Histidine-rich Glycoprotein Specifically Binds to Necrotic Cells via Its Amino-terminal Domain and Facilitates Necrotic Cell Phagocytosis*

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Cells that become necrotic or apoptotic through tissue damage or during normal cellular turnover are usually rapidly cleared from the circulation and tissues by phagocytic cells. A number of soluble proteins have been identified that facilitate the phagocytosis of apoptotic cells, but few proteins have been defined that selectively opsonize necrotic cells. Previous studies have shown that histidine-rich glycoprotein (HRG), an abundant (≈100 μg/ml) 75-kDa plasma glycoprotein, binds to cell surface heparan sulfate on viable cells and cross-links other ligands, such as plasminogen, to the cell surface. In this study we have demonstrated that HRG also binds very strongly, in a heparan sulfate-independent manner, to cytoplasmic ligand(s) exposed in necrotic cells. This interaction is mediated by the amino-terminal domain of HRG and results in enhanced phagocytosis of the necrotic cells by a monocytic cell line. In contrast, it was found that HRG binds poorly to and does not opsonize early stage apoptotic cells. Thus, HRG has the unique property of selectively recognizing necrotic cells and may play an important physiological role in vivo by facilitating the uptake and clearance of necrotic, but not apoptotic, cells by phagocytes.

Cell death is vital for the morphological shaping of tissues during development and for the sculpting of functionally appropriate cellular repertoires as well as for protecting an individual from viral infections and pathogenic microorganisms (1, 2). Selective cell death continues to play a role in the homeostasis of mature tissues, such as the deletion of immune cells in the attenuation of an immune response (3) and the elimination of cells that have become functionally inappropriate, including virally infected and transformed cells (4). Apoptosis and necrosis represent two different forms of cell death and are characterized by distinct morphologies. Rapid and efficient phagocytic removal of dying cells is a key feature of apoptosis, whereas the role and extent of phagocytosis in the clearance of necrotic cells is not well documented. It is thought, however, that by engulfing necrotic and apoptotic cells phagocytes of the innate immune system not only provide a first line of defense against microbial pathogens but also dispose of self-antigens that are released from dying cells (5).

Apoptosis is characterized by an orderly sequence of internal events, including chromatin condensation that precedes the loss of cellular integrity (6). Apoptotic cells also display phosphatidylserine and altered membrane carbohydrates on their surface. Multiple ligands and receptors have been implicated in the recognition and uptake of apoptotic cells by phagocytes prior to membrane lysis, thus preventing release of potentially toxic and immunogenic intracellular substances into tissues. In addition, the binding and/or uptake of apoptotic cells inhibits proinflammatory cytokine production (7). When there are disturbances in either apoptosis or the phagocytosis of apoptotic cells, antibodies against subsequently exposed nucleosomes may be formed, leading to the development of autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis. In contrast to apoptosis, necrotic cell death has usually been defined as a disordered mode of cell death, occurring either in cases of severe and acute injuries such as sudden shortage of nutrients and abrupt axon injury or in extreme injuries such as exposure to heat, detergents, strong bases, and irradiation (8, 9). More recently, the existence of a necrotic-like cell death pathway regulated by an intrinsic death program distinct from that of apoptosis has also become apparent (9, 10). Necrotic cell death is characterized by the rapid and disorganized swelling and rupture of a cell (11). As with apoptotic cells, necrotic cells are usually rapidly cleared from the circulation by phagocytic cells. A large number of soluble extracellular proteins have been described (12) that bind to apoptotic cells and facilitate their uptake by macrophages. In contrast, few proteins have been identified that can specifically opsonize necrotic cells.

Histidine-rich glycoprotein (HRG) (13) is a 75-kDa plasma glycoprotein (≈100 μg/ml) that binds to numerous ligands, including heparan sulfate on the cell surface and in extracellular matrices (14–16), plasminogen (17–20), thrombospondin (21–23), tropomyosin (24, 25), IgG (26, 27), C1q (26), and Fc receptor (28, 29). HRG is a multidomain molecule consisting of an amino-terminal domain composed of two cystatin-like modules (N1N2), a central histidine-rich region, and a carboxyl-terminal domain (30, 31). Such a molecular structure allows the molecule to act as an adaptor protein that cross-links different ligands in solution or on cell surfaces (13). Furthermore, the histidine-rich region of HRG has recently been shown to mediate anti-angiogenic effects (32, 33), with HRG also being implicated in plasminogen activation (20, 34), particularly on cell surfaces (19, 34), and in immune complex clearance (28, 35). A recent study by Gorgani et al. (29) also suggested that HRG can potentiate the ingestion of late stage apoptotic cells by macrophages.

