Dimeric Galectin-1 Induces Surface Exposure of Phosphatidylserine and Phagocytic Recognition of Leukocytes without Inducing Apoptosis*

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Received for publication, June 23, 2003, and in revised form, July 9, 2003
Published, JBC Papers in Press, July 9, 2003, DOI 10.1074/jbc.M306624200

We report that human galectin-1 (dGal-1), a small dimeric β-galactoside-binding protein, induces phosphatidylserine (PS) exposure, measured by Annexin V staining, on human promyelocytic HL-60 cells, T leukemic MOLT-4 cells, and fMet-Leu-Phe-activated, but not resting, human neutrophils. This effect of dGal-1 on HL-60 and MOLT-4 cells is enhanced by pretreatment of the cells with neuraminidase, but treatment of resting neutrophils with neuraminidase does not enhance their sensitivity to dGal-1. Although the induction of staining with Annexin V is often associated with apoptosis, the dGal-1-treated HL-60 cells, MOLT-4 cells, and activated neutrophils do not undergo apoptosis, and there is no detectable DNA fragmentation. HL-60 and MOLT-4 cells treated with dGal-1 continue to grow normally. By contrast, camptothecin-treated HL-60 cells, etoposide-treated MOLT-4 cells, and anti-Fas-treated neutrophils exhibit extensive DNA fragmentation and/or cell death. Lactose inhibits the dGal-1-induced effects, indicating that dGal-1-induced signaling requires binding to cell surface β-galactosides. The dimeric form of Gal-1 is required for signaling, because a monomeric mutant form of Gal-1, termed mGal-1, binds to cells but does not cause these effects. Importantly, dGal-1, but not mGal-1, treatment of HL-60 cells and activated human neutrophils significantly promotes their phagocytosis by activated mouse macrophages. These dGal-1-induced effects are distinguishable from apoptosis, but like apoptotic agents, prepare cells for phagocytic removal. Such effects of dGal-1 may contribute to leukocyte homeostasis.

It is believed that the turnover of neutrophils and other leukocytes in tissues involves programmed cell death (apoptosis) and then phagocytosis by tissue macrophages (1–4). However, the factors regulating turnover of leukocytes are unclear. Although Fas and Fas ligand (FasL) induce apoptosis of mature granulocytes (5–7), mice deficient in FasL (gld) or Fas (lpr) have essentially normal numbers of circulating mature granulocytes (5). FasL/Fas-mediated apoptosis is not essential in regulating the clearance of neutrophils during inflammation (9). Fas and FasL may promote, rather than decrease, inflammatory responses in vivo (10–12). In transgenic mice expressing bel-2 in mature neutrophils, apoptosis of circulating cells is inhibited, but neutrophil homeostasis is unaltered, and macrophage-mediated phagocytosis of neutrophils is normal (13). Phagocytosis is required for resolution of the inflammatory process and leukocyte homeostasis in vivo (14–16). These results suggest that factors not yet defined may regulate leukocyte turnover in tissues.

Such observations led us to explore whether the basement membrane and extracellular matrix might harbor other proteins capable of binding to leukocytes and inducing their apoptosis or phagocytic recognition. A candidate protein is the β-galactoside-binding protein termed galectin-1 (dGal-1), which binds to most leukocytes. dGal-1 is a widely expressed dimeric protein (subunit ~14.6 kDa), which is a member of the galectin family of lectins (17–19). It is secreted by many cell types, including human endothelial cells (20, 21), and is found in the basement membrane and extracellular matrices around capillary walls (22, 23). dGal-1 has been reported to have various biological activities, including effects on neurite outgrowth (24, 25), growth inhibition of non-neural cells (26–28), cell growth stimulation (29, 30), and apoptosis of immature thymocytes (31, 32), and to activate human T cells and T cell lines (33, 34).

To explore the biological activity of dGal-1 toward leukocytes, we prepared a recombinant form of dimeric human dGal-1 and a mutated, monomeric form of galectin-1 (mGal-1). We explored the interactions of these lectins with HL-60 cells, MOLT-4 cells, and both resting and activated human neutrophils. Our results show that dGal-1, but not mGal-1, rapidly enhances surface staining with Annexin V (phosphatidylserine (PS) exposure) in desialylated HL-60 cells, desialylated MOLT-4 cells, and activated, but not resting, human neutrophils. The exposure of PS is often associated with apoptosis.
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(35–37). However, dGal-1 does not induce apoptosis, because no DNA fragmentation is observed in dGal-1-treated cells, and HL-60 cells and MOLT-4 cells continue to grow normally. Importantly, dGal-1-treated, but not mGal-1-treated, cells are actively phagocytosed in vitro by activated mouse peritoneal macrophages. Thus, dGal-1-induced effects are distinguishable from apoptosis yet prepare cells for phagocytic recognition. These results suggest that dGal-1 could induce surface changes that play a role in leukocyte turnover in tissues independently of apoptosis.

