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Eosinophils Oxidize Damage-Associated Molecular Pattern Molecules Derived from Stressed Cells

Ramin Lotfi,* Gloria Isabelle Herzog,* Richard Anthony DeMarco,† Donna Beer-Stolz,‡ James Joseph Lee,§ Anna Rubartelli,‖ Hubert Schrezenmeier,* and Michael Thomas Lotze‡

Eosinophils (Eos) are found at increased numbers within necrotic areas of tumors. We show that necrotic material from cell lysates containing damage-associated molecular pattern molecules induce eosinophil degranulation (release of major basic protein and eosinophil peroxidase) and enhance their oxidative burst while the stimulatory capacity of cell lysates is significantly diminished following oxidation. High mobility group box 1 (HMGB1), a prototypic damage-associated molecular pattern molecule, released following necrosis but not apoptosis, induced a similar effect on Eos. Additionally, we demonstrate that HMGB1 enhances eosinophil survival and acts as a chemoattractant. Consistently, we show that Eos express an HMGB1 receptor, the receptor for advanced glycation end product, and that anti-receptor for advanced glycation end product could diminish the HMGB1-mediated effects. Of all tested biologic activities, Eos respond most sensitively to the presence of necrotic material including HMGB1 with generation of peroxide. We postulate that Eos “sense” necrotic cell death, migrating to and responding to areas of tissue injury/necrosis. Oxidation of cell lysates reduces their biologic activity when compared with native lysates. We postulate that eosinophil-associated modulation of immunity within tumor and other damaged tissues may be primarily by promoting oxidative degradation of necrotic material. Novel therapeutic strategies may be considered by advancing oxidative denaturation of released necrotic material using Eos or other aerobic strategies. The Journal of Immunology, 2009, 183: 5023–5031.

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1 R.L. and M.T.L. designed and conducted the research, analyzed the data and wrote the article; G.I.H. performed the oxidative burst and degranulation assays; D.B.S. produced electron microscopic and confocal images; R.A.D. purified HMGB1 and produced specific antibodies to HMGB1; A.R., J.J.L., and H.S. helped in writing the article and provided important advice; and all authors contributed to critical review of the article.

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3 Abbreviations used in this paper: DAMP, damage associated molecular pattern molecule; HMGB1, high mobility group box 1; Eos, eosinophil; Gr, granulocyte; MSC, mesenchymal stem cell; F/T, freeze/thaw; EPO, eosinophil peroxidase; RAGE, receptor for advanced glycation end product; MBP, major basic protein; DC, dendritic cell.

Eosinophils (Eos) are found at increased numbers within necrotic areas of tumors. We show that necrotic material from cell lysates containing damage-associated molecular pattern molecules induce eosinophil degranulation (release of major basic protein and eosinophil peroxidase) and enhance their oxidative burst while the stimulatory capacity of cell lysates is significantly diminished following oxidation. High mobility group box 1 (HMGB1), a prototypic damage-associated molecular pattern molecule, released following necrosis but not apoptosis, induced a similar effect on Eos. Additionally, we demonstrate that HMGB1 enhances eosinophil survival and acts as a chemoattractant. Consistently, we show that Eos express an HMGB1 receptor, the receptor for advanced glycation end product, and that anti-receptor for advanced glycation end product could diminish the HMGB1-mediated effects. Of all tested biologic activities, Eos respond most sensitively to the presence of necrotic material including HMGB1 with generation of peroxide. We postulate that Eos “sense” necrotic cell death, migrating to and responding to areas of tissue injury/necrosis. Oxidation of cell lysates reduces their biologic activity when compared with native lysates. We postulate that eosinophil-associated modulation of immunity within tumor and other damaged tissues may be primarily by promoting oxidative degradation of necrotic material. Novel therapeutic strategies may be considered by advancing oxidative denaturation of released necrotic material using Eos or other aerobic strategies. The Journal of Immunology, 2009, 183: 5023–5031.