While investigating the interaction of HRG with different cell populations.

§This work was supported in part by a National Health and Medical Research Council program grant. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†Recipient of a Viertel Senior Medical Research fellowship.

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The abbreviations used are: HRG, histidine-rich glycoprotein; DVA, ovalbumin; N1N2, amino-terminal domain of histidine-rich glycoprotein; CHO, Chinese-hamster ovary; GAG, glycosaminoglycan; FCS, fetal calf serum; PBS, phosphate-buffered saline; BSA, bovine serum albumin; mAb, monoclonal antibody; SNARF, carboxy-semiaffthor-hodafuor; CFSE, carboxy-fluorescein diacetate succinimidyl ester.

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lations, it became apparent that the molecule bound strongly to dead cells; thus we undertook a detailed study of the interaction of HRG with necrotic, apoptotic, and viable cells. It was found that HRG binds avidly to necrotic cells, but not to early stage apoptotic cells, and aids the uptake of necrotic cells by a phagocytic cell line. Although HRG interacts with cell surface heparan sulfate on viable cells, additional experiments revealed that HRG binds via its N1N2 domain to a cytoplasmic ligand within necrotic cells that is not heparan sulfate. These data demonstrate, for the first time, that HRG represents a novel plasma protein that specifically facilitates the ingestion of necrotic cells by phagocytes and thus may play a key role in maintaining the efficient clearance of necrotic cells and necrotic cell debris from the circulation.

EXPERIMENTAL PROCEDURES

Cell Lines—THP-1 and Jurkat cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum (FCS). pgsA-745 cells are a mutant form of the CHO-K1 parent cell line and are unable to express any cell surface heparan sulfate due to a deficiency in xylosyltransferase. Both CHO-K1 (GAG+ve) and pgsA-745 (GAG-ve) cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 21 μg/ml l-proline and 10% FCS. Mammalian cell lines were incubated at 37 °C in a humidified atmosphere containing 5% CO2. The Spodoptera frugiperda-derived insect cell line (SF-9) was cultured in SF-900 II serum-free medium (Invitrogen) at 27 °C.

Purification of HRG—HRG was purified from human plasma based on a previously described method (16, 36). Briefly, human plasma was passed through a phosphocellulose column equilibrated with 0.5 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 6.8. Bound HRG was eluted with 2.0 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 6.8. Recombinant full-length HRG (507 amino acids) (30) and the N1N2 amino-terminal domain (amino acids 1–112) consisting of two cystatin-like modules, were produced in insect SF-9 cells using a baculovirus expression system as previously described (16).

Immunofluorescence Flow Cytometry—Cells were analyzed for HRG binding by immunofluorescence flow cytometry. Typically, plasma-purified or recombinant HRG and recombinant N1N2 domain (100 μg/ml) were added to 5 × 10^6 cells in PBS/0.1% BSA ± 20 μM ZnCl2 for 60 min at 4 °C and washed three times with PBS/0.1% BSA. Cell-bound HRG or N1N2 domain was detected using the HRG-specific mAb HRG-4 (AGEN, Brisbane, Australia). Cell surface heparan sulfate expression was detected by the heparan sulfate-specific mAb F58–10E4 (Amrad Biotech). Slides were mounted with coverslips and examined by confocal microscopy using a Nikon Eclipse TE 300 confocal microscope with a Nikon super high pressure mercury lamp power supply (Nikon Corp., Tokyo, Japan) and a Radiance 2000 laser scanning system (Bio-Rad).