EXPERIMENTAL PROCEDURES

Preparation of dGal-1 and mGal-1—The cDNA for human dGal-1 was cloned by PCR from the published sequence (38, 39). The recombinant human dGal-1 protein was purified as described previously (40) employing affinity chromatography on lactosyl-Sepharose, and the protein was eluted with lactose. The column was washed with 5 column volumes of PBS (0.01 M NaH2PO4, 0.01 M Na2HPO4, 0.85% NaCl, pH 7.4) containing 2-ME (14 mM), and the bound dGal-1 and mGal-1 were eluted with PBS containing 2-ME (14 mM) and lactose (0.1 M). To confirm that the purified protein quantitatively retained carbohydrate-binding activity, the protein was re-chromatographed on lactosyl-Sepharose. Elution profile of the re-purified dGal-1 revealed a single peak of protein that was quantitatively eluted with lactose (data not shown). In some studies we heat-inactivated the dGal-1 by heating the protein at a concentration of 1 mg/ml to 100 °C for 10 min. The treated sample remained in suspension, although cloudy, and was inactive, because it was unable to bind lactosyl-Sepharose.

To generate a monoclonal form of the protein (mGal-1), we used PCR primer-directed mutagenesis as described for the hamster-derived Gal-1 (41) to place a Ser residue codon at the Cys-2 position and an Asp residue at the Val-5 position. The cDNAs for dGal-1 and mGal-1 were ligated into the BamHI and HindIII sites of the plasmid PEQ-50 (Qiagen). Both dGal-1 and mGal-1 were expressed at high levels in transformed Escherichia coli. dGal-1 and mGal-1 were purified from sometreated E. coli cells extract on columns of lactosyl-Sepharose as described above. Human dGal-1 at a concentration from 2–80 μM behaves as a dimeric protein on size-exclusion chromatography, whereas mGal-1 behaves as a monomeric species under the same conditions, using experimental approaches previously described (41). The detailed characterization of the binding properties and kinetics of subunit association of human dGal-1 and mGal-1 will be described elsewhere.

Cell Lines and Enzymatic Desialylation—HL-60 and MOLT-4 cells were obtained from the American Type Culture Collection and maintained at 37 °C and 5% CO2 in complete RPMI 1640 media containing 10% fetal calf serum, glutamine (2 mM), penicillin (100 milliunits/ml), and streptomycin (100 μg/ml). To desialylate the cells, HL-60 cells or MOLT-4 cells (3 × 107 cells) were treated with 100 units of Antibacterial neuraminidase (Sigma) in 500 μl of complete RPMI for 1 h at 37 °C. The treated cells were washed twice with RPMI before use.

Isolation, Activation, and Desialylation of Neutrophils—Heparinized blood was obtained from normal donors. Neutrophils were isolated by dextran sedimentation, hypotonic lysis, and density gradient centrifugation on Histopaque-1077 (Sigma) as described previously (42). Typically, the polymorphonuclear cells were >90% neutrophils by Wright Giemsa staining and >90% CD16+ as assessed by flow cytometry. Neutrophils were activated in cell culture with PMA (Sigma, 10 μM) for 10 min at 37 °C in 1 ml of HBSS/BSA (Ca2+/Mg2+-free Hanks’ balanced salt solution with 0.2% BSA) at 2 × 109/ml. Activated neutrophils were then washed twice with PBS and incubated in a 100-μl volume of lactose (20 mM) in 100 μg/ml final concentration) at room temperature for 4 h. The treated neutrophils were then used in subsequent experiments.

Flow Cytometry—HL-60 and MOLT-4 cells were examined by flow cytometry. In a FACSCalibur equipped with a 488-nm argon laser (BD Biosciences), cells were incubated with FITC-conjugated Annexin V (Roche Applied Science, 2 μl of conjugate per 106 of resuspended cells, as described by the manufacturer) and PI (Molecular Probes, 1 μg/ml final concentration) at room temperature for 15 min. Into this tube was pipetted 300 μl of HBSS buffer, and the sample was analyzed by flow cytometry in a FACSCalibur. In the case of dGal-1, we used biotinylated forms of the lectins (10 μg/ml) for 30 min at 37 °C. Cells were then washed twice with HBSS before resuspending them in 100 μl of HBSS/BSA for flow cytometric analysis.

Confocal Microscopy—At the end of incubations with galectins, cells were washed with 20 μl of lactose in PBS and incubated for 20 min at 37 °C in PBS with the DNA stain Hoechst 33342 (1 μg/ml, Molecular Probes). These cells were then stained with a mixture of FITC-conjugated Annexin V and PI and analyzed by confocal microscopy on a Leica TCS NT Microscope.

DNA Fragmentation Assays—To detect DNA fragmentation, two approaches were used. In the first approach, cells were subjected to the TUNEL assay. Cells were treated with or without dGal-1 (20 μM for 3–4 h). The TUNEL assay was conducted by incubating the cells for 1 h at 37 °C with 50 μl of TUNEL reaction mixture. The mixture was then washed and analyzed by flow cytometry.