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Materials and Methods

Cellular preparation

In the context of an Institutional Review Board-approved protocol, and with the permission and supervision of the Local Ethical Committee, human granulocytes (Gr) were purified from whole blood by density gradient centrifugation using Ficoll-Paque Plus (Amersham Biosciences) followed by lysis of red cells using either ammonium chloride solution (155 mM NH4Cl, 10 mM KHCO3, and 0.1 mM EDTA) or hypotonic distilled sterile water. Human Eos were negatively separated from other Gr using MACS-separation (Miltenyi Biotec) following the manufacturer’s instructions. The purity was assessed by H&E staining and was at least 95%. Cells were kept in RPMI 1640 (CellGro; Mediatech) supplemented with 10% FBS (Invitrogen) and containing 100 U/ml penicillin-G, 0.25 μg/ml amphotericin B, 100 μg/ml streptomycin (CellGro; Mediatech) in a humidified atmosphere at 37°C with 5% CO2. Generation of cell lysates

Two individual colorectal tumor cell lines, human colorectal tumor-116 and cancer of the colon-2 cells (American Type Culture Collection), or bone marrow-derived human mesenchymal stem cells (MSCs) were resuspended in PBS at a concentration of 1 × 106 cells/ml and lysed by 3–5 cycles of freeze-thawing (F/T) (~80 to 37°C). The viability following treatment was assessed using trypan blue exclusion and was always <0.1%. For colorimetric measurement of eosinophil peroxidase (EPO) release, lysates were spun down hard (16,300 × g) and the soluble supernatant was used at given concentrations.

HMGB1

_Escherichia coli_ expressed recombinant HMGB1 was provided by Biovitan and purchased from R&D Systems. Affinity-purified (natural) HMGB1 was obtained from lysed HeLa cells or human liver as previously described (14). In brief, HeLa cells or human liver cells were lysed in a buffer containing PBS with 1% Igepal CA-630 (Nonidet P-40), 1 mM N-ethylmaleimide, 5 mg/ml 6-aminohexanoic acid, 1 mM 4-(2-amino-ethyl) benzenesulfonyl fluoride hydrochloride, 500 mM aprotinin, 50 μM leupeptin, 100 μM E64, 36 μM phosphoramidon, 40 μM bestatin, 1 mM benzamidine, 16 μM antipain, 10 μg/ml trypsin inhibitor, and 1 mM EDTA (Sigma-Aldrich). The lysate was centrifuged for 15 min at 16,000 × g to pellet nuclei and insoluble material. Supernatants were pooled and filtered through an Acrodisc 0.45-μm syringe filter ( Pall Corporation).

HMGB1 polyclonal Ab

This Ab was obtained from New Zealand white rabbits immunized with the peptide sequence KSEAGKKGPGRPTGS corresponding to amino acids 166–181 of HMGB1. The affinity purification of the polyclonal anti-HMGB1 Ab was performed as previously reported (15). In brief, HeLa cells or human liver cells were lysed in a buffer containing PBS with 1% Igepal CA-630 (Nonidet P-40), 1 mM N-ethylmaleimide, 5 mg/ml 6-aminohexanoic acid, 1 mM 4-(2-amino-ethyl) benzenesulfonyl fluoride hydrochloride, 500 mM aprotinin, 50 μM leupeptin, 100 μM E64, 36 μM phosphoramidon, 40 μM bestatin, 1 mM benzamidine, 16 μM antipain, 10 μg/ml trypsin inhibitor, and 1 mM EDTA (Sigma-Aldrich). The lysate was centrifuged for 15 min at 16,000 × g to pellet nuclei and insoluble material. Supernatants were harvested and frozen at −80°C.

Specific inhibition of HMGB1 effects

Specific blocking of HMGB1-mediated effects was performed by pretreating the lysate or HMGB1 itself with either anti-HMGB1 Abs (MBL) for 2 h or by pretreating Eos with anti-receptor for advanced glycation end product (RAGE) polyclonal Abs (R&D Systems) for 20 min before stimulation. The anti-HMGB1 Abs used were a mixture of three mAbs used at a working concentration of 3 μg/ml for each mAb. Goat polyclonal anti-human RAGE Ab was used at a working concentration of 20 μg/ml. Mouse IgG1, IgG2a, and goat IgG (R&D Systems) were used as isotype controls.