Phagocytosis Assay—Equal volumes of SNARF-1-labeled THP-1 cells (1 × 10^6 cell/ml) and CFSE-labeled Jurkat cells (1 × 10^7 cell/ml) in RPMI 1640/10% FCS were mixed and incubated (37 °C, 5% CO2, 0–80 min) with either ovalbumin (OVA) (100 μg/ml; Sigma) or different concentrations of HRG in the presence or absence of 12.5-kDa bovine lung heparin (100 μg/ml), porcine muscle tropomyosin (200 μg/ml; Sigma), a CD32 (FcγRII) mAb (clone 8.26) (37), and a CD64 (FcγRI) mAb (clone 10.1) (BD Biosciences) in a pre-warmed 96-well plate. Cells were then immediately placed on ice and analyzed by flow cytometry. Rate of phagocytosis was calculated as the percentage of SNARF−THP-1 that were CFSE+. Proper inter- and intralayer compensations were determined using single color controls before each experimental run.

Binding of HRG and OVA to Intracellular Ligands—Recombinant full-length HRG or the N1N2 domain of HRG (20 μl, 100 μg/ml) or OVA (20 μl, 100 μg/ml) diluted in PBS was incubated with fixated monolayers of HEp-2 human epithelial cells (HEp-2 slides; INOVA Diagnostics, San Diego, CA) for 30 min. The slides were subsequently washed with PBS before detection of bound HRG using a HRG-specific mAb, HRG-4 (AGEN), or OVA using an OVA-specific mAb (Sigma) followed by secondary detection with sheep anti-mouse Ig fluorescein isothiocyanate (Amrad Biotech). Slides were mounted with coverslips and fluorescent mounting medium (DakoCytomation, Carpinteria, CA) before viewing immediately using an Olympus fluorescence microscope (Olympus Optical Co. Ltd, Tokyo, Japan). In some experiments HRG (100 μg/ml) was co-incubated with 12.5-kDa bovine lung heparin (100 μg/ml; Sigma) or porcine muscle tropomyosin (200 μg/ml; Sigma). In other experiments, slides were treated with Escherichia coli-derived...
RNase I (100 µg/ml; Promega Corp., Madison, WI) or bovine pancreas-derived RQ1 DNase I (100 units/ml, Promega) for 30 min at 37 °C prior to HRG binding and detection.

RESULTS

Binding of HRG to Necrotic Cells Is Heparan Sulfate-independent and Is Mediated by Its N1N2 Domain—Our laboratory recently demonstrated that HRG binds to cell surface heparan sulfate on viable cells via its N1N2 domain and that this binding is almost entirely abolished by high concentrations of heparin (16). While investigating the binding of HRG to transfected cell lines exposed to selective antibiotics, it was noted that the late stage apoptotic cells produced by antibiotic selection exhibited enhanced binding of HRG that was not inhibited by heparin (data not shown). A previous report suggested that HRG interacts with late stage apoptotic cells (29), but we wished to further investigate the molecular basis of this phenomenon and determine whether HRG can

FIGURE 1. Ability of HRG to bind specifically to viable and necrotic CHO cells. A, CHO-K1 cells were induced into a necrotic state by exposure of the cells to hyperthermic conditions (56 °C) for 45 min. Cells were analyzed by immunofluorescence flow cytometry for necrosis using the DNA intercalating dye 7-AAD. Filled histogram represents 7-AAD uptake by viable cells; open histogram depicts 7-AAD uptake by necrotic cells. B, binding of plasma-derived HRG (100 µg/ml) in PBS/0.1% BSA/20 µM Zn2+ (pH 7.2) to viable (top panels) or hyperthermia-induced necrotic (bottom panels) GAG+ve or GAG–ve CHO cells in the presence or absence of 100 µg/ml of 12.5-kDa bovine lung heparin. HRG binding was detected using the HRG-specific mAb HRG-4. Representative flow cytometry histograms are shown, with filled histograms representing background binding of the HRG-specific mAb to cells in the absence of HRG and open histograms representing HRG binding in the presence (blue histogram) and absence (red histogram) of heparin. C, quantitative comparison of the binding of HRG to viable and necrotic cells in the presence or absence of heparin, with data being expressed as fold increase in HRG binding (median fluorescence) relative to background. Error bars represent S.E. (n = 3). D, confocal microscopy of HRG binding to viable CHO GAG+ve cells and necrotic CHO GAG–ve cells. Binding of HRG (100 µg/ml) was detected using the HRG-specific mAb HRG-4. Cells were also incubated with the DNA intercalating dye 7-AAD to indicate cell viability.
HRG Opsonizes Necrotic Cells