In the second approach, we examined DNA fragmentation by gel electrophoresis. After treatments of cells with galectins or other reagents as described above, 1 × 107 cells were lysed with 1% Nonidet P-40 in EDTA (20 μM), Tris-HCl (50 mM), pH 7.5, and then microcentrifuged to remove pelleted, insoluble material. The supernatants were digested with RNase A and DNase I (10 μg/ml, 25 μg/ml, respectively) for 2 h at 37 °C, followed by digestion with proteinase K (2.5 μg/ml) for 2 h at 37 °C. A half volume of ammonium acetate (10 μl) was added, and DNA was precipitated with 2.5 volumes of absolute ethanol. DNA was recovered by centrifuging at 12,000 × g for 10 min and dissolved in gel loading buffer. Finally, DNA was separated by electrophoresis in a 1.6% agarose gel containing ethidium bromide (44).

Determination of HL-60 and MOLT-4 Cell Growth in the Presence of dGal-1—Desialylated HL-60 cells (1.8 × 106/ml) or MOLT-4 cells (1.5 × 106 cells/ml) were incubated with or without dGal-1 (20 μM) in complete RPMI in 12-well plates (2 ml/well). Cells were collected at 24-h intervals, washed with lactose (20 μM), pelleted by centrifugation, and resuspended in PBS. Live and dead cells were determined by propidium iodide exclusion and counting with a hemacytometer in duplicate assays.

Phagocytosis Assays—The standard macrophage phagocytosis assay was utilized as described (43, 44). Briefly, peritoneal macrophages were cultured in vitro from mice treated with thioglycollate (Difo, 4%), and the isolated macrophages were cultured in a 24-well plate. The macrophage monolayer was washed with PBS, before use. dGal-1 or mGal-1-treated cells, untreated cells (negative control), in vitro aged human neutrophils that had been incubated in vitro for 4 h at 37 °C in HBSS/BSA (positive control), or camptothecin-treated HL-60 cells (positive control) were added with different macrophages and incubated for 1 h at 37 °C. After incubation, each well was washed in ice-cold PBS and treated briefly with trypsin (0.25%) to remove lightly adherent cells. The macrophages were fixed with paraformaldehyde (1.8%), and phagocytosis was microscopically evalu-
ated by counting 200 macrophages/well in each duplicate well and noting those macrophages that had visibly enlarged and contained visibly phagocytosed cells. Results were then expressed as the percentage of macrophages that had phagocytosed cells (% phagocytosis), as described by Fadok et al. (45) and as modified by Zhuang et al. (46).

RESULTS

Binding of dGal-1 and mGal-1 to HL-60 Cells, MOLT-4 Cells, Resting Human Neutrophils, and Activated Human Neutrophils—The human recombinant galectin-1 preparation was purified by affinity chromatography on lactosyl-Sepharose, as described under “Experimental Procedures.” The activity and stability of the purified protein was demonstrated by the quantitative rebinding of the material to a column of lactosyl-Sepharose (data not shown). SDS-PAGE analysis of fractions demonstrated that the protein was apparently homogeneous and migrated with a molecular mass of ~14.9 kDa (data not shown). These results demonstrate that the protein used in the studies below was quantitatively active and pure. Similar purification and activity profiles were obtained with mGal-1, the monomeric mutant form of galectin-1 (data not shown), prepared as described under “Experimental Procedures.” Human dGal-1 at a concentration from 2–80 μM behaves as a dimeric protein on size-exclusion chromatography, whereas mGal-1 behaves as a monomeric species under the same conditions, using experimental approaches previously described (41) (data not shown).

We examined the binding of dGal-1 and mGal-1 to HL-60 cells, a human promyelocytic cell line. Both dGal-1 and mGal-1 bound to HL-60 cells, but enzymatic desialylation of the cells significantly enhanced the binding of both lectins (Fig. 1, A and B, respectively). The binding of both dGal-1 and mGal-1 was inhibitable by lactose. We confirmed the effectiveness of enzymatic desialylation by examining the binding of the sialic acid-specific lectin MAL, which recognizes NeuAcβ2-3Galβ1-4GlcNAc-R linkages (43). Enzymatic desialylation of HL-60 cells (dsHL-60 cells) decreased the binding of MAL (Fig. 1C). These results demonstrate that both dGal-1 and mGal-1 bind equivalently to HL-60 cells and that enzymatic desialylation of the cells exposes more binding sites for both lectins. We then assessed the binding of dGal-1 to normal human leukocytes. For these studies we used resting neutrophils, enzymatically desialylated resting neutrophils, and fMet-Leu-Phe-activated neutrophils. dGal-1 bound to resting neutrophils, but it bound at higher levels to activated neutrophils (Fig. 1D). The binding of dGal-1 was largely inhibitable by inclusion of lactose (20 mM) (Fig. 1D). dGal-1 binding was enhanced to enzymatically desialylated resting neutrophils (Fig. 1D). Similar results were obtained using mGal-1 (data not shown).
shown). The enzymatic desialylation of resting neutrophils was effective, as shown by the higher binding of MAL to resting neutrophils than to enzymatically desialylated resting neutrophils (Fig. 1E). We also observed that activation of neutrophils without treatment of the cells with neuraminidase, also slightly reduced their binding to MAL, consistent with a possible loss or redistribution of sialic acid-containing glycoconjugates upon activation (Fig. 1E). In control studies, we confirmed the activation of neutrophils, because activation resulted in up-regulation of MAC-1 expression and down-regulation of L-selectin expression (47) (Fig. 1F). These results demonstrate that dGal-1 binds to resting neutrophils but binds slightly more to activated neutrophils.