Oxidation of lysates

Cell lysates were oxidized by incubating in 0.08 mM H2O2 for 2 h at room temperature. In other experiments, various concentration of H2O2 were used to discern the concentrations necessary to effect oxidation and limit direct effect on responding cells, allowing us to select this concentration as the most effective.

Cell surface staining and flow cytometry

Fixed cells using 2% paraformaldehyde were blocked for at least 10 min with whole IgG (Jackson ImmunoResearch Laboratories) of the same species which the specific Ab was generated in. Specific staining was performed for at least 1 h in room temperature with rabbit anti-human RAGE (R&D Systems). The anti-RAGE Ab was conjugated to Alexa 546 (Molecular Probes, Invitrogen) following the manufacturer’s instructions. The fluorescence of stained cells was assessed using a BD FACS Array (BD Biosciences) and the data were analyzed using FACS Diva software (BD Biosciences). At least 20,000 events were acquired in all flow cytometric analyses of membrane and intracellular proteins.

Microscopic imaging

Confocal images were obtained on 2% paraformaldehyde-fixed cells that were pelleted onto slides (Cytospin 3, Shandon), washed three times in PBS, then three times with PBS supplemented with 0.5% BSA and 0.15% glycine (PBSG). Cells were permeabilized in 0.1% Triton X-100 in PBS for 20 min, then washed once with PBG. After blocking in 20% normal goat serum (for goat secondary antibody) or 20% normal donkey serum (for donkey secondary Abs) in PBG for 30 min, cells were washed once with PBG. Primary Abs were added to cells in PBG (rabbit anti-HMGB1 1/500; goat anti-RAGE 1/100) and incubated at room temperature for 1 h. After four washes in PBG, cells were incubated for 1 h in secondary Abs (goat anti-rabbit Cy3, donkey anti-goat Cy3 1/1000, Jackson ImmunoResearch Laboratories; Cy5-phallolidin, 1/250 dilution, Molecular Probes) with the nuclear counterstain, Draq5 (1/2000, Biostatus). After three washes in PBG followed by PBS, cells were mounted using gelvatol (23 g poly(vinyl alcohol) 2000, 50 ml glycerol, 0.1% sodium azide to 100 ml PBS), then viewed on a confocal scanning fluorescence microscope (Olympus Fluoview 1000).

Survival assay

Cell viability was assessed by staining with Annexin-V (labeled with Alexa 647) and SYTOX-Orange (Molecular Probes) following the manufacturer’s instructions. Incubation in recombinant human GM-CSF (100 ng/ml) (Leukine, Immunex Corporation) served as a positive control for survival. SYTOX-Orange/Annexin-V double negative cells were considered as viable. At least 20,000 events were acquired in all flow cytometric analyses using BD FACS-Array (BD Biosciences).

Migration and adherence assays

For migration and adherence assays, we used 96-well ChemoTx system (Neuro Probe) with a membrane pore size of 5 μm, alternatively we used BD Falcon HTS Fluoroblot Insert systems with a pore size of 8 μm. Cells were stained with CFSE (CellTrace, Molecular Probes, Invitrogen), and 0.5–1 × 106 CFSE-stained cells were added on the top of the membrane of each migration well. The assay was performed for 40–45 min at 37°C. Cell rows of migrating cells (lower chamber) and cells adhering to the membrane were calculated based on the standard curve for CFSE fluorescence. Fluorescence was measured using a microplate reader (Safire, Tecan, or POLARStar Omega, BMG Labtech). To compare results from individual migration experiments, we normalized the results by setting 0% as the nonspecific migration toward middle amount and defining the migration toward positive control as 100%. Alternatively, for experiments using HTS Fluoroblot Insert systems, we subtracted background fluorescence of the plate and then calculated the ratio of fluorescence, following 45 min of incubation, divided by fluorescence at the beginning of the experiment (T0) for each condition. Recombinant human IL-8 was used at 100 ng/ml (Bio-source) and eotaxin at 100 ng/ml (R&D Systems). These served as positive controls for migration assays on granulocyte whole population and eosinophilic granulocytes, respectively.