FIGURE 2. Ability of the N1N2 domain of HRG to bind to viable and necrotic CHO cells. A, binding of the N1N2 domain of HRG (50 μg/ml) in PBS/0.1% BSA/20 μM Zn2+ (pH 7.2) to viable (top panels) or hyperthermia-induced necrotic (bottom panels) GAG−ve CHO cells in the presence or absence of 100 μg/ml of 12.5-kDa bovine lung heparin. N1N2 binding was detected using the HRG-specific mAb HRG-4. Representative flow cytometry histograms are shown, with filled histograms representing background binding of the HRG-4 mAb to cells in the absence of N1N2 and open histograms representing N1N2 binding in the presence (blue histogram) or absence (red histogram) of heparin. B, quantitative comparison of the binding of the N1N2 domain to necrotic and viable cells in the presence or absence of heparin, with data being expressed as fold increase in N1N2 binding relative to background. Error bars represent S.E. (n = 3).

levels 2–3-fold higher than to viable GAG+ve CHO cells. Binding to necrotic GAG−ve CHO cells was at least 100-fold higher than binding to viable GAG−ve CHO cells (Fig. 1, B and C). Necrotic cell binding was only partially inhibited by heparin (~20–30%), suggesting that the majority of HRG binding to necrotic cells is mediated through a heparan sulfate-independent ligand. We have previously shown that heparan sulfate-mediated HRG binding to viable cells is highly dependent on the presence of physiological concentrations of free Zn2+ (20 μM) (16). Interestingly, HRG binding to necrotic cells is not dependent on the presence of physiological concentrations of free Zn2+ (data not shown), indicating that HRG binding to necrotic cells is Zn2+-independent. Confocal microscopy was used to visualize the binding of HRG to both viable and hyperthermia-induced necrotic CHO cells using the HRG-specific mAb HRG-4 (Fig. 1D). Viable GAG+ve CHO cells exhibited HRG binding (green) that was predominantly localized to the outer surface of all viable cells. As expected, viable GAG−ve CHO cells did not exhibit HRG binding (data not shown). Conversely, HRG binding to necrotic GAG+ve (data not shown) and GAG−ve CHO cells was localized within the cytoplasm, but not the nucleus (Fig. 1D). Necrotic cells exhibited positive nuclei staining for the DNA intercalating dye 7-AAD, whereas viable cells were negative for 7-AAD staining (Fig. 1D).

Recombinant full-length and the amino-terminal (N1N2) domain of HRG were produced in insect cells using the baculovirus expression system as previously described (16). Flow cytometry studies were performed to determine whether the N1N2 domain of HRG also binds to necrotic cells. In agreement with our previous studies (16), the N1N2 domain bound to viable GAG+ve CHO cells, binding that was completely inhibited by 12.5-kDa bovine lung heparin (100 μg/ml) (Fig. 2, A and B). Consistent with this observation, the N1N2 domain of HRG did not bind to GAG-deficient CHO cells (Fig. 2, A and B). However, the N1N2 domain of HRG exhibited comparable binding to both necrotic GAG+ve and GAG−ve CHO cells, binding that was not blocked by heparin (Fig. 2, A and B). Again, binding was not affected by the presence of physiological concentrations of Zn2+ (data not shown). Collectively, these data indicate that the N1N2 domain of HRG interacts with necrotic cells and that the necrotic cell ligand is unrelated to heparan sulfate.

