

Phosphatidylserine-dependent phagocytosis of apoptotic glioma cells by normal human microglia, astrocytes, and glioma cells

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Apoptotic cells display signals that trigger phagocytic removal by macrophages or neighboring cells. To better understand the signals triggering phagocytosis of apoptotic glioma cells, and to identify the cells that might be involved in the phagocytic process, U-251 MG glioma cells were made apoptotic by etoposide (25 $\mu\text{g/ml}$) treatment and were incubated with normal human astrocytes (NHA), glioma cells, or microglia. Extent of phagocytosis was assessed by an *in vitro* phagocytosis assay. After 3 h of incubation with apoptotic cells, phagocytes tested were washed to remove nonengulfed cells, then fixed, stained, and counted to determine phagocytosis index (PI). NHA, glioma cells, and microglia all phagocytosed apoptotic, but not nonapoptotic, glioma cells. Microglia, however, had a PI approximately 4-fold higher than did either NHA or glioma cells. Binding of phosphatidylserine (PS) on apoptotic glioma cell membranes by annexin-V inhibited phagocytosis by 90% in both microglia and NHA. The activity of an enzyme (scramblase) that moves PS from the inner cell membrane to the outer cell membrane was also increased in apoptotic glioma cells. These results suggest that a variety of cells present in and near gliomas *in vivo* can remove glioma cells in a PS-dependent scramblase-mediated fashion. Manipulation of scramblase and/or PS

exposure in glioma cells may therefore be a means of triggering phagocytic removal of glioma cells. *Neuro-Oncology* 2, 174-183, 2000 (Posted to Neuro-Oncology [serial online], Doc. 00-016, May 30, 2000. URL <neuro-oncology.mc.duke.edu>)

Apoptosis, or programmed cell death, is a physiologic mechanism designed to eliminate unwanted cells from the body (Raff et al., 1992). Considerable evidence has shown that apoptotic cells can be recognized and engulfed by either surrounding cells (Gobe et al., 1987) or professional phagocytes such as macrophages (Wyllie et al., 1980). Phagocytosis of apoptotic cells has been suggested to be a safe way to remove unwanted cells without exposing the adjacent tissues to inflammatory molecules. For example, the engulfment of apoptotic lymphocytes by macrophages prevents the release of proinflammatory tumor necrosis factor- α and instead stimulates the release of immunosuppressive interleukin-10 from macrophages (Voll et al., 1997). The phagocytic clearance of apoptotic bodies is important in several physiologic events including inflammatory resolution (Savill et al., 1989), thymic involution (Surh et al., 1994), and tissue homeostasis (Savill et al., 1993).

Despite the physiologic importance of phagocytosis of apoptotic cells, the events that trigger phagocytosis remain largely unknown. Phagocytosis appears to require the expression of specific receptors on the phagocytes as well as changes in the membrane of the soon-to-be-engulfed apoptotic cells. The receptors thought to be involved in phagocytic recognition of apoptotic cells are varied and include lectin (Dini et al., 1995), CD14 (Devitt et al., 1998), vitronectin $\alpha_v\beta_3$ /CD36 (Fadok et al., 1998), and ATP-binding cassette 1 systems (Luciani et al., 1996). Changes on the cell membranes of apoptotic cells suggested to be key in inducing phagocytosis are also varied and include the presence of glycosylation (Dini et al., 1995), creation of thrombospondin-1 bind-

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²Abbreviations used are as follows: AT, aminophospholipid translocase; DMEM, Delbecco's modified Eagle's medium; FITC, fluorescein isothiocyanate; LDL, low-density lipoprotein; NBD-PC, 1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]caproyl]-sn-glycero-3-phosphocholine; NHA, normal human astrocytes; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PI, phagocytosis index; PS, phosphatidylserine; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

