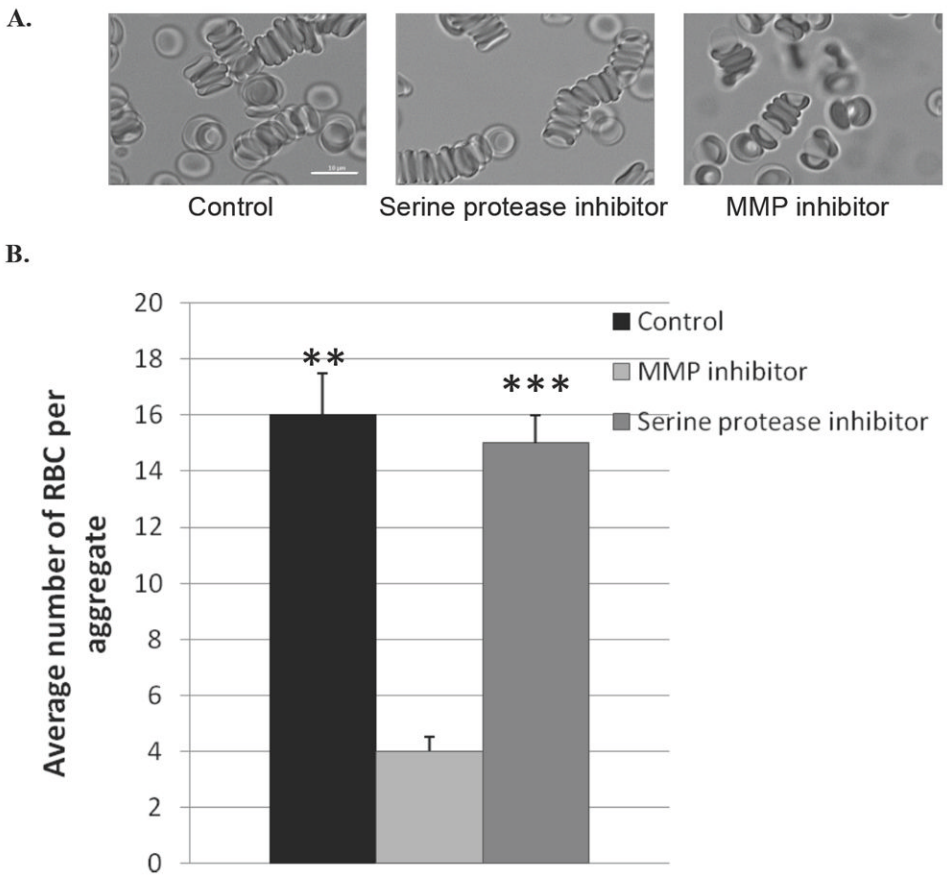

Figure 3.

A. WKY RBC aggregation induced by dextran after centrifugation and plasma removal (250 G, 5 minutes, 20°C) followed by 30 minutes of SHR plasma exposure (WKY RBCs: SHR plasma at 7:3 volume ratio) without inhibitor, with MMP inhibitor (GM6001, 100μM), or with serine protease inhibitor (ANGD, 370μM), and 20 minutes suspension in dextran 70 kDa (15 $g.L^{-1}$). Blood was diluted 1:20 by volume in dextran in PBS-albumin buffer. Length bar is 10μm. **B.** Histogram of WKY and SHR aggregate size. ** $p < 0.01$: control vs.MMP inhibitor. *** $p < 0.01$: serine protease inhibitor vs. MMP inhibitor.