HRG Binds Poorly to Early Stage Apoptotic Cells—The capacity of HRG to bind to early stage (6 h) apoptotic (pre-necrotic) cells was then investigated. Jurkat T cells were induced into early stage apoptosis (1 μM camptothecin, 6 h, 37 °C), and the cells were analyzed by flow cytometry for Annexin-V binding and uptake of the DNA intercalating dye 7-AAD. Viable cells were defined as 7-AAD− and Annexin-V-negative, early stage apoptotic cells (30% of camptothecin-treated cells) were classified as Annexin-V-positive, 7-AAD-negative, and necrotic cells (hyperthermia treatment) were identified as 7-AAD− and Annexin-V-negative (Fig. 3A). The ability of HRG to bind to early stage apoptotic cells, compared with viable or necrotic cells, and the effect of heparin on this binding was then assessed. In contrast to viable cells, early stage apoptotic cells exhibited very low levels of HRG binding that was marginally affected by heparin, whereas necrotic cells bound high levels of HRG that was also only slightly affected by heparin (Fig. 3, B and C). Based on reactivity with a heparan sulfate-specific mAb, early stage apoptotic cells were also shown to exhibit very low levels of cell surface heparan sulfate (data not shown). Thus the predominant cell surface ligand for HRG is lost from early stage apoptotic cells, a process that results in these cells reacting very weakly with HRG. Furthermore, Jurkat cells behave in a similar manner to GAG+ve CHO cells in that they are necrotic they bind HRG in a heparan sulfate-independent manner. These binding data also suggest that the ligand recognized by HRG

interact with necrotic and early stage apoptotic cells. CHO cell lines that either express cell surface GAGs (CHO-K1) or lack cell surface GAGs (pgsA-745) due to a deficiency in xylosyltransferase (38, 39) were used in these initial experiments. GAG+ve and GAG−ve CHO cells were induced into necrosis by exposure of the cells to hyperthermic conditions (56 °C) for 45 min. Cell death was confirmed as >99% using the DNA intercalating dye 7-AAD with viability being assessed by flow cytometry (Fig. 1A). Both viable and necrotic GAG+ve and GAG−ve CHO cells were analyzed for their capacity to bind HRG. In agreement with previous findings (16), viable GAG+ve CHO cells bound human HRG (100 μg/ml), binding that was almost entirely abolished by the presence of 12.5-kDa bovine lung heparin (100 μg/ml) (Fig. 1, B and C). In contrast, HRG exhibited little binding to viable GAG−ve CHO cells either in the presence or absence of heparin (Fig. 1, B and C). On the other hand, HRG bound strongly to necrotic GAG+ve CHO cells at

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in necrotic cells is not exposed until the cell membrane has become permeable, allowing HRG access to cytoplasmic ligands within necrotic cells. Early stage apoptotic cells have intact cell membranes and thus do not appear to allow HRG access to the cytoplasmic ligands.

HRG Binds Specifically to an Intracellular Cytoplasmic Ligand via Its N1N2 Domain — Based on the above data, experiments were undertaken to characterize the interaction of HRG with intracellular ligands within necrotic cells. Our confocal studies (Fig. 1D) indicated that HRG interacts with necrotic cells via a cytoplasmic ligand. To confirm this observation, we examined the binding of HRG to monolayers of fixed human HEp-2 epithelial cells. This fixed epithelial cell line is routinely used clinically to screen for anti-nuclear antibodies that are present in the sera of systemic lupus erythematosus patients. It was found that both recombinant full-length HRG and the N1N2 domain of HRG bound uniformly to the cytoplasm of the HEp-2 cells, with no binding being detected in the cell nuclei (Fig. 4A), suggesting that the HRG ligand is evenly distributed throughout the cytoplasm of cells. Treatment of the slides with RNase (100 μg/ml), DNase I (100 units/ml), and co-incubation of HRG with 12.5-kDa bovine lung heparin (100 μg/ml) or tropomyosin (200 μg/ml) did not modify the intensity or binding pattern of HRG or the N1N2 domain (data not shown), suggesting that the cytoplasmic ligand for HRG is unrelated to RNA, DNA, heparin-like molecules, or tropomyosin. As a specificity control, the binding of an irrelevant protein (OVA) to the HEp-2 cells was examined. OVA did not show any detectable binding to the fixed epithelial cells when incubated at similar concentrations to HRG (Fig. 4B), indicating that HRG binding is specific.