ing sites (Savill et al., 1992), and loss of membrane phospholipid asymmetry (Fadok et al., 1992). PS,² an anionic aminophospholipid normally located at the inner leaflet of the plasma membrane, is currently the best characterized marker for phagocytic recognition. The exposure of PS at the outer membrane leaflet appears to be a general feature of apoptosis (Martin et al., 1995) and the exposed PS plays an essential role in phagocytic recognition. For example, phagocytosis of erythrocytes and apoptotic lymphocytes can be inhibited by the preincubation of macrophages with liposomes containing PS, but is not affected by liposomes containing other anionic phospholipids such as PC (Fadok et al., 1992). Apoptotic vascular smooth muscle cells pretreated with annexin-V, which specifically binds to PS, are not phagocytosed by normal smooth muscle cells (Bennet et al., 1995). The exposure of PS is attributed to the coordinated activities of AT and the enzyme scramblase in apoptotic cells (Beverly et al., 1999). AT, as its name suggests, specifically recognizes aminophospholipids as its substrates, transferring PS, and to some extent, phosphatidylethanolamine, from the outer leaflet to the inner leaflet (Zachowski et al., 1986). Scramblase, on the other hand, mediates the bidirectional transverse movement of all plasma phospholipids across the membrane bilayer in a nonspecific fashion, including the rather inert PC, which usually resides at the outer leaflet of the membrane (Basse et al., 1996). During apoptosis, scramblase activity is increased, whereas AT activity is suppressed, resulting in PS exposure at the cell surface. Furthermore, the loss of AT activity alone is not sufficient to induce PS appearance during apoptosis when scramblase activity is blocked, suggesting that the induction of scramblase activity is critical for PS exposure (Bratton et al., 1997).

Although the processes of apoptosis and phagocytosis appear to be intimately linked, recent evidence suggests that the pathways regulating apoptosis and phagocytosis are in fact independent and separable. For example, human monocytic THP.1 cells treated with inhibitors blocking PS externalization can still undergo apoptosis without being phagocytosed by macrophages (Zhuang et al., 1998). Conversely, in transgenic mice that overexpress bcl-2, phagocytic removal of aging neutrophils by macrophages occurs even though the apoptosis of neutrophils is blocked (Lagasse et al., 1994). These data suggest that, although apoptosis normally precedes phagocytosis, the removal of cells can proceed in the absence of apoptotic signals.

The potential for removing unwanted cells, such as tumor cells, by phagocytosis is of therapeutic interest, particularly in the treatment of gliomas. Gliomas are among the most malignant tumors, with a median survival rate of less than 2 years. Chemotherapy in conjunction with surgical removal and irradiation to treat gliomas is of marginal use (Hosli et al., 1998). Gliomas, however, are surrounded by NHA and are known to be infiltrated with microglia, the CNS equivalent of macrophages (Lorusso et al., 1997). As both NHA and microglia have been shown to help remove cell debris and myelin in certain CNS disorders (Smith, 1999; Thomas et al., 1992; and Vinorel et al., 1993), the possibility exists that the phagocytic ability of these cells could

be directed toward glioma cells, even in the absence of apoptosis of the glioma. To address this possibility, we first examined whether astrocytes and microglia had phagocytic activity. We then addressed the specificity of the phagocytosis process and the signals involved in triggering phagocytosis of apoptotic glioma cells.

Materials and Methods

Cell Line and Cell Culture

NHAs were obtained from Clonetics (Walkersville, Md.). U-87 MG, U-251 MG, and SF268 cell lines were obtained from Brain Tumor Research Center, University of California at San Francisco. NHAs were cultured in the media made from an AGM Bullet Kit (Clonetics), and the media was changed every other day. Other glioma cells were cultured in DMEM supplemented with 20% bovine calf serum, 20 mM HEPES, and penicillin, streptomycin, and gentamicin (University of California at San Francisco Cell Culture Facility). Cells were maintained in a 37°C, 5% CO₂, humidified incubator.

Microglial Culture

Fresh human brain tissue was obtained from a patient undergoing epilepsy surgery. There was no evidence of neoplastic cells on pathologic examination of the tissue obtained at the time of surgery. Brain tissue was minced and digested for 90 min at 37°C in DMEM containing 0.25% trypsin, 50 µg/ml DNase, and 50 µg/ml gentamicin. The cell suspension was filtered through a 100-µm nylon mesh, and then the cells were pelleted by centrifugation at 1500 rpm for 10 min. The cell pellet was resuspended with 30% Percoll (Sigma, St. Louis, Mo.) in PBS and centrifuged at 17,500g for 30 min to form a gradient. The upper layer of the gradient was discarded, while the central part containing mainly microglia and oligodendrocytes was collected and washed twice with PBS. Cells were then cultured in DMEM with 20% bovine calf serum, 20 mM HEPES, and penicillin, streptomycin, and gentamicin. After 48 h of incubation, nonadherent oligodendrocytes were discarded. Attached microglial cells were washed with PBS 3 times and replenished with fresh culture media. The cells were then positively identified as microglia by the uptake of fluorescent di-acylated LDL (Molecular Probes, Eugene, Ore.) as follows: cells were split and 10⁴ cells were put into a 4-well chamber slide overnight to allow attachment. Then cells were washed twice with PBS, followed by the addition of 15 µg/ml di-acylated LDL in 0.1% bovine serum albumin in DMEM. Internalization of di-acylated LDL was allowed to occur for 3 h at 37°C, after which the cells were washed, fixed in 4% paraformaldehyde, and monitored using a fluorescent microscope for the uptake of fluorescent di-acylated LDL. Only those cells able to internalize di-acylated LDL were used in further studies. All microglial cells in culture displayed an ameboid morphology.