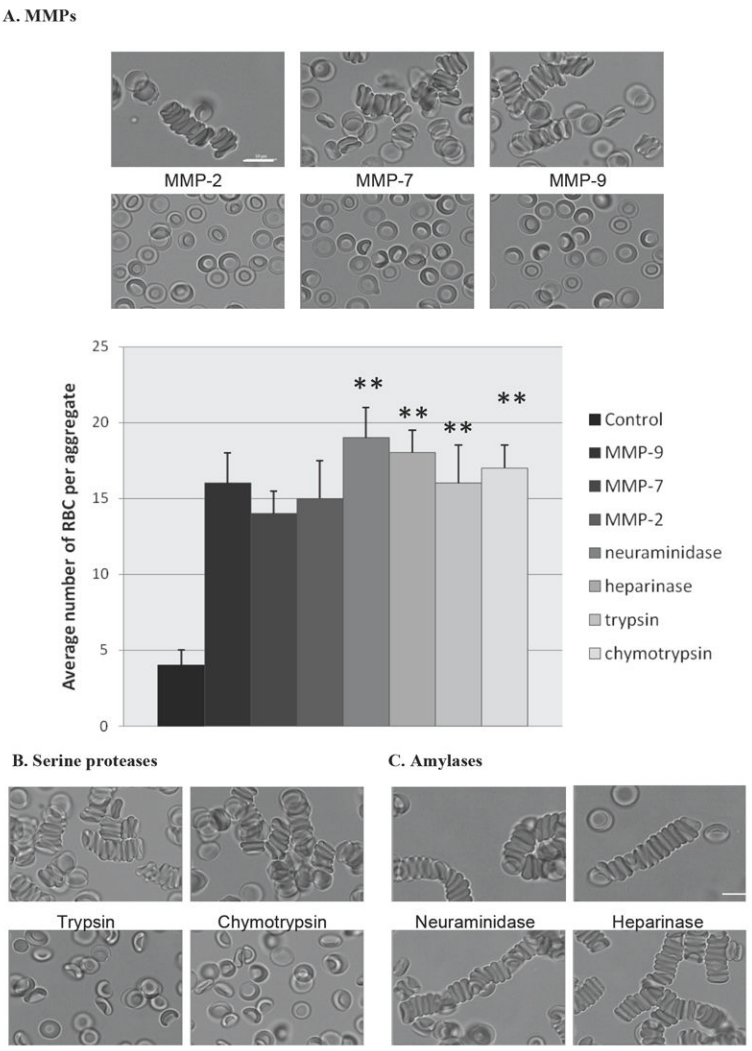

Figure 4.

WKY RBC induced aggregation after centrifugation and plasma removal (250 G, 5 minutes, 20°C) followed by **A.** 20 minutes of MMP-2, MMP-7, or MMP-9 treatment (120 nM), **B.** 20 minutes of trypsin or chymotrypsin treatment (0.1 $g.L^{-1}$), or **C.** 60 minutes of neuraminidase or heparinase treatment (15 $mg.L^{-1}$), and 20 minutes suspension in fibrinogen (6 $g.L^{-1}$, lower images) or dextran 70 kDa (15 $g.L^{-1}$, upper images). ** $p < 0.01$: MMP-2, MMP-7, MMP-9, neuraminidase, heparinase, trypsin, and chymotrypsin vs. control, respectively. Length bar represents 10μm. Histogram in part **A** shows the average number of RBCs per aggregate from dextran-induced aggregations for each group.

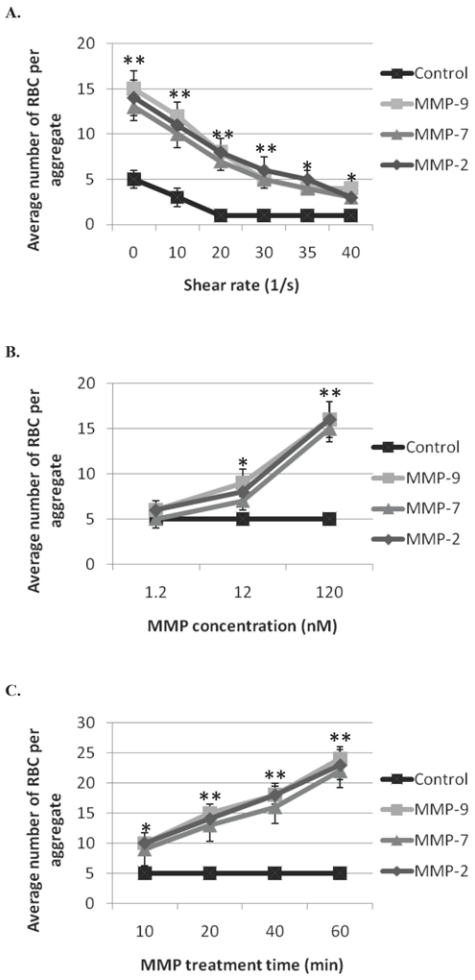

Figure 5.