**PS Exposure on Leukocytes Induced by Treatment with dGal-1**—Based on observations by others that dGal-1 appears to induce apoptosis in some leukocytes (31–34), we assessed whether dGal-1 could induce apoptosis of neutrophils by assessing the staining of cells by Annexin V. Apoptosis is often accompanied by increased exposure of cell surface PS, which is recognized by Annexin V (35–37). dGal-1 treatment of resting human neutrophils did not significantly induce PS exposure (Fig. 2A). In addition, dGal-1 treatment of enzymatically desialylated resting neutrophils, while increasing their binding to dGal-1 (Fig. 1D), did not significantly induce surface exposure of PS (Fig. 2A). By contrast, dGal-1 treatment of fMet-Leu-Phe-activated neutrophils markedly enhanced their staining with Annexin V (Fig. 2B). Lactose inhibited the effects of dGal-1 on activated neutrophils (Fig. 2B), demonstrating that carbohydrate ligands on the cell surface are required for the dGal-1-induced staining of these cells with Annexin V (Table I and Fig. 2, A and B). dGal-1-treated neutrophils stained with Annexin V remained impermeable, as demonstrated by the lack of staining with PI (Fig. 2B), demonstrating that changes induced by dGal-1 are not associated with membrane leakage. These results demonstrate that dGal-1 induces surface PS exposure in activated but not resting neutrophils.

Although human neutrophils required activation but not enzymatic desialylation in order for dGal-1 to induce surface PS exposure, HL-60 cells and the human T leukemic MOLT-4 cells responded differently. dGal-1 treatment of HL-60 and MOLT-4 cells induced PS exposure in a lactose-inhibitable manner, but enzymatic desialylation of the cells markedly enhanced their sensitivity to dGal-1-induced PS exposure (Table I). As observed for HL-60 cells, we found that enzymatic desialylation of MOLT-4 cells enhanced their binding to dGal-1 and mGal-1 (Fig 1, A and B, and data not shown). Both HL-60 cells and MOLT-4 cells treated with dGal-1 and induced to stain with Annexin V remained intact and impermeable, as demonstrated by the lack of staining with PI (data not shown). Thus,
dGal-1 induces PS exposure in both HL-60 and MOLT-4 cells and enzymatic desialylation of the cells enhances their sensitivity to the lectin. 

To further assess the ability of dGal-1 to induce PS exposure on treated cells, we used confocal microscopy to analyze dsHL-60 cells and resting and activated human neutrophils treated with dGal-1. Representative fields of dsHL-60 cells and activated human neutrophils staining with Annexin V follow-treatment with dGal-1. Representative fields of dsHL-60 cells and resting and activated human neutrophils on treated cells, we used confocal microscopy to analyze flow cytometry. The data shown represent averages of duplicate determinations where the S.E. was <5%.

**Table I**

| Treatment, Experiment 1 | % of cells staining with Annexin V<sup>a</sup> | Activated neutrophils |
|-------------------------|---------------------------------------------|----------------------|
|                         | HL-60 | dsHL-60 | MOLT-4 | dsMOLT-4 |
| No treatment (control)  | 7.1   | 5.1     | 2.9    | 7.8      |
| dGal-1                  | 44.2  | 71.1    | 11.6   | 50.1     |
| dGal-1 plus lactose     | 7.5   | 11.2    | 1.3    | 11.5     |
| mGal-1                  | —     | 6.4     | —      | —        |
| mGal-1 plus lactose     | —     | 5.1     | —      | —        |