Apoptosis Induction and Measurement

U-251 MG cells were made apoptotic by treatment with 25 µg/ml etoposide (Clontech) for 24 h. NHA, U-87 MG, and SF268 cells were resistant to etoposide-induced apo-

ptosis and were made apoptotic by incubation with 1 mM H_2O_2 for 24 h. Cells were collected and assessed for apoptosis using both annexin-V-FITC binding and TUNEL assays. The annexin-V-FITC binding assay was done using an ApoAlert Annexin V kit (Clontech). After drug treatment, cells were washed with PBS and trypsinized. Cells were rinsed with and then suspended in 1× binding buffer (200 μ l/ 10^6 cells). Five microliters of annexin-V-FITC was then added to the cell suspension and the solution was incubated at room temperature for 15 min in the dark. Binding buffer (1×) was added to bring the final volume to 0.5 ml, and the cell suspension was analyzed by flow cytometry to assess the binding of the fluorescent annexin-V-FITC to the cell surface. For fluorescence microscopy observation of apoptosis, cells were grown directly on glass slides and rinsed with 1× binding buffer. Five microliters of annexin V-FITC and 10 μ l of propidium iodide in 200 μ l of 1× binding buffer were then added onto the slide and incubated for 15 min in the dark. Cells were observed under a fluorescent microscope, with apoptotic cells being considered to be those cells which stained positive for annexin V-FITC at the cell membrane. Propidium iodide, a membrane-impermeable DNA-binding dye, was used to identify the early and late stages of apoptotic cells. Early apoptotic cells, which still have intact membranes, exclude propidium iodide, whereas late apoptotic cells, in which the membrane integrity is partially lost, should stain positive for propidium iodide at the nucleus.

The TUNEL assay was done using a ApoAlert DNA fragmentation assay kit (Clontech) and was based on the ability of terminal deoxynucleotidyl transferase to catalyze the incorporation of fluorescent dUTP-FITC at the free 3'-hydroxy ends of fragmented DNA in the nucleus. Cells were grown on glass slides and, after apoptosis induction, were washed with PBS and then fixed with fresh 4% formaldehyde/PBS at 4°C for 25 min. Cells were then washed with PBS 3 times and permeabilized with prechilled 0.2% Triton X-100/PBS on ice for 10 min. Slides were then washed with PBS 3 times and equilibrated in 100 μ l of equilibration buffer for 10 min at the room temperature. A solution composed of 1 μ l terminal deoxynucleotidyl transferase, 5 μ l of nucleotide mix containing FITC-conjugated dUTP, and 45 μ l of equilibration buffer was then added on a slide. The tailing reaction was performed by placing the slides in a dark humidified 37°C incubator for 60 min. Slides were immersed in 2× saline-sodium citrate to terminate the tailing reaction after which the attached cells were washed with PBS 4 times and stained with propidium iodide (0.5 μ g/ml) or '6-diamidino-2-phenylindole (250 ng/ml) at room temperature for 10 min. Cells were then washed with PBS twice and with water twice. A drop of antifade (2.3% 1,4-diazobicyclo[2.2.2]octane, 90% glycerol in PBS) was added on top of each slide, and cells were observed immediately under a fluorescent microscope to look for the appearance of the green fluorescent dUTP-FITC at the nucleus.