A. WKY RBC induced aggregation by dextran after centrifugation and plasma removal (250 G, 5 minutes, 20°C) followed by no treatment or 20 minutes of MMP-2, MMP-7, or MMP-9 treatment (120 nM), 20 minutes suspension in dextran 70 kDa (15 $g.L^{-1}$), and 1 minute shear at different shear rates (10, 20, 30, 35, or 40 s^{-1}). * $p < 0.05$, ** $p < 0.01$: MMP-2, MMP-7, and MMP-9 vs. control. **B.** WKY RBC induced aggregation after centrifugation and plasma removal (250 G, 5 minutes, 20°C) followed by no treatment or 20 minutes of MMP-2, MMP-7, or MMP-9 treatment at different concentrations (1.2, 12, or 120 nM) and 20 minutes suspension in dextran 70 kDa (15 $g.L^{-1}$); * $p < 0.05$, ** $p < 0.01$: MMP-2, MMP-7, and MMP-9 vs. control, respectively. **C.** WKY RBC induced aggregation after centrifugation and plasma removal (250 G, 5 minutes, 20°C) followed by no treatment or MMP-2, MMP-7, or MMP-9 treatment (120 nM) for different durations (10, 20, 40, and 60 min) and 20 minutes suspension in dextran 70 kDa (15 $g.L^{-1}$); * $p < 0.05$, ** $p < 0.01$: MMP-2, MMP-7, and MMP-9 vs. control, respectively.

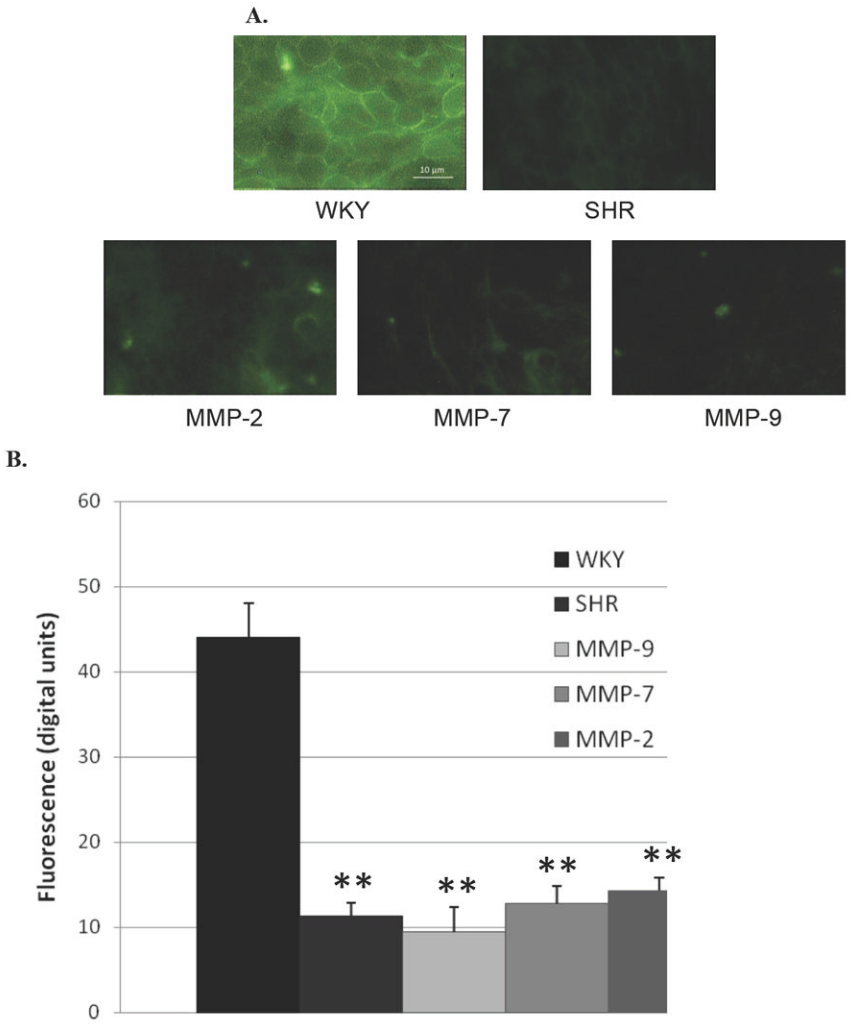

Figure 6.