HRG Enhances the Phagocytic Uptake of Necrotic Cells by THP-1 Cells — Because HRG strongly binds to necrotic cells and is known to interact with Fc receptor on macrophages, the effect of HRG on the uptake and phagocytosis of necrotic cells by phagocytic THP-1 cells was investigated. A phagocytic assay was developed whereby THP-1 phagocytic cells were labeled with the intracellular dye SNARF (red fluorescent) and Jurkat T cells were labeled with the intracellular dye CFSE (green fluorescent) before induction into necrosis. Labeled cells were then mixed at a ratio of ∼10:1 necrotic cells:phagocytic cells and incubated.
HRG Opsonizes Necrotic Cells

FIGURE 4. HRG specifically binds to cytoplasmic ligands in necrotic cells via its N1N2 domain. Recombinant full-length HRG and the N1N2 domain of HRG (100 μg/ml) (A) and OVA (100 μg/ml) (B) were incubated with a monolayer of fixed human HEp-2 epithelial cells for 30 min, with HRG binding being detected using the HRG-specific mAb HRG-4 and OVA binding being detected using an OVA-specific mAb. The control slides represent binding detected using HRG-4 or OVA mAb in the absence of HRG or OVA, respectively. Slides were analyzed by fluorescence microscopy for HRG and OVA binding patterns.

with or without plasma-derived HRG (100 μg/ml) at 37 °C for up to 80 min. Cells were then stored at 4 °C to inhibit further ingestion before immediate analysis of the cells by flow cytometry (Fig. 5A). At time 0, the majority of cells were either CFSE- or SNARF-positive, indicating that no interaction had taken place between the cells. As time progressed, cells gradually became positive for both CFSE and SNARF, indicating that SNARF-labeled THP-1 cells were binding and ingesting the labeled necrotic Jurkat T cells and becoming double positive cells. The rate of phagocytosis was calculated as percentage of THP-1 cells that were CFSE+/SNARF+. It was found that the presence of physiological concentrations of HRG (100 μg/ml) resulted in a significantly higher percentage of THP-1 cells binding and ingesting necrotic cells at all time points tested (Fig. 5A). HRG not only increased the overall number of THP-1-containing necrotic cells from ~40 to 75% but also accelerated the rate of ingestion by ~2-fold (Fig. 5B) in a HRG concentration-dependent manner (Fig. 5C). Furthermore, the presence of heparin (100 μg/ml), porcine muscle tropomyosin (200 μg/ml), or blocking antibodies against FcγRII and FcγRII (CD64 and CD32) did not interfere with the ability of HRG to enhance the phagocytosis of necrotic cells (Fig. 5D). Interestingly, the recombinant N1N2 domain of HRG, despite binding strongly to necrotic cells (Figs. 2 and 4), failed to enhance the uptake of necrotic cells by THP-1 cells (data not shown). The explanation of this result requires further investigation. Control experiments revealed that in either the presence or absence of HRG (100 μg/ml) there was negligible binding of viable Jurkat T cells to THP-1 cells after 80 min of incubation at 37 °C (3–4%), indicating that HRG selectively opsonized necrotic cells for ingestion by THP-1 cells (Fig. 5D). Furthermore, inclusion of an irrelevant protein (OVA, 100 μg/ml) in the assay did not enhance the phagocytosis of necrotic cells (data not shown). HRG was also unable to enhance the uptake of early stage apoptotic cells by THP-1 cells (data not shown), a not unexpected result because HRG exhibits little or no binding to early stage apoptotic cells (Fig. 3). Finally, confocal microscopy was used to unequivocally demonstrate that the THP-1 cells contained ingested necrotic cells. Necrotic Jurkat cells were labeled with the intracellular dye CFSE and THP-1 cells were labeled with the membrane dye PKH26 to allow good visualization of the green ingested cells within the red membrane phagocytic cells. Fig. 6 depicts early stages of necrotic cell uptake when the necrotic cells adhere to the THP-1 cells (Fig. 6, A and B) and initial stages of ingestion are evident (Fig. 6B). At later stages, entire necrotic cells are ingested by the THP-1 cells (Fig. 6, C–F).