In Vitro Phagocytosis Assay

Two days before the assay, 10^4 of the phagocytes to be tested (NHA, microglia, or glioma cells) were placed into a 35-mm tissue culture dish. Meanwhile, glioma cells

were treated with various apoptosis-inducing agents (etoposide or H_2O_2 as described above). Apoptotic bodies were collected, washed 3 times with PBS, and resuspended in culturing media with serum (4×10^5 cells/ml). One milliliter of apoptotic cell suspension was then added onto each plate containing approximately 10^4 phagocytes. For experiments with annexin-V preincubation, recombinant annexin-V (PharMingen, San Diego, Calif.) at a concentration of 0.25 μ g/ 10^4 cells was added to the apoptotic bodies 30 min before loading apoptotic bodies onto the phagocyte monolayer. Phagocytosis was allowed to proceed in a 37°C humidified incubator for 3 h. The nonphagocytosed cells were then washed off with PBS 5 times. Adherent cells were then fixed with 4% formaldehyde/PBS for 30 min, washed with PBS twice, and then stained with crystal violet solution (0.5% crystal violet in 20% methanol) for visualization under a microscope. A minimum of 100 phagocytes were counted in random fields from at least 4 independent areas of each dish. PI was determined by multiplying the percentage of phagocytes that had engulfed apoptotic bodies by the average number of phagocytosed bodies per phagocyte.

Scramblase Activity

The activity of scramblase was determined by the internalization of fluorescent NBD-PC (Avanti, Alabaster, Ala.) during the course of apoptosis of glioma cells. One microgram of NBD-PC was dissolved in 20 μ l of HEPES-buffered saline (137 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 5 mM glucose, 10 mM HEPES pH 7.4, and 2 mM $CaCl_2$) with 200 μ M fresh phenylmethylsulfonyl fluoride and 0.25% lipid-free bovine serum albumin (Sigma). Cells were collected at different time points after apoptosis induction, washed with PBS once, and resuspended in HEPES-buffered saline (10^7 cells/ml). The cell suspension (50 μ l) was then incubated with 2 μ l of NBD-PC lipid solution for 10 min at room temperature. Cells were pelleted by centrifugation at 3000 rpm for 5 min, and the supernatant was discarded. Albumin extraction of the plasma membrane outer leaflet was performed by adding 50 μ l of 1% bovine serum albumin/HEPES-buffered saline to the cells for 5 min at the room temperature to remove fluorescent lipid that had not entered the cells. Cells were pelleted by centrifugation at 3000 rpm for 5 min and the supernatant was removed. Ice-cold HEPES-buffered saline (0.9 ml) was then added to the cell pellets, and flow cytometry analysis was performed within 2 h to measure the ability of scramblase to transfer the exogenous fluorescent NBD-PC into the cells.

Western Blotting

SF268, U-87 MG, and U-251 MG cells were lysed using radioactive immuno-protection assay solution (1% Nonidet P-40 [NP-40], 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate in PBS) with freshly added phenylmethylsulfonyl fluoride, leupeptin, aprotinin, sodium orthovanadate, and phosphatase inhibitor. Cell lysates were centrifuged at 14,000 rpm for 10 min and the supernatants were collected. Cellular proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis

(25 µg/lane), and the protein samples were transferred onto a nitrocellulose membrane (Millipore, Marlborough, Mass.). The membrane was incubated with the blocking solution (5% nonfat dry milk in Tris-buffered saline with 0.5% NP-40) for 1 h. Then the membrane was incubated with the rabbit antiscramblase antibody (Oncogene, Cambridge, Mass.) at a concentration of 2 µg/ml for 2 h. The membrane was washed 3 to 4 times for 10 min in Tris-buffered saline with 0.5% NP-40. Subsequently, the membrane was incubated with horseradish peroxidase-conjugated secondary donkey antirabbit IgG (1:5000) (Jackson ImmunoResearch, Ft. Lauderdale, Fla.) for 90 min. The membrane was washed 3 times in Tris-buffered saline with 0.5% NP-40 and was then treated with enhanced chemiluminescence reagent (NEN, Boston, Mass.) for 1 min. The proteins were detected by autoradiography. Equal protein loading was confirmed by Coomassie blue staining of the polyacrylamide gel and by Ponceau S staining of the blotted membrane.