<sup>a</sup> HL-60 cells or MOLT-4 cells (10<sup>6</sup> cells) (either untreated or desialylated by neuraminidase treatment, ds) were treated with dGal-1 (20 µM) or mGal-1 (20 µM) in the presence or absence of lactose (20 mM) for 4 h at 37 °C. The cells were then washed with lactose (20 mM) and stained with Annexin V-FITC and PI. The dashed line signifies lack of treatment. The percentage of Annexin V-positive/PI-negative cells was determined by flow cytometry.
Lack of DNA Fragmentation Induced by dGal-1 Treatment of HL-60 Cells, MOLT-4 Cells, and Activated Human Neutrophils—The dGal-1-induced exposure of PS in cells without cell shrinkage suggested that dGal-1 treatment does not induce apoptosis. To further explore whether apoptosis might be induced, we looked for evidence of DNA fragmentation. dGal-1 treatment did not induce DNA fragmentation in dsHL-60 and dsMOLT-4 cells, as measured by the TUNEL assay (Fig. 6A).
By contrast, treatment of HL-60 and MOLT-4 cells with camptothecin induced DNA fragmentation (Fig. 6A). Similarly, no DNA fragmentation was detected in activated human neutrophils treated with dGal-1 (Fig. 6B), although significant DNA fragmentation was induced in cells treated with anti-Fas (Fig. 6B). As another method to assess DNA fragmentation, we examined dGal-1-treated dsHL-60 cells and activated human neutrophils for DNA laddering by acrylamide gel electrophoresis and ethidium bromide staining. Camptothecin treatment of HL-60 cells and anti-Fas treatment of activated human neutrophils induced DNA laddering, but treatment with dGal-1 did not (Fig. 6C). These results demonstrate that dGal-1 does not induce DNA fragmentation in these cells.

Normal Growth of dGal-1 Treated HL-60 and MOLT-4 Cells—The lack of DNA fragmentation indicated that dGal-1 does not induce apoptosis. To further critically test this conclusion, we examined the growth of both HL-60 cells and MOLT-4 cells in the continuous presence of dGal-1 over 2 days. Desialylated cells were treated with dGal-1 (20 μM) before being placed in growth media. The treated and untreated cells were assessed for PS exposure by staining with Annexin V after 6 h of incubation in the growth media. Annexin V stained ~60% of the cells treated with dGal-1, similar to that seen in Table I, whereas untreated cells were not significantly stained with Annexin V (data not shown). As positive controls, dsHL-60 cells were treated with camptothecin and dsMOLT-4 cells were treated with etoposide, which is also an inhibitor of topoisomerase and is an inducer of apoptosis. The cells were then examined for growth in the continuous presence of dGal-1 (20 μM) over a 72-h period. The growth rates for dsHL-60 cells and dsMOLT-4 cells were unaffected by dGal-1 treatment (Fig. 7). By contrast, there was quantitative apoptosis and cell loss for cells treated with either camptothecin (Figs. 6A and 7A) or etoposide (Fig. 7B). The normal growth rates and the lack of DNA fragmentation observed in these cells treated with dGal-1 demonstrate that dGal-1 does not induce cell death.

dGal-1 Treatment of HL-60 Cells Enhances Their Phagocytosis by Macrophages—The surface exposure of PS on cells as measured by Annexin V staining has been associated with their phagocytosis by macrophages (52–56). A standard assay for phagocytosis is to place cells in contact with a macrophage monolayer, allow phagocytosis to proceed, and then remove the treated cells and count the numbers of macrophages with visibly engulfed cells (45). To assess whether treatment of cells with dGal-1 promotes their phagocytic recognition, dsHL-60 cells or resting or activated human neutrophils were incubated...
with dGal-1 or mGal-1. As positive controls, we used HL-60 cells incubated with camptotheacin and in vitro aged human neutrophils, which spontaneously undergo apoptosis that is correlated with Annexin V staining. The cells were washed with lactose to remove galectin-1 following treatment and then immediately placed in chambers containing activated mouse peritoneal macrophages. dGal-1-treated dsHL-60 cells, camptotheacin-treated dsHL-60 cells, and in vitro aged human neutrophils were phagocytosed by activated macrophages (Fig. 8A). Inclusion of lactose inhibited the dGal-1-induced effects and reduced measurable phagocytosis. Treatment of activated neutrophils with dGal-1 stimulated their phagocytosis by activated macrophages to a level comparable to that observed with in vitro aged neutrophils (Fig. 8B). The inclusion of lactose during treatment of the cells with dGal-1 blocked the ability of dGal-1 to signal changes resulting in subsequent phagocytosis (Fig. 8B). Importantly, mGal-1 treatment of dsHL-60 cells (Fig. 8A) or activated human neutrophils (Fig. 8B) did not promote their phagocytic recognition, indicating that galectin dimerization is important in signaling the changes leading to phagocytic recognition. This lack of induction by mGal-1 of phagocytic recognition of the cells correlated with its inability to induce PS exposure of treated cells (Table I). Thus, dGal-1 treatment of dsHL-60 cells and activated human neutrophils induces membrane surface changes that are recognized by activated macrophages, causing the cells to be phagocytosed.

**DISCUSSION**

Our study shows that interaction of dGal-1 with carbohydrate determinants on leukocytes exposes binding sites for Annexin V, which binds with high affinity (−1 μM) and specificity to phosphatidylserine (PS) (57, 58). Importantly, dGal-1-enhanced exposure of Annexin V binding sites on HL-60 cells, MOLT-4 cells and activated, but not resting, human neutrophils is not associated with apoptosis. Our results demonstrate, however, that the exposure of binding sites for Annexin V is associated with enhanced phagocytic recognition of the dGal-1-treated cells. Thus, the effects of dGal-1 on these cells are non-apoptotic, but prepare cells for phagocytic recognition. A key finding of our study is that activation of neutrophils is required for the dGal-1-induced surface exposure of PS and corresponding binding of Annexin V. The mechanism by which neutrophil activation enhances dGal-1 binding and signaling is likely to be complex. Among the many possibilities are movement of new receptors to the cell surface or enhanced affinity of existing membrane receptors for dGal-1.