DISCUSSION

This study examined in detail the interaction of HRG with viable, early stage apoptotic and necrotic cells. We found that physiological concentrations of human HRG bound strongly to necrotic cells in a heparan sulfate-independent manner and specifically increased the phagocytosis of necrotic Jurkat cells by the monocytic cell line THP-1, thus implicating a role for HRG in regulating the binding, uptake, and clearance of necrotic cells in vivo, with previous studies suggesting that HRG can also opsonize late stage apoptotic cells (29). Necrotic cell death occurs in cases of severe and acute injury due to a shortage of nutrients or exposure to heat, detergents, strong bases, irradiation, or abrupt anoxia (9, 40). Usually necrotic cells are extremely efficiently cleared from the circulation; however, if they are not rapidly cleared, as occurs in pathological tissue, they can induce an inflammatory response (41, 42). Interestingly, the mechanisms by which necrotic cells are so efficiently cleared from the circulation are not well understood.

HRG was shown to exhibit heparin-inhibitable binding to viable GAG+ve CHO cells and negligible binding to viable GAG−ve CHO cells (Fig. 1, B and C), confirming previous results that heparan sulfate is the predominant cell surface ligand for HRG on viable cells (16). Interestingly, however, HRG was shown to bind strongly to both necrotic GAG+ve and GAG−ve CHO cells (Fig. 1, B and C). Thus, despite GAG−ve CHO cells lacking cell surface GAGs due to a deficiency in xylosyltransferase, HRG exhibited high levels of binding to necrotic GAG−ve cells, binding that was also not abolished by the presence of 100 μg/ml of 12.5-kDa bovine lung heparin (Fig. 1, B and C). Although heparin did partially reduce (~20%) the binding of HRG to necrotic cells, the remaining HRG binding in the presence of heparin was still significantly greater than HRG binding to viable cells. Subsequent confocal fluorescence microscopy studies revealed that, unlike viable cells where HRG primarily bound to the cell surface, HRG binding to necrotic CHO-K1 cells appeared to be localized intracellularly, being predominantly in the cell cytoplasm rather than in the cell nuclei (Fig. 1D). In addition, analysis of the binding of HRG to monolayers of fixed human epithelial cells revealed that both the N1N2 domain of HRG and full-length HRG bound specifically and uniformly to cytoplasmic ligand(s) and showed no reactivity with cell nuclei (Fig. 4A). In contrast, an irrelevant protein, OVA, did not exhibit any detectable binding to fixed HEp2 cells (Fig. 4B), indicating that HRG binding is specific.

In addition to characterizing the interaction of HRG with necrotic cells, we also investigated the interaction between HRG and early stage apoptotic cells. We used Jurkat T cells for these experiments because this cell line provides a good model for studying apoptotic cells. HRG binding studies were carried out on viable, early stage apoptotic and necrotic Jurkat T cells (Fig. 3A). Interestingly, HRG binding to early stage apoptotic cells appeared significantly lower than HRG binding to viable cells (Fig. 3, B and C). In addition, the residual HRG binding to
early stage apoptotic cells appeared to be unaffected by the presence of heparin, whereas HRG binding to viable cells was almost entirely abolished by heparin. It is well established that there are substantial changes in the extracellular composition of the plasma membrane of early stage apoptotic cells (6), with this study detecting a loss of cell surface heparan sulfate (data not shown). In contrast to early stage apoptotic cells, HRG binding to necrotic cells was significantly higher than HRG binding to viable cells and also was only partially inhibited by heparin. Thus, HRG interacts differently with viable early stage apoptotic and necrotic cells. Binding to viable cells is heparin inhibitable and is mediated by interaction of the N1N2 domain of HRG with cell surface heparan sulfate. In contrast, early stage apoptotic cells have undergone cell membrane changes, including exposure of phosphatidylserine and alteration of membrane carbohydrates, including heparan sulfate, and thus exhibit low levels of HRG cell surface binding (Fig. 3C). However, once cells become necrotic, either by becoming late stage apoptotic cells or following exposure to toxic conditions such as hyperthermia, the plasma membrane becomes permeable and allows high level binding of HRG.