A. Lectin fluorescence after SHR RBC centrifugation and plasma removal (250 G, 5 minutes, 20°C) followed by no MMP treatment or WKY RBC centrifugation and plasma removal (250 G, 5 minutes, 20°C) followed by no MMP treatment or 20 minutes of MMP-2, MMP-7, or MMP-9 treatment (120 nM) with FITC BS-I lectin (80 $mg.L^{-1}$) and centrifugation (250 G, 5 minutes, 20°C) with supernatant removal; 40% hematocrit or packed red cells; excitation: 490 nm, emission: 525 nm, microscope image exposure time: 4 seconds. Length bar represents 10μm. **B.** Histogram of fluorescent intensity from RBCs due to lectin labeling. ** $p < 0.01$: SHR, MMP-2, MMP-7, and MMP-9 vs. WKY, respectively.

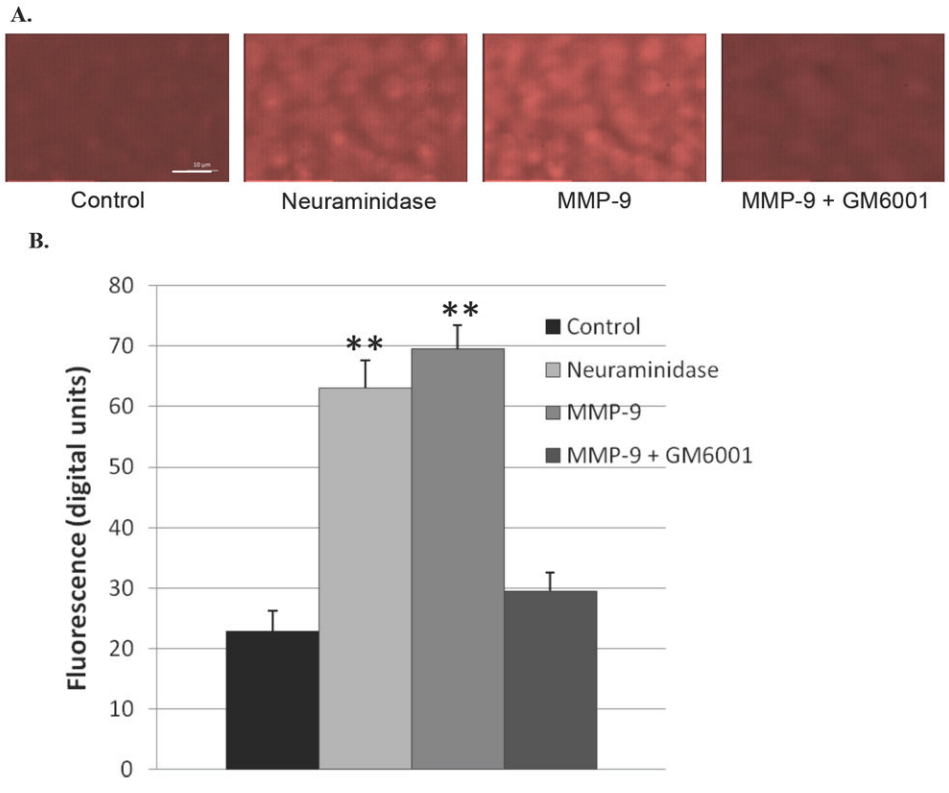

Figure 7.