We considered the obvious possibility that neutrophil activation results in desialylation of existing glycoproteins, thereby enhancing binding of dGal-1 and promoting the induction of PS exposure. It was previously proposed that neutrophil activation is accompanied by cell surface desialylation that is associated with mobilization of an endogenous sialidase to the plasma membrane, as evidenced by blocking the effects with a specific inhibitor of neuraminidase, 2,3-dehydro-2-deoxy-N-acetylmuraminic acid (59). However, we found that inclusion of this inhibitor did not alter dGal-1 binding to or signaling of activated neutrophils (data not shown). Our results are consistent with those of others (60), who showed that activation of neutrophils in the presence of a sialidase inhibitor did not suppress the binding of *M. amurense* lectin II, which recognizes surface sialic acid. Clearly, much remains to be done in identifying the dGal-1 ligands on leukocytes and the precise mechanisms of PS exposure induced by dGal-1 in cultured cells and activated neutrophils.

dGal-1-induced exposure of PS on HL-60 and MOLT-4 cells is enhanced by enzymatic desialylation of the cells, suggesting that sialic acid is likely to be a capping glycan for the dGal-1 ligands in these cells. Preliminary results also suggest that PS exposure induced in HL-60 cells by dGal-1 requires extracellular Ca\(^{2+}\). This is interesting, because treatment of Jurkat T-cells with human dGal-1 increases their intracellular [Ca\(^{2+}\)] (61). dGal-1-induced exposure of PS likely requires cross-linking of membrane receptors, because monomeric mGal-1 binds similarly to cells, but does not induce PS exposure. This is the initial demonstration that the signaling effects of human dGal-1 on cells require a dimeric form of the protein. Taken together, the different effects of dGal-1 on activated neutrophils *versus* desialylated cell lines indicates that there are different mechanisms operative in human neutrophils *versus* HL-60 and MOLT-4 cells in terms of their responsiveness to dGal-1 and possibly in regard to the ligands recognized by dGal-1. Future studies will be focused on identifying the potentially different mechanisms and endogenous ligands for dGal-1 in human neutrophils *versus* cell lines.

It has been reported that dGal-1 can induce cells to undergo apoptosis (31, 33). However, the potential pro-apoptotic property of dGal-1 remains unclear in the literature. For example,

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3 S. Karmakar, M. Dias-Baruffi, H. Zhu, R. D. Cummings, and R. P. McEver, unpublished observations.
FIG. 6. dGal-1 does not induce DNA fragmentation in dsHL-60 and dsMOLT-4 cells and activated neutrophils. A, TUNEL assay of dsHL-60 and dsMOLT-4 cells. dsHL-60 and dsMOLT-4 cells (10^6 cells) were either left untreated (top panels) or treated in complete RPMI at 37 °C with dGal-1 (20 μM) (bottom panels) for 8 h. Middle Panels, HL-60 or MOLT-4 cells (10^6 cells) were treated with camptothecin (15 μM) for 4 h. All cells were then fixed in PBS-buffered 1% paraformaldehyde, permeabilized with 70% ethanol on ice, stained for DNA fragmentation by the TUNEL assay, washed, and analyzed by flow cytometry. The region designated M1 represents positive staining. The experiments were performed in duplicate, but only one experimental profile is shown, and the S.E. values are indicated.

B, TUNEL assay of activated neutrophils. fMet-Leu-Phe-activated neutrophils (10^6 cells) were either left untreated or treated in complete RPMI at 37 °C with dGal-1 (20 μM) or anti-Fas (100 ng/ml) for 8 h. Neutrophils were activated by treatment with fMet-Leu-Phe (1.0 μM) for 10 min at 37 °C in 1 ml of HBSS/HSA. The cells were fixed in PBS-buffered 1% paraformaldehyde, permeabilized with 70% ethanol on ice, and stained for DNA fragmentation by the TUNEL assay. The cells were then washed and analyzed by flow cytometry. The region designated M1 represents positive staining. The experiments were performed in duplicate, but only one experiment profile is shown, and the S.E. values are indicated.