FIGURE 5. Effect of HRG on the phagocytosis of necrotic Jurkat T cells by the monocytic THP-1 cell line. A, a phagocytosis assay was developed using the intracellular fluorescent dyes SNARF and CFSE. THP-1 cells were labeled with SNARF (red fluorescence); necrotic Jurkat T cells were labeled with CFSE (green fluorescence). Phagocytic cells (THP-1) were mixed with necrotic cells (Jurkat) at a ratio of 1:10 and incubated at 37°C for up to 80 min in the presence or absence of plasma-derived HRG (100 μg/ml) before analysis by flow cytometry. Representative density plots are shown at time 0 and 60 min in the presence and absence of HRG. Rate of phagocytosis was determined as the number of CFSE<sup>−</sup>/SNARF<sup>−</sup> cells as a percentage of total THP-1 cells. B, numerical representation of the phagocytosis rate in the presence (▲) or absence (■) of plasma-derived HRG (100 μg/ml), expressed as the percentage of CFSE<sup>−</sup>/SNARF<sup>−</sup> THP-1 cells. Error bars represent S.E. (n = 3). C, the effect of HRG concentration (3–200 μg/ml) on the phagocytosis of necrotic Jurkat T cells following incubation for 60 min at 37°C, with data expressed as the percentage of CFSE<sup>−</sup>/SNARF<sup>−</sup> THP-1 cells. D, effect of 12.5-kDa bovine lung heparin (100 μg/ml), porcine muscle tropomyosin (200 μg/ml), and a mixture of Fc<sub>γRI</sub> and Fc<sub>γRII</sub> blocking antibodies on HRG-enhanced uptake of necrotic Jurkat T cells by phagocytic THP-1 cells (80 min, 37°C). The uptake of viable cells by phagocytic THP-1 cells is also shown. Results are shown as the percentage of THP-1 cells that are CFSE<sup>−</sup>/SNARF<sup>−</sup> (% phagocytosis).
via its N1N2 domain, to unidentified cytoplasmic ligands that are not heparan sulfate related (Figs. 1, C and D, 2B, and 4A).

Not only did HRG vary in its interaction with viable apoptotic and necrotic cells but, in an in vitro phagocytosis assay, HRG had a striking and selective effect on the phagocytosis of necrotic cells, increasing both the level and rate of uptake of the cells by ~2-fold (Fig. 5, B and C). Such results are in agreement with the general findings of Gorgani et al. (29), who showed that HRG binds and opsonizes late stage apoptotic cells. However, one of the key features arising from this study is that HRG binds and opsonizes necrotic cells, and not early stage apoptotic cells, highlighting the fact that the cell membrane needs to become permeable to allow HRG to bind to intracellular ligands. Thus, theoretically, HRG may play a role in the uptake and clearance of both necrotic and late stage apoptotic cells, although in reality late stage apoptotic cells rarely exist in vivo as there are highly efficient clearance mechanisms for eliminating early stage apoptotic cells (8, 12). Thus, a major in vivo function of HRG is probably to facilitate the clearance of necrotic cells and necrotic cell debris by macrophages, providing the individual with some protection against the development of autoimmune antibodies and resultant autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis.

The molecular basis of the opsonization of necrotic cells by HRG remains to be determined. Clearly, based on the data presented in this study, the cytoplasmic ligand for HRG in necrotic cells is not heparan sulfate. A recent study reported that HRG can interact with the cytoskeletal protein tropomyosin, which is expressed on the surface of FGF-2-activated human endothelial cells (25). However, the histidine-rich region of HRG has been implicated in tropomyosin binding (24), whereas the N1N2 domain of HRG interacts with necrotic cells (Figs. 2 and 4A). Furthermore, addition of high concentrations of soluble tro-
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Summary: This study investigated the role of HRG in the uptake of necrotic cells by macrophages. The authors found that HRG selectively opsonizes necrotic and late stage apoptotic cells, with a pattern of CD32-specific mAbs recognizing this process. The failure of HRG to opsonize apoptotic cells may interact with HRG (29). The failure of HRG to interact with the nuclei of necrotic/fixed cells and may not play a major role in the HRG-mediated opsonization observed in this study. In contrast, Gorgani et al. (29) reported that an anti-FcRII mAb partially blocks the HRG-mediated uptake of late-stage apoptotic cells by human monocyte-derived macrophages. This discrepancy may be because of differences in the dead cells and macrophage populations used in the two studies.

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