Elevated Expression of Phosphatidylserine in the Outer Membrane Leaflet of Human Tumor Cells and Recognition by Activated Human Blood Monocytes

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Abstract

We determined whether the presence of phosphatidylserine (PS) in the outer membrane leaflet of human tumor cells correlated with their recognition by activated human monocytes. Three tumorigenic cell lines, A375 melanoma and A431 and Colo-16 carcinomas, and a normal human epidermal keratinocyte line (NHEK) were incubated with monocytes activated to the tumoricidal state by γ-interferon and lipopolysaccharide. Activated human monocytes bound to and lysed all tumorigenic targets, while the nontumorigenic NHEK were neither bound nor killed. Semi-quantitative analysis of PS in the outer leaflet of the cells revealed that the tumorigenic cells expressed 3-7-fold more PS than did the nontumorigenic NHEK. To determine whether enhanced PS expression on the tumor cells was responsible for their recognition by activated monocytes, NHEK were supplemented with exogenously supplied analogues of PS and phosphatidylcholine. PS-labeled NHEK but not phosphatidylcholine-labeled nor control NHEK bound to activated human monocytes. These results suggest a role for PS in monocyte recognition of tumor cells.

Introduction

The macrophage plays many roles in diverse physiological processes; it recognizes, phagocytoses, and ultimately disposes of effete cells, cellular debris, and foreign invaders (1). Macrophages also play an important role in host defense against cancer (2) and infections (3). Normal, nontumotoxic blood monocytes or tissue macrophages can be activated to become tumoricidal subsequent to interaction with lymphokines, bacterial products, or both (2). Although tumor cells are heterogeneous with regard to many characteristics (4), they seem to share susceptibility to destruction by activated macrophages. In fact, activated macrophages can lyse tumor cells that are resistant to other immune effector cells such as T-cells or natural killer cells or to chemotherapeutic drugs (1, 2). Moreover, activated macrophages can discriminate between tumorigenic cells, which they lyse, and nontumorigenic cells, which they do not, even under cocultivation conditions (5).

Studies in many tumor systems have indicated that macrophage-mediated tumor cell lysis is independent of such tumor cell characteristics as surface receptors, transplantation antigens, tumor antigens, species-specific antigens, cell cycle time, expression of endogenous C type viruses, and metastatic potential (for a review, see Ref. 1). The exact mechanism that regulates macrophage discrimination between normal and tumorigenic cells is not known. The broad spectrum of tumor cell susceptibility to macrophage-mediated lysis suggests, however, that a uniform surface moiety could be involved in target cell recognition.

Membrane phospholipids are known to be asymmetrically distributed between the two leaflets of the bilayer (6–9). Whereas particular membrane phospholipids may show some preference for either leaflet, PS (10) is localized exclusively in the inner leaflet of cells (6–10). The preservation of PS in the inner leaflet of cells may play an important role in cell physiology (11, 12) since its exposure in the outer leaflet of, e.g., sickled RBC (13, 14) is associated with their recognition by mononuclear phagocytes (15). PS is also found in the outer leaflet of activated platelets (16) and regulates hemostasis by serving as a procoagulant surface (11, 12). In addition, RBC expressing PS in the external leaflet (15, 17–19) or liposomes containing PS (20) are rapidly bound to and phagocytosed by macrophages. Collectively, these findings suggest that the maintenance of PS asymmetry in cell membranes represents a homeostatic mechanism that may differentiate normal from abnormal cells.

We have recently reported that tumorigenic, undifferentiated murine erythroleukemia cells express 7–8-fold more PS in their outer leaflet than do their differentiated counterparts (21). These observations are extended here to the human system by using three tumorigenic (melanoma, two squamous cell carcinomas) and one normal (human epidermal keratinocytes) cell line. Activated human blood monocytes bound to all three tumorigenic lines to produce target cell lysis. In contrast, human blood monocytes did not bind to or lyse normal keratinocytes. Semiquantitative analyses of PS in the outer leaflet of the cells by prothrombinase activity (21) showed that tumorigenic cells expressed significantly higher levels of PS than did normal keratinocytes.