Assessment of major basic protein (MBP) release

Degranulation of Eos was assessed based on both assessment of loss of intercellular MBP from Eos, and by in-house established ELISA for MBP in supernatants. After indicated times of stimulation the supernatants were harvested and frozen at −30°C for later ELISA measurements while Eos were fixed and permeabilized using Cytofix/Cytoperm-solution (BD Pharmingen) according to the manufacturer’s instructions. After blocking nonspecific binding sites for 30 min with whole goat IgG (Jackson ImmunoResearch Laboratories), they were fixed and permeabilized. Eos were incubated an hour with murine anti-human MBP Ab (BD Pharmingen) as primary and for an hour with PE-labeled goat anti-mouse IgG (Jackson ImmunoResearch Laboratories).
IgG as a secondary Ab (Jackson ImmunoResearch Laboratories). Alternatively, we detected intracellular MBP directly with fluorescent labeled murine anti-human MBP. Abs were labeled with Alexa Fluor 546 (Molecular Probes, Invitrogen) according to the manufacturer’s instructions. When using labeled Abs, we blocked nonspecific binding sites for 30 min with whole murine IgG (Jackson ImmunoResearch Laboratories). Degranulation was assessed by comparing the fluorescence from nonstimulated vs stimulated Eos.

**EPO release assay**

Peroxidase activity in standard and test supernatant samples was determined as previously described. In brief, o-phenylenediamine (Pierce) was dissolved in stable peroxide buffer (Pierce) to a final concentration of 0.5–1.0 mg/ml. Of this o-phenylenediamine solution 100 µl was added to 100 µl of an Eos suspension of 2 × 10^5 cells/ml in a 96-well plate. The reaction was stopped by adding 100 µl of a 1M sulfuric acid solution, the OD at 492 nm was assessed using a microplate reader (Safire, Tecan). The standard curve was constructed based on the OD obtained from a serial dilution of lysed Eos. The quantity of EPO in supernatants was expressed as the equivalent amount of lysed Eos generating this amount of EPO.

**Oxidative burst**

Oxidative burst of eosinophils and neutrophils was measured by adding 1 µM 2,7’-H2DCFDA (Invitrogen Molecular Probes) to 2 × 10^5 cells (0.75 × 10^5 cells/ml), the cells were then stimulated by indicated concentrations of lysate or GM-CSF.

After incubation at various times in a humidified atmosphere at 37°C with 5% CO2, fluorescence from nonstimulated vs stimulated cells was assessed using FACS-SCAN (BD Biosciences). For some kinetics experiments, a POLARStar Omega fluorescence plate reader (BMG Labtech) was used; alternatively 5 × 10^5 cells were resuspended in 200 µl of phenol-red free medium and added to a 96-well plate. fMLP (Sigma–Aldrich) served as a positive control. Intracellular MBP was assessed by flow cytometric analysis after staining with specific Abs for detection. Shift to the left correlates with degranulation (i.e., MBP-release). EPO release (B) in supernatant was assessed using a colorimetric assay as described in *Materials and Methods*. Shown is the arithmetic mean ± SD from four individual experiments. Lysates from both cell lines induce MBP and EPO release from eosinophils. The degranulation correlated with the number of lysed cells. C demonstrates dose-dependent degranulation of eosinophils (decreasing content of intracellular MBP) with increasing concentration of cell lysate. Shown is mode fluorescence intensity ± coefficient of variation of intracellular MBP. D demonstrates dose-dependent release of EPO (arithmetic mean ± SD) from eosinophils stimulated with lysate from HCT-116 cells. Time dependent degranulation of eosinophils responding to lysates from HCT-116 cells is shown in E.

To exclude the possibility of missing the oxidative burst of neutrophils at time points earlier than 5 min, we repeated our measurements beginning 10 s following stimulation and still could not identify enhancement of the neutrophil oxidative burst. (Fig. 2C). When using lysates from normal, nonneoplastic human MSCs, we could observe similar effects (Fig. 2D), indicating that the effect was not limited to tumor cell lines.