C, dsHL-60 cells (10^7 cells) were either left untreated or treated with dGal-1, as in A. Resting neutrophils (10^7 cells) were treated with anti-Fas as in B. DNA laddering was examined by gel electrophoresis as described under “Experimental Procedures.” DNA size markers are indicated. The results are representative of three independent experiments.
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![Graph](image)

**Fig. 7.** Treatment of cells with dGal-1 does not cause cell death. Growth curves of dsHL-60 (A) and dsMOLT-4 (B) cells upon various treatments are shown. dsHL-60 cells (1.8 × 10⁵/ml) and dsMOLT-4 cells (1.5 × 10⁵/ml) were treated with 20 μM dGal-1 in the presence or absence of 20 mM lactose and cultured in complete RPMI in a 12-well plate (2 ml/well). (Approximately 70% of the dsHL-60 cells and 55% of dsMOLT-4 were stained with Annexin V within 6 h of this treatment.) Untreated dsHL-60 or dsMOLT-4 cells were used as negative controls. dsHL-60 cells treated with camptothecin (20 μM) or dsMOLT-4 treated with etoposide (20 μM) were used as positive controls. At various times, cells were washed with lactose (20 mM) in RPMI, and the cell numbers were determined by direct counting in a hemacytometer. Greater than 90% of all cells were intact as determined by trypan blue stain staining. The results shown are duplicate analyses, and the ranges are indicated by the error bars. The results are representative of three independent experiments.

One study reported that dGal-1 does not induce apoptosis of CEM cells (33), a T lymphoblastoid cell line, as demonstrated by lack of cell staining with 7-aminoactinomycin D. In another study it was concluded that dGal-1 induces apoptosis in CEM cells based on an induction of Annexin V staining in the treated cells (62). Similarly, it was reported that MOLT-4 cells are stimulated to undergo apoptosis upon treatment with dGal-1, primarily based upon staining with Annexin V (34, 62). In studies on other cells it was observed that dGal-1 treatment induced Annexin V staining, which was interpreted to represent apoptosis (32, 62, 63). Although we also found that dGal-1 induces PS exposure and Annexin V staining of MOLT-4 cells, our analysis demonstrated that this induction is not accompanied by DNA fragmentation or loss of cell viability (Table I and Figs. 6 and 7). Although exposure of Annexin V binding sites has been used as a marker of apoptosis (35, 36, 62), PS exposure is not necessarily associated with other features of apoptosis, such as DNA fragmentation, caspase activity, or cytoskeletal organizational changes (46, 64-67). In addition, it has been reported that PS exposure can be induced by N-ethylmaleimide treatment of cells without inducing other indicators of apoptosis and that this induction causes phagocytic recognition of the cells by macrophages (64). Interestingly, Shi et al. (16) showed that, in a rat endotoxemia model, Kupffer cells could phagocytose neutrophils that did not display DNA fragmentation, but stained in vivo with Annexin V, suggesting that neutrophils ingested by Kupffer cells were not apoptotic. In a similar fashion, our results show that dGal-1 induces PS exposure on leukocytes (Table I and Figs. 6 and 3) without DNA degradation (Fig. 6) and cell loss (Fig. 7) and support our conclusion that these cells were being phagocytosed independently of apoptotic events. Taken together, these data also show that the appearance of Annexin V binding sites is not a reliable marker of apoptosis.

In addition, the effects of dGal-1 on leukocytes in terms of inducing PS exposure independently of apoptosis may be related to recent findings on aged human platelets. The loss-of-function in plasma-derived platelets is associated with biochemical and morphological features resembling granulocyte apoptosis, including cytoplasmic condensation, plasma membrane changes, including exposure of PS, and recognition by phagocyte scavenger receptors via a novel caspase-independent process (68).

![Graph](image)

**Fig. 8.** dGal-1 induces murine macrophage phagocytic recognition of dsHL-60 cells and activated neutrophil. A. dsHL-60 cells (3 × 10⁵) were left untreated (negative control) or treated with dGal-1 (20 μM) or mGal-1 (20 μM) in the presence or absence of lactose (20 mM) for 6 h. All cells were then washed with lactose (20 mM). As positive controls, both HL-60 cells treated for 4 h with camptothecin (15 μM) and in vitro aged human neutrophils (24 h) were used. All cells were washed in RPMI, placed in complete RPMI, and then added to each well containing macrophages and incubated for 1 h at 37 °C. The percentage of macrophages that had visibly phagocytosed cells was determined by counting 200 macrophages/well. Results are expressed as the percentage of macrophages that had visibly phagocytosed cells (% phagocytosis), as described by Fadok et al. (45) and as modified by Zhuang et al. (46). Similar results were obtained in three different experiments with duplicate samples. B, activated neutrophils (3 × 10⁶) were treated with dGal-1 (20 μM) or mGal-1 (20 μM) in the presence or absence of lactose (20 mM) for 2 h. After treatment the cells were washed by lactose (20 mM), washed in RPMI, and placed in complete RPMI. The phagocytosis of these untreated or treated cells was determined as for A. Results shown are the means of duplicate wells, and the ranges are indicated by the error bars.
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PS to the outer leaflet and equilibrates its distribution (73), and aminophospholipid translocase, which moves PS from the outer to the inner leaflet (52). Increases in intracellular Ca2+ up-regulate phospholipid scramblase but down-regulate aminophospholipid translocase, but other factors may also be important. Scramblase-associated elevations of surface PS exposure can be induced by different pathways involving either Ca2+ or apoptosis (74).