Materials and Methods

Reagents. Eagle’s minimal essential medium, HBSS, and fetal bovine serum were purchased from M. A. Bioproducts (Walkersville, MD). Recombinant human γ-interferon was the gift of Genentech, Inc. (South San Francisco, CA). Activated Factor X (Factor Xa), l-serine, and LPS were purchased from Sigma Chemical Co. (St. Louis, MO). Thrombin and the thrombin-sensitive chromophore, S2238, were from Helena Laboratories (St. Louis, MO), and prothrombin (Factor II) was obtained from Calbiochem (San Diego, CA). NBD-PC was purchased from Avanti Polar Lipids (Birmingham, AL). NBD-PS was prepared from NBD-PC by phospholipase D-catalyzed base exchange in the presence of l-serine (22) and purified by thin-layer chromatography. Factor V was isolated from bovine plasma (21) and was activated by incubation with subcatalytic amounts of thrombin (0.02 unit) for 3 min at 37°C. Small unilamellar vesicles were prepared from PC by sonication (20). Radioisotopes were obtained from New England Nuclear (Boston, MA). All the reagents except LPS were free of endotoxins as...
determined by the Limulus amoebocyte lysate assay (sensitivity limit of 0.125 ng/ml; Associates of Cape Cod, Woods Hole, MA).

Cell Cultures. The A375 human melanoma cell line (23) and human squamous cell carcinoma cell lines Colo-16 (24) and A431 (25) were maintained as monolayer cultures in Eagle's minimal essential medium supplemented with 5% fetal bovine serum, sodium pyruvate, nonessential amino acids, 2 mM l-glutamine, 2% vitamin solution, penicillin, and streptomycin (complete Eagle's minimal essential medium). The cells were incubated at 37°C in a humidified atmosphere of 5% CO2 in air. NHEK and keratinocyte growth medium were purchased from Clonetics Corporation (San Diego, CA). All cell lines were examined for and were found to be free of Mycoplasma contamination.

Isolation and Culture of Human Peripheral Blood Monocytes. Mononuclear cells were separated by Ficoll-Hypaque centrifugation fromuffy coats (Gulf Coast Regional Blood Center, Houston, TX) obtained from 400 ml of blood from healthy donors. The monocytes were isolated from the mononuclear cells by counterflow centrifugal elutriation (26) with a Beckman JE-6B elutriation rotor. Fractions containing monocytes were obtained at a speed of 3500 rpm and a flow rate of 42 to 48 ml/min. These fractions were washed with HBSS and were resuspended in complete Eagle’s minimal essential medium. More than 97% of the cells were monocytes as determined by morphology, nonspecific esterase staining, and positive staining with monoclonal anti-human monocyte antibody Leu-M3 (Becton Dickinson, Mountain View, CA). Cell viability was >98% as determined by trypan blue dye exclusion.

Binding Assay. Monocytes (5 × 105/well) were plated into 38-mm2 wells of round-bottomed plates. After 90 min, nonadherent cells were removed by washing. The monocyte monolayers (monocyte purity, >98%) were then incubated at 37°C for 18-24 h with 0.2 ml of medium (control monocytes) or medium containing LPS (0.1 µg/ml) and γ-interferon (10 units/ml) (activated monocytes). Target cells (A375, A431, Colo-16, NHEK) in exponential growth were incubated for 24 h with medium containing 0.3 µCi/ml of [3H]iododeoxyuridine (specific activity, 2200 Ci/mmol). The radiolabeled cells were washed twice with HBSS to remove unincorporated radioisotope and then harvested by brief trypsinization. After washing, 0.1 ml of cells (2 × 105/ml) in medium without serum was added to control or to activated monocyte monolayers. Target cells were also plated into plastic wells as an additional control. The cultures were incubated at 37°C for 30 min and then placed on a Mini-Orbital shaker (Belco Biotechnology, Vineland, NJ). The cells were shaken for 1 min at an instrument setting of 5 and washed twice with PBS. Adherent cells were lysed with 0.1 ml of 0.1 M NaOH. The lysates were absorbed onto cotton swabs and radioactivity was monitored in a gamma counter. Monocytes and target cells were also processed for scanning electron microscopy (27).