**Oxidation reduces the capacity of lysates to induce Eos degranulation**

Having shown that Eos were quite sensitive to necrotic material obtained from lysed cells, responding to as low as 10^5 lysed tumor cells/ml and that this effect is specific for Eos, we sought to determine whether oxidation of lysates would influence their biologic activity. H2O2 at a concentration of 0.125 mM is readily detectable within inflammatory tissues (16, 17). H2O2 below this concentration as reported by others (17) and in our experience is not cytotoxic (data not shown). Preincubation of HCT-116 and CACO-2 cell lysates with H2O2 at nontoxic concentrations (0.08 mM) for 2h at room temperature markedly diminished the lysate-induced EPO release from Eos (Fig. 3A). We confirmed the neutralizing effect of H2O2 on lysates by assessing MBP release (Fig. 3B).
Eos express RAGE

F/T lysis of cells as we had performed is a form of necrotic cell death leading to release of DAMPs. To confirm that the observed effect is due to necrosis as opposed to apoptosis and to characterize candidate DAMPs at least in part responsible for the stimulatory effect of lysates, we focused on an evolutionary conserved protein, HMGB1, which is released following cellular stress/necrosis but not with programmed/apoptotic cell death (5). The RAGE is one of the first defined receptors for HMGB1 (18, 19). We demonstrated expression of RAGE by flow cytometry on Gr and Eos (Fig. 4A). Confocal microscopy demonstrated expression of intracellular as well as membrane bound RAGE on these cells (Fig. 4A).

In degranulation assays, preincubation of Eos with anti-RAGE before stimulation with HCT or MSC lysate or preincubation of these lysates with anti-HMGB1 diminished the lysate-induced degranulation of Eos (Fig. 4C), indicating that RAGE participates in the lysate-induced stimulatory effect on Eos and supported our hypothesis that HMGB1 is a potential candidate for the DAMP activity found within necrotic material. In oxidative burst assays, we could confirm our observations with the inhibitory effect of anti-RAGE (Fig. 4D).

HMGB1 induces Eos degranulation

Consistent with the effect of tumor cell lysates on Eos, the addition of HMGB1 (the prototypic DAMP) prompted a significant decrease in the intracellular content of MBP which could be blocked by using anti-HMGB1 Abs (Fig. 5A). We could also observe a dose-dependent release of EPO from Eos following stimulation with individual concentrations of HMGB1 (Fig. 5B). The degranulation-inducing effect of HMGB1 could be demonstrated by both natural and recombinant HMGB1. The most consistent stimulatory effect could be shown at the highest concentration of HMGB1 used, 10 μg/ml, similar to the concentrations we used to stimulate TNF-α or IL-6 production. HMGB1 concentrations higher than 10 μg/ml were not tested. Degranulation could be observed within 30 min and was highest following 2 h of stimulation (data not shown).

HMGB1 specifically enhances the oxidative burst of Eos when compared with neutrophils

Eos, but not neutrophils, respond to HMGB1 in a dose-dependent manner with a maximum oxidative burst discerned following 60 min of incubation (Fig. 6). In contrast with the concentration needed to induce degranulation (at least 1000 ng/ml), the concentration necessary to enhance the oxidative burst of Eos is only 10 ng/ml or thus 100-fold lower.

HMGB1 promotes Eos and Gr migration and adhesion

The chemotactic capacity of necrotic tumor cells has been previously demonstrated for Eos in vitro and in vivo (6, 7). Both recombinant as well as natural HMGB1 (1 and 10 μg/ml) promoted significant (p < 0.05) recruitment of Gr within 40 min (Fig. 7A). IL-8 served as a positive control for Gr migration. We obtained similar results for Eos (Fig. 7B) with eotaxin serving as a positive control; all concentrations were significant (p ≤ 0.05) except that of 1 μg/ml recombinant HMGB1 (p = 0.14). The number of migrating cells was calculated based on the standard curve for fluorescent-stained cells. Results from independent experiments were normalized by subtracting the nonspecific migration toward medium and defining the migration toward positive control as 100%. HMGB1 was tested negative for endotoxin, additionally we could...
diminish any putative endotoxin-induced effects by adding polymyxin B to all medium. Using similar experimental procedures we could demonstrate that HMGB1 also induces adherence of Gr and Eos (although to a lesser extent) to the migration membrane (Fig. 8). Consistent with our results with inactivation of necrotic material by oxidation and that in published data concerning the biologic activity of reduced vs oxidized HMGB1, we could significantly decrease the chemotactic activity of HMGB1 on eosinophils by oxidizing this protein (Fig. 7). In accordance with our results in Fig. 4, the HMGB1 effect could also be blocked by pretreating Eos with anti-RAGE Abs (Fig. 7).