A. Rhodamine B isothiocyanate dextran fluorescence after WKY RBC centrifugation and plasma removal (250 G, 5 minutes, 20°C) followed by no treatment, 60 minutes of neuraminidase treatment (15 $mg.L^{-1}$), 20 minutes of MMP-9 treatment (120 nM), or 20 minutes of MMP-9 (120 nM) and GM6001 (100μM) treatment, 20 minutes suspension in rhodamine B dextran 70 kDa (15 $g.L^{-1}$), and centrifugation (250 G, 5 minutes, 20°C) with supernatant removal; excitation: 540 nm, emission: 625 nm, microscope image exposure time: 4 seconds. Length bar represents 10μm. A negative control with WKY RBC treated for 20min with GM6001 (100μM) alone also showed relatively low fluorescence labeling (not shown). **B.** Histogram of fluorescent intensity over RBCs due to rhodamine dextran labeling. ** $p < 0.01$: neuraminidase and MMP-9 vs. control.

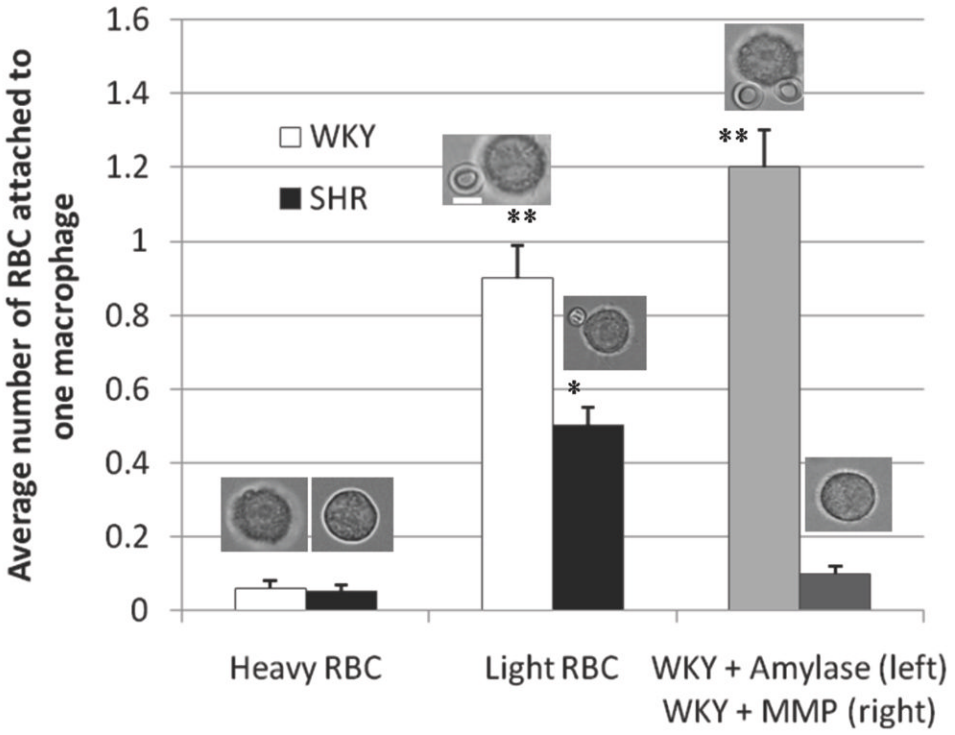

Figure 8.

Average number of RBC attached to one macrophage for light and heavy WKY and SHR RBCs after centrifugation, plasma removal (250 G, 5 minutes, 20°C), and resuspension in a solution of macrophages in dextran 70 kDa (15 $g.L^{-1}$), and for WKY RBC after centrifugation, plasma removal (250 G, 5 minutes, 20°C), followed by 20 minutes of MMP-9 treatment (120nM) or 60 minutes of neuraminidase (15 $mg.L^{-1}$) treatment, and resuspension in a solution of macrophages in dextran 70 kDa (15 $g.L^{-1}$) at 0.5% hematocrit or 1:80 dilution of blood in solution. The image inserts show individual macrophages with single red cell attachments in the case of light and amylase treated RBCs. * $p < 0.05$: light SHR RBC vs. heavy WKY RBC, ** $p < 0.01$: light WKY RBC and WKY RBC + amylase vs. heavy WKY RBC, respectively. The scale bar represents 10μm.