Monocyte-mediated Cytotoxicity Assay. Monocyte-mediated cytolysis was assessed by measuring the release of radiation from DNA of target cells as described previously (28, 29). Briefly, monocytes were plated at the density of 1 × 103/38-mm2 well of flat-bottomed Microtest III plates (Falcon Plastics, Oxnard, CA) and allowed to adhere for 1.5 h at 37°C. At that time, nonadherent cells were removed by washing with medium. The purity of the adherent monocytes was >98%. The monocytes were activated as described above. Radiolabeled target cells were harvested as described above and resuspended in medium, and then 105 cells were plated into wells containing adherent monocytes. After 72 h the cultures were washed twice with HBSS. Adherent viable cells were then lysed with 0.1 ml of 0.1 M NaOH. The lysates were absorbed onto cotton swabs and radioactivity was monitored in a gamma counter. The percentage of specific cytotoxicity mediated by monocytes was calculated as

\[
\text{% of specific cytotoxicity} = \frac{A - B}{A} \times 100
\]

where A is cpm in cultures of untreated monocytes and target cells and B is cpm in cultures of activated monocytes and target cells.

Determination of NBD-PS/RBC Standard Curve. RBC collected from healthy donors were washed twice, resuspended in PBS (2 × 105 RBC/ml), and treated with 2 mM pyridyldithioethylamine for 30 min at 4°C to inhibit translocation of NBD-PS (30, 31). The cells were then washed with cold buffer and incubated with increasing concentrations of NBD-PS for 20 min at 4°C. After washing, the amount of cell-associated NBD-PS was quantified by fluorescence. The fraction of PS in the outer leaflet was determined by its ability to be removed by “back-exchange” with small unilamellar acceptor vesicles as described previously (21, 30, 31). Aliquots of these NBD-PS containing RBC were used as a standard for analysis of endogenous PS in the outer leaflet of cells by prothrombinase assay.

Prothrombin-converting Activity Assay. NBD-PS containing RBC (2 × 105/ml), tipuor cells (0.5-1 × 105 cells/ml) and normal cells (1-2 × 105 cells/ml) were incubated at 37°C with CaCl2 (6 mM), Factor Xa (0.2 unit), Factor Va (12 nm), and prothrombin (0.8 unit) in Tris-NaCl buffer (50 mM Tris-120 mM NaCl, pH 7.8; final volume, 600 µl). After 3 min, the reaction was stopped by the addition of EDTA to 15 mM. The thrombin-dependent chromophore S2238 was then added (to 0.4 µm), and the rate of chromogen formation was monitored at 405 nm with a Gilford response spectrophotometer using appropriate kinetic software. The initial rate of thrombin conversion activity, which is directly proportional to the amount of PS present on the catalytic cell surface, was determined from the slopes of the absorbance. The absolute amount of PS present was determined by comparing the rates of thrombin production to the rates generated by known amounts of NBD-PS in RBC. To rule out that the trypsin treatment might produce errors, A375 melanoma cells were incubated with 0.25% trypsin for periods ranging from 1 to 10 min. PS content determined with the prothrombinase activity assay was not affected as compared to attached cells by the trypsin treatment (data not shown).

Insertion of Exogenous Fluorescent PS or PC into NHEK and A375 Melanoma Cells. Target cells were labeled with 0.2 mCi 35Cr for 1 h at 37°C and washed with PBS. To inhibit transmembrane movement of the exogenously added phospholipids, the cells were then incubated with 2 mM pyridyldithioethylamine for 20 min on ice (30, 31). Aliquots of 1 × 105 cells in 2 ml of PBS were mixed with 4 µg of NBD-PS or NBD-PC in 20 µl of ethanol (17). After 30 min incubation at 4°C, the cells were washed and resuspended in PBS. At this time, cell viability was 90% (trypan blue exclusion).

The fraction of cell-associated NBD-lipid remaining in the outer leaflet of the cells was determined as described for RBC (see above) except that 1% bovine serum albumin was substituted for vesicles.