HMGB1 promotes eosinophil and neutrophil survival

Gr-including Eos are postmitotic, differentiated cell types having a half life of 8–12 h within the circulation. Incubation of Gr (Fig. 9, A and C) and Eos (Fig. 9, B and D) with either natural (HeLa-derived) or recombinant HMGB1 promoted survival when tested at either 24 or 48 h. These findings are dose dependent and significant (p ≤ 0.05) at 10 μg/ml HMGB1 for both time points and both cell populations.

Discussion

Although eosinophilia is often observed in the peripheral blood and within tumors of patients with cancer (1), little attention has been paid to identifying the mechanisms underlying their traffic into neoplastic tissue and their role within this setting. The prevailing opinion associates Eos with allergic and autoimmune diseases as well as with helminthic infections. In these settings, Eos are considered to be responsible for tissue damage by virtue of their highly cytotoxic basic and oxidizing granules, which they can release following activation (13). The favorable prognosis of patients with colorectal malignancies with tumor-associated tissue

FIGURE 4. The receptor for advanced glycation end products, RAGE is expressed and functional on eosinophils. Granulocyte whole population (consisting of >95% of neutrophils) and eosinophils with an overall viability of >95% were fixed and stained for advanced glycation end-product (RAGE), a receptor for HMGB1. Confocal microscopy (A) demonstrates the expression of intracellular as well as membrane bound RAGE in granulocytes (left) and eosinophils (right). Flow cytometric analysis (B) confirms the results obtained from confocal microscopy. Preincubation of eosinophils with anti-RAGE Abs before stimulation with lysate as well as preincubation of lysate with anti-HMGB1 Abs diminishes the effect of lysate on eosinophils in terms of degranulation (C) and oxidative burst (D).

FIGURE 5. Human Eos degranulate following HMGB1 treatment. A. Approximately 1 × 10⁶ Eos/ml were cultured for 2 h in medium (gray) or with 10 μg/ml HMGB1 (open). Intracellular MBP was assessed by flow cytometric analysis after staining with specific Abs for detection (upper panel). Shift to the left correlates with degranulation (i.e., MBP-release). A representative study is shown with similar results obtained in six other experiments with rHMGB1 and three experiments performed with natural HMGB1. Described HMGB1 effect could be diminished by using specific Abs. B shows colorimetric assessment of released EPO in the supernatant of HMGB1 stimulated eosinophils, the HMGB1 effect is dose dependent. PMA served as positive control for degranulation. Shown is arithmetic mean (±SD).

FIGURE 6. HMGB1 specifically enhances the oxidative burst of Eos when compared with neutrophils. Eos and granulocytes (consisting of >95% neutrophils) at a concentration of 10⁶/ml were stimulated with individual concentrations of recombinant HMGB1 for the indicated time periods. Oxidative burst was measured by a fluorescence plate reader. Shown is mean fluorescence intensity. HMGB1 specifically enhances the oxidative burst of eosinophils but not of neutrophils with a maximum following ~60 min.
ties of natural (f) and recombinant (H18554) HMGB1.

stained granulocytes were added to the upper chamber above a 5-µm pore size membrane. The migration assay was stopped after 40 min. The number of migrating cells was calculated based on the standard curve for stained cells. A representative standard curve demonstrating excellent linear correlation between cell count and fluorescence with sensitivity to 400 cells is shown in inset of C. The migration was assessed at individual concentrations of natural (A) and recombinant (B) HMGB1. A and B, Migration of whole granulocytes as well as separated eosinophils, respectively; IL-8 and eotaxin (100 ng/ml) served as positive controls for granulocytes and Eos, respectively. Results from independent experiments are normalized by subtracting the nonspecific migration toward medium and defining the migration toward positive control as 100%. Shown is the average and SEM of five and seven individual experiments for granulocytes and eosinophils, respectively. HMGB1 induced dose-dependent adherence for both cell populations; p values (compared with adherence when using medium alone) are indicated on the top of each bar. All medium were supplemented with 10 µg/ml polymyxin B (Sigma-Aldrich) to block any effects of contaminating endotoxin. Chemotactic effects of HMGB1 on eosinophils could be inhibited by oxidizing HMGB1 or pretreating eosinophils with anti-RAGE Abs (C).