Our results indicate that the PS exposure on HL-60 cells induced by dGal-1 is reversible. However, the exposure of PS during apoptotic death is irreversible, due to decreased backward movement of PS to the inner leaflet of the lipid bilayer (75). The mechanism of the reversibility of PS exposure seen in dGal-1-treated cells is presently unknown. Frasch et al. (76) recently demonstrated that scramblase activity is transiently active in non-apoptotic, activated neutrophils, although there is no evidence that this transient activation results in significant PS exposure. This transient activation of scramblase may be involved in the reversible effects of PS exposure induced by dGal-1, but further studies will be needed to evaluate the role of scramblase or phospholipid translocase in dGal-1 induction of PS exposure.

It is interesting that we observed a punctate staining pattern of Annexin V in dGal-1-treated cells and activated human neutrophils treated with dGal-1 (Fig. 3). Others have also observed a unique punctate pattern of Annexin V staining in dGal-1-treated MOLT-4 and CEM cells (34). The nature and function(s) of this clustering of PS in dGal-1-treated cells, as revealed by Annexin V staining, is unknown. Clearly, the clustering does not result from the simple binding of Annexin V to PS, because, in cells stimulated to undergo apoptosis by anti-Fas or camptothecin treatment (Fig. 3), there is exposure of PS but no punctate staining observed with Annexin V. It will be interesting in future studies to identify the cause and consequence of the PS clustering induced by dGal-1 treatment.

Our studies of galectin-1 were prompted by efforts to better understand factors regulating the turnover of human neutrophils. Selectins, integrins, and other adhesion molecules are required for neutrophil adhesion, activation, and extravasation from the circulation (77), after which extravasated neutrophils are removed by tissue macrophages (78). There is substantial evidence that macrophages recognize and phagocyte apoptotic neutrophils in vitro, leading to the proposition that apoptosis initiates neutrophil turnover in vivo (79, 80). However, the role of apoptosis in neutrophil turnover is uncertain. Neutrophils lacking detectable DNA fragmentation can be phagocytosed by Kupffer cells in vivo (16). Fas/FasL can stimulate neutrophil apoptosis in vitro, leading to macrophage recognition and engulfment, but mice deficient in FasL (gld) or Fas (lpr) have normal levels of circulating mature granulocytes (8, 9). Neutrophils lose expression of bcl-2 as they mature, but in transgenic mice expressing bcl-2 in mature neutrophils, spontaneous apoptosis of circulating cells is inhibited; yet, homeostasis of the neutrophil population is unaffected and macrophage-mediated phagocytosis of neutrophils is near normal (13). Giudice et al. (10) reported that neutrophil homeostasis was maintained in the Fas-defective mice. In addition, there is increasing evidence that Fas or FasL may actually promote inflammatory responses in vivo (11, 81). All of these studies suggest that non-apoptotic mechanisms may be important in regulating leukocyte turnover.

Our results raise the possibility that dGal-1 is involved in leukocyte turnover and thus serves an anti-inflammatory role. dGal-1 is expressed in the basement membrane and extracellular matrices of most tissues, and is specifically concentrated around blood vessels (20). Inflammatory cytokines also increase the synthesis of dGal-1 in vascular endothelial cells (21). Thus, activated leukocytes might encounter dGal-1 during their migration from the circulation through the basement membrane to the extravascular space at sites of inflammation. Of interest in this regard, galectin-1 and other galectins may be useful therapeutically in regulating inflammation (82, 83). Galectins and possibly other factors may initiate PS exposure of extracellular neutrophils, leading to their turnover by tissue macrophages in vivo. The gross phenotypes of mice deficient in galectin-1 so far appear normal (84, 85), although sensory olfactory axons of galectin-1-deficient mice failed to project to their correct target sites in the caudal olfactory bulb (25). However, the responses of galectin-1-deficient mice to inflammatory stimuli or the turnover of neutrophils in inflamed tissues have not been studied. Mice deficient in galectin-3 mobilize fewer neutrophils into the peritoneum in response to an inflammatory challenge compared with normal mice (86). The binding of dGal-1 and perhaps other galectins to migrating leukocytes may induce PS exposure that targets their phagocytic removal by tissue macrophages, thus limiting the inflammatory response. Our finding that dGal-1 primes activated leukocytes for phagocytosis suggests a possible pathway to maintain leukocyte homeostasis that functions independently of apoptosis. Whether dGal-1 or related proteins directly regulate neutrophil turnover in vivo should be explored in future studies.

Acknowledgments—We thank Drs. Paul Kincade, A. Kwame Nyame, Ron Bowditch, Joan Conaway, and James Morrissey for helpful discussions. We also thank Jim Henthorn from the Flow Cytometry and Confocal Microscopy Laboratory, William K. Warren Medical Research Institute, University of Oklahoma Health Sciences Center, for help in the flow cytometry and confocal microscopy experiments and Chanda Hill for help in the galectin purification.

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