Results

The interaction of tumorigenic or normal human cells with activated monocytes was examined by measuring the ability of monocytes to bind and lyse the target cells. In the first set of experiments, the ability of activated human monocytes to bind to tumorigenic or nontumorigenic cells was determined. As

<table>
<thead>
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<th>Table 1</th>
<th>PS content of the outer leaflet of target cell membrane and binding to and lysis by human monocytes</th>
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<tr>
<td></td>
<td>Binding to activated monocytes (%)</td>
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<tr>
<td>Cells</td>
<td></td>
</tr>
<tr>
<td>A375</td>
<td>48.1 ± 1.8</td>
</tr>
<tr>
<td>A431</td>
<td>23.9 ± 7.4</td>
</tr>
<tr>
<td>Colo-16</td>
<td>36.2 ± 1.8</td>
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<tr>
<td>NHEK</td>
<td>6.9 ± 0.8</td>
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* In vitro binding of tumor or normal cells to activated monocytes was measured after a 30-min incubation period using [3H]iododeoxyuridine-labeled target cells as described in “Materials and Methods.” Data are the mean ± SD of a representative experiment of five.

¹ Target cells (1 × 10⁵) labeled with [3H]iododeoxyuridine were plated into triplicate wells containing activated monocytes. The cultures were terminated 72 h after plating of target cells. Data are the mean ± SE of 3 independent experiments.

² PS content of the outer leaflet was determined by the prothrombinase activity assay as described in “Materials and Methods.” Data are the mean ± SE of 3 independent experiments normalized to cell surface area.

³ Average surface area calculated from measurement of the diameter of 200 cells/group/experiment.
shown in Table 1, all tumorigenic cells exhibited higher binding to activated monocytes than that found for the normal NHEK cells. Control experiments, where target cells were incubated under the same conditions with control-nonactivated monocytes or in the absence of any monocytes, demonstrated low level binding (data not shown). The binding of suspended monocytes to adherent A375 melanoma and NHEK cells and the binding of suspended A375 melanoma and NHEK cells to adherent monocytes was examined by scanning electron microscopy. By 30 min of incubation, human blood monocytes plated onto adherent target cells bound to the A375 melanoma (Fig. 1, A and B) but not to the NHEK cells (Fig. 1C). Similar findings were obtained when suspended target cells were plated onto adherent monocytes. By 30 min of incubation, A375 melanoma cells bound to the monocytes (Fig. 1D), whereas NHEK cells did not (Fig. 1E).

To determine whether binding of macrophages to tumor cells correlates with cytotoxicity, the ability of activated human monocytes to lyse tumorigenic and nontumorigenic target cells was determined. The data in Table 1 show that nontumorigenic NHEK cells were not lysed (2% cytotoxicity), whereas A375 melanoma, A431, and Colo-16 squamous cell carcinoma cells were lysed by activated monocytes with specific cytotoxicities of 51, 22, and 15%, respectively.

The amount of PS present in the outer leaflet of target cell membranes was assessed by measuring the initial rates of thrombin production initiated by standard NBD-PS containing RBC, A375, A431, Colo-16, and NHEK cells simultaneously.

Fig. 1. Scanning electron microscopy of monocyte binding to target cells. (A, B) Monocytes binding to adherent A375 melanoma cells after 30 min of incubation. Note a large number of round monocytes clustering on and around adherent melanoma cells. (C) Monocyte binding to adherent NHEK after 30 min of incubation. Note the lack of monocytes adhering to the NHEK. (D) Binding of suspended A375 cells (arrows) to adherent monocytes after 30 min of incubation. (E) Absence of binding of suspended NHEK to adherent monocytes after 30 min of incubation.
EXPRESSION OF MEMBRANE PS BY TUMOR CELLS

The amount of PS, based on results obtained from the standard curve shown in Fig. 2, was corrected for cell surface area. This compensation for differences in cell size normalized the data to PS density (see Ref. 21). The results shown in Table 1 indicate that the tumorigenic cells express 3–7 times more PS than do nontumorigenic NHEK.

To further investigate the involvement of PS in monocyte-target cell recognition, 51Cr-labeled NHEK and A375 melanoma cells were supplemented with NBD-PS or NBD-PC, and their propensity to be bound by activated monocytes was then assessed. Back-exchange of NBD-lipid-treated cells at the initiation of the binding experiments revealed that approximately 50% of the cell-associated lipid was localized at the outer leaflet. As can be seen in Fig. 3, the binding of NBD-PS-labeled NHEK to macrophages (31%) was markedly higher than that found for NHEK labeled with NBD-PC (12%) or control NHEK (8%) (Fig. 3A). In contrast, no discernible differences in binding to macrophages were found among control A375 melanoma cells (35%) and those labeled with NBD-PS (40%) or NBD-PC (33%) (Fig. 3B).