FIGURE 7. Both natural and recombinant HMGB1 serve as chemoattractants for human granulocytes and Eos. Approximately 1 × 10⁵ CFSE-stained granulocytes were added to the upper chamber above a 5-µm pore size membrane. The migration assay was stopped after 40 min. The number of migrating cells was calculated based on the standard curve for stained cells. A representative standard curve demonstrating excellent linear correlation between cell count and fluorescence with sensitivity to 400 cells is shown in inset of C. The migration was assessed at individual concentrations of natural (□) and recombinant (■) HMGB1. Top and bottom demonstrate adherence of whole granulocytes as well as separated eosinophils, respectively. Shown is the average and SEM of five and seven individual experiments for granulocytes and eosinophils, respectively. HMGB1 induced dose-dependent adherence for both cell populations; p values (compared with adherence when using medium alone) are indicated on the top of each bar. All medium were supplemented with 10 µg/ml polymyxin B (Sigma-Aldrich) to block any effects of contaminating endotoxin.

FIGURE 8. Both natural and recombinant HMGB1 promote human granulocyte and eosinophil adherence. Approximately 1 × 10⁵ CFSE-stained granulocytes were added to the upper chamber above a 5-µm pore size membrane. After 40 min, the membrane was washed twice with PBS and the number of adhering cells to the membrane was calculated based on the standard curve for stained cells. The adherence was assessed at individual concentrations of natural (□) and recombinant (■) HMGB1. The migration toward positive control as 100%. Shown is the average and SEM of six experiments.

In addition, local and systemic eosinophilia is also found following effective immunotherapy of patients with cancer. The antitumor effects of successful cytokine therapy of cancer patients with IL-2 is associated with the identification of degranulating Eos within the tumor (1, 22, 23). Sosman (24) and colleagues found that IL-4 therapy of cancer patients also induced systemic Eos degranulation with increased MBP in serum (p = 0.018) and urine (p = 0.031). The increase in serum MBP was IL-4 dose-dependent (p = 0.001). Thus, we (J.J.L.) have suggested (20) that T cell-induced immune responses associated with the inflammatory response against tumor, acute rejection of transplanted organs, the accumulation/differentiation of Ag specific T cell populations, and the immune modulation of the gastrointestinal tract mucosa may ultimately each be causatively linked to tissue-specific recruitment of activated effector T cells by Eos accumulating at these sites. The major role of RAGE-expressing recruited cells is nominally for wound repair (25) whereas TLR ligation by pathogen associated molecular patterns promotes further tissue destruction.

Three findings alerted us to the possible role of DAMPs, including the prototypic DAMP, HMGB1: 1) Neoplastic cells, with prominent defects in apoptotic death and with induced autophagy, often undergo a necrotic death allowing release of DAMPs such as HMGB1; 2) Eos are activated and attracted by necrotic epithelial cells in vitro and in vivo (6, 7); and 3) HMGB1 activates and...
recruits Gr, increasing the nuclear translocation of NF-κB, thereby enhancing the expression of proinflammatory cytokines in Gr (26).

To convince ourselves that necrotic tissue can mediate direct activation of Eos, we repeated some of the experiments previously performed by Stenfeldt and colleagues (7). We studied two colorectal cancer cell lines, CACO-2 and HCT-116, which have not been previously studied in this setting. We could confirm that Eos responded to factors derived from lysed cells. The two most dominant effector functions ascribed to Eos results from the production of reactive oxygen species (i.e., oxidative burst) and the release of cationic granule proteins (i.e., degranulation), thus we examined both of these properties and demonstrated that necrotic material from lysate, among all tested biologic responses to necrotic material. EPO acts as an enzyme catalyzing the oxidation of chloride, bromide, and thiocyanate to their respective hypohalous acids with even higher oxidative capacity than peroxide itself, consistent with the oxidative role of Eos within stressed tissues.