Discussion

The main function of macrophages is to discriminate between “self” and “altered self” by recognizing, phagocytosing, and disposing of effete cells, cellular debris, and foreign invaders (1). How mononuclear phagocytes discriminate between young and old cells, healthy and damaged cells, and nontumorigenic and tumorigenic cells is not known. Several observations suggest that the expression of PS on the outer membrane leaflet of cells could serve as a recognition moiety for macrophages. For example, the insertion of an exogenous fluorescent PS analogue (NBD-PS) into the outer leaflet of RBC facilitates their uptake by macrophages (17) and clearance from the circulation after i.v. injection (18). Furthermore, experiments using sickled RBC demonstrated that upon removal of oxygen, PS is localized in both the inner and outer leaflets of the membrane (13, 14), and these cells exhibited increased binding to monocytes as compared with that of oxygenated, sickled RBC (PS only in the inner leaflet) (15). Similarly, the recognition of undifferentiated leukemic mouse cells by macrophages could also be due to PS. Terminal differentiation of mouse erythroleukemia cells is associated with a marked reduction of binding to activated mouse macrophages (21, 32) and correlates with a decrease in the expression of PS in the cells’ outer leaflet (21). Collectively, these data suggest that the expression of PS in the outer leaflet of the cell may play a role in recognition and subsequent removal by phagocytes (33).

In the present report, we determined whether a correlation exists between the PS expression and interaction of human tumor cells with activated human monocytes. Our results indicate that by 30 min, all three tumorigenic cell lines bound to activated human blood monocytes, albeit to different degrees. These results may be due to inter- and intratumoral heterogeneity. A375 melanoma cells and A431 squamous carcinoma cells were also lysed by the monocytes. Although Colo-16 squamous carcinoma cells bound to activated monocytes, they were relatively resistant to lysis. These results agree with previous reports (3, 33) showing that all tumorigenic cells bound to activated monocytes but binding does not always result in subsequent lysis (34). In contrast, nontumorigenic NHEK cells did not bind to nor were they lysed by tumoricidal human blood monocytes. Since PS expression in the outer leaflet of the 3 tumorigenic cell lines was 3–7-fold higher than the expression of PS exposed on the outer membrane leaflet of the NHEK cells, these data suggest a correlation between expression of PS on the outer leaflet of target cell membranes and their recognition by monocyte-macrophages.

To investigate whether the inclusion of exogenous PS in the outer leaflet bilayer of NHEK results in their recognition by human monocytes, NBD-PS-labeled NHEK were incubated

Fig. 2. Standard curve generated from NBD-PS-labeled RBC. PDA-treated RBC were labeled with increasing amounts of NBD-PS. The amounts of outer leaflet NBD-PS in these cells were determined by direct fluorescence assay versus the initial rates of thrombin production (correlation of 0.98).

Fig. 3. Binding of suspended NHEK (A) and A375 (B) cells to activated adherent monocytes.
with activated monocytes. NHEK labeled with exogenous NBD-PC served as controls. We used NBD-PC because PC has a positive charge at its N-substitution methylation site, and this is the exact control for NBD-PS with a primary amine that is also strongly positive (NH$_3^+$). The results indicate that the presence of PS, but not PC, on the outer leaflet of NHEK directly correlated with binding to macrophages. Surprisingly, the addition of PS to A375 cells did not increase the already enhanced binding to monocytes. These results might suggest that the recognition of tumor targets by monocytes ensues above a critical threshold, similar to that observed for recognition of PS-containing RBC (18).

In conclusion, the data presented here suggest that PS can serve as a signal for triggering macrophage recognition as manifested by binding to target cells (33). Although the binding site on the macrophage surface responsible for recognition of PS is not known, several possibilities exist. These include the participation of autologous cytophilic antibodies (35, 36), components of the clotting cascade (16), and specific phospholipases (37, 38). These findings do not imply any lack of importance for other and more developed mammalian recognition systems, such as proteins and carbohydrate-regulated processes (1), but do suggest that a relatively simple recognition mechanism involving phospholipids may also be important, a process that has endured at least several steps of evolutionary development.

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References


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