Examinin the possible mechanisms underlying the effect of necrotic material, we focused on HMGB1 for two reasons. We had confirmed that HMGB1 is released from necrotic but not apoptotic tumor cells (5), and could demonstrate that Eos express RAGE, one of the receptors for HMGB1. By using anti-HMGB1 and anti-RAGE Abs, we could diminish the stimulatory effect of necrotic material and HMGB1 itself on Eos even though the inhibitory effect was not 100% and differed between HCT and MSC lysates (Fig. 4 and 5), indicating that there may be a qualitative or quantitative difference between components of lysates from different origin. Similar to necrotic material obtained from lysates, HMGB1-induced dose dependent degranulation of Eos and enhanced their oxidative burst. Our findings that HMGB1 promotes survival of Gr and Eos is consistent with the increase of nuclear translocation of NF-κB in such treated Gr (26). HMGB1 not only serves as a chemoattractant but also promotes retention of both Eos and Gr within necrotic and inflammatory tissue, as well as the survival of both cell types.

Consistent with our observations for lysates, oxidized HMGB1 loses its biologic activity (32, 33). In accordance with our findings for necrotic material from lysate, among all tested biologic responses to HMGB1, the Eos oxidative burst was the most sensitive, thus giving
and thus degrade DAMPs, inactivating them. Although MBP boosts neutrophils’ oxidative burst leading to O2−-production, release of peroxidase from eosinophils (EPO) induces generation of additional reactive oxygen species (ROS). Together, these ROS oxidize and thus degrade DAMPs, inactivating them.

In this study, we propose a mechanism for Eos trafficking into necrotic tumor tissue, mediated in part by HMGB1 and suggest a role for Eos in sensing cell stress and damage, amplifying immune response, and oxidizing necrotic material, thus regulating immune responses (Fig. 10). The hypothesis we have proposed of necrosis-associated tumorigenesis and tumor proliferation is based on the capacity of released necrotic material including DAMPs to induce tissue proliferation, and most recently (data not shown) to limit apoptosis (34). Eosinophils may be capable of breaking this vicious circle by oxidation, thus inactivating factors released from necrotic cells. Understanding the relationship between necrosis/tumor derived factors (such as HMGB1) and Eos is important to discern their role within the tumor microenvironment. Previously, we demonstrated that Eos interact with DCs promoting their maturation and thus modulating immunity (21). Only 1 percent of Eos are found within the bloodstream with the remainder residing in tissues directly interfacing with the environment, primarily within the gastrointestinal and respiratory tracts. These are sites of rapid tissue turnover with substantial chronic exposure to microbial flora and their associated mutagenic agents.

Most contemporary studies in tumor immunology have focused primarily on lymphoid effectors such as T cells, and NK cells, and myeloid cells including macrophages and DCs. The role of granulocytes, comprising neutrophils, Eos, basophils, and mast cells, has been understudied even though these cells belong to the first set of leukocytes recruited into inflammatory sites, thus initiating and modifying the subsequent immune response. DAP10- or DAP12-associated receptors as well as ST2, the receptor for IL-33 are candidate molecules regulating recruitment of inflammatory cells such as eosinophils and the wound healing phenotype within tissues (35, 36). Even in the setting of metastasis, the ability of tumor-derived factors such as DAMPs likely due to necrosis to promote “metastatic” inflammation has been overlooked (37–39). Inflammation associated with chronic allergic respiratory provocation and substantial local eosinophil accumulation (38) is also capable of significantly enhancing metastasis to the lung in a murine tumor model. Thus, distant inflammatory events independent of or directly attributable to tumor necrosis, significantly contributes to the “seeding” of metastatic cells regardless of their intrinsic genotype. Characterizing the role of innate immune cells, specifically Eos, within tumors and their metastases has broad implications in the development of agents capable of modulating chronic inflammation, particularly within the tumor microenvironment and suggests that they might represent novel targets for developing successful therapies for patients with cancer.

References