Results

Apoptotic Profile of Glioma Cells

Etoposide (25 µg/ml) was used to induce apoptosis of U-251 MG glioma cells, whereas H₂O₂ (1 mM) was used to induce apoptosis in U-87 MG and SF268 cell lines. Both annexin-V-FITC and TUNEL assays were used to measure apoptosis after 24 h of drug administration in various glioma cells. annexin-V-FITC labeling was monitored by both fluorescence microscopy and flow cytometry, whereas the results from TUNEL assay were assessed by fluorescence microscopy. After 24 h of etoposide treatment, apoptotic U-251 MG cells showed strong green fluorescent annexin-V-FITC staining at the plasma membrane (Fig. 1A). Both early (propidium iodide-negative) and late (propidium iodide-positive, with strong nuclear staining of red fluorescence) stages of apoptotic cells were observed (Fig. 1A). Annexin-V-FITC staining was also analyzed by flow cytometry, with the data plotted as cell number (*y*-axis) against the green annexin-V-FITC fluorescence (*x*-axis). After etoposide treatment, U-251 MG cells showed a sharp peak of annexin-V-FITC fluorescence as compared with low annexin-V-FITC intensity in untreated U-251 MG cells (Fig. 1B). TUNEL assay showed that apoptotic U-251 MG cells exposed to etoposide for 24 h incorporated dUTP-FITC and stained green (Fig. 1C, left panel). Similarly, after 24 h of H₂O₂ addition, both annexin-V-FITC and TUNEL assays demonstrated that apoptosis occurred in both U-87 MG cells (Fig. 1D and E, respectively) and in SF268 cells (data not shown). These apoptotic cells were collected and used in *in vitro* phagocytosis assays.

Phagocytosis of Apoptotic Glioma Cells by NHA, Microglia, and Glioma Cells

Next, we assessed the ability of NHA, U-251 MG, and microglial cells to recognize and phagocytose apoptotic U-251 MG cells. PI was calculated as described, and the PI of NHA was normalized to 100% and used as the con-

trol. When incubated with apoptotic U-251 MG cells, both NHA and microglia phagocytosed apoptotic glioma cells (Fig. 2 A and B). We then compared the phagocytic ability of NHA with that of microglia. As shown in Fig. 2C, the PI of microglia was approximately 4-fold higher than that of NHA, demonstrating that microglia were more efficient in phagocytosing apoptotic glioma cells. This is probably due to the ability of microglia to take up more apoptotic bodies per cell than NHA (Fig. 2A and 2B). On the other hand, U-251 MG cells had little phagocytic activity against their apoptotic selves, with a PI of only 4% (Fig. 2D). One possible explanation is that glial cells, including normal cells and cancerous cells, are blocked in their ability to phagocytose apoptotic cells of the same type. Alternatively, glioma cells may lack the ability to phagocytose apoptotic cells. To address the first possibility, NHA were made apoptotic by treatment with H₂O₂ for 24 h at a concentration of 1 mM. Apoptosis was confirmed by both TUNEL and annexin-V binding assays (data not shown). The PI value of NHA engulfing apoptotic NHA, however, was not significantly different from that of NHA phagocytosing apoptotic glioma cells (Fig. 3), ruling out the possibility that glial cells can't phagocytose apoptotic cells of the same type. To investigate whether all glioma cells lack the ability to perform phagocytosis, U-87 MG cells and SF268 cells were presented with apoptotic U-251 MG cells. Both U-87 MG and SF268 glioma cell lines recognized and engulfed apoptotic U-251 MG cells (PI = 54% for SF268 and 62% for U-87 MG), although their ability to phagocytose was not as great as that of NHA (Fig. 4A). In contrast, the U-251 MG cells incubated with a variety of apoptotic cells showed low PI values compared with that of the negative control group (*in vitro* phagocytosis assay of normal U-251 MG cells in the absence of apoptotic cells, Fig. 4B), indicating that U-251 MG cells were unable to perform phagocytosis. These results demonstrate that NHA, microglia, and the glioma cells tested (except U-251 MG cells) were able to phagocytose apoptotic bodies, with microglia being the most efficient, followed by NHA, and then glioma cells. U-251 MG cells were unique in their inability to phagocytose a variety of apoptotic cells.

Phagocytosis by NHA and Microglia is Specific for Apoptotic Cells

As microglia, and to some extent NHA, are considered to be scavenger cells, we questioned whether phagocytosis by these cells was specific to apoptotic cells or was simply a generalized response to dead cells. To address this issue, nonadherent, dead, floating U-251 MG cells were collected after cells were plated. Annexin-V binding and TUNEL assays both showed that these floating cells were nonapoptotic dead cells (data not shown). These nonapoptotic dead cells were then used to test the phagocytosis specificity of NHA and microglia. Fig. 5 shows that both NHA and microglia showed some phagocytic activity against nonapoptotic dead glioma cells. The PI values using nonapoptotic cells were approximately 80% less than the values obtained using apoptotic cells. Thus, NHA and microglia phagocytosed apoptotic cells far better than they phagocytosed nonapoptotic dead cells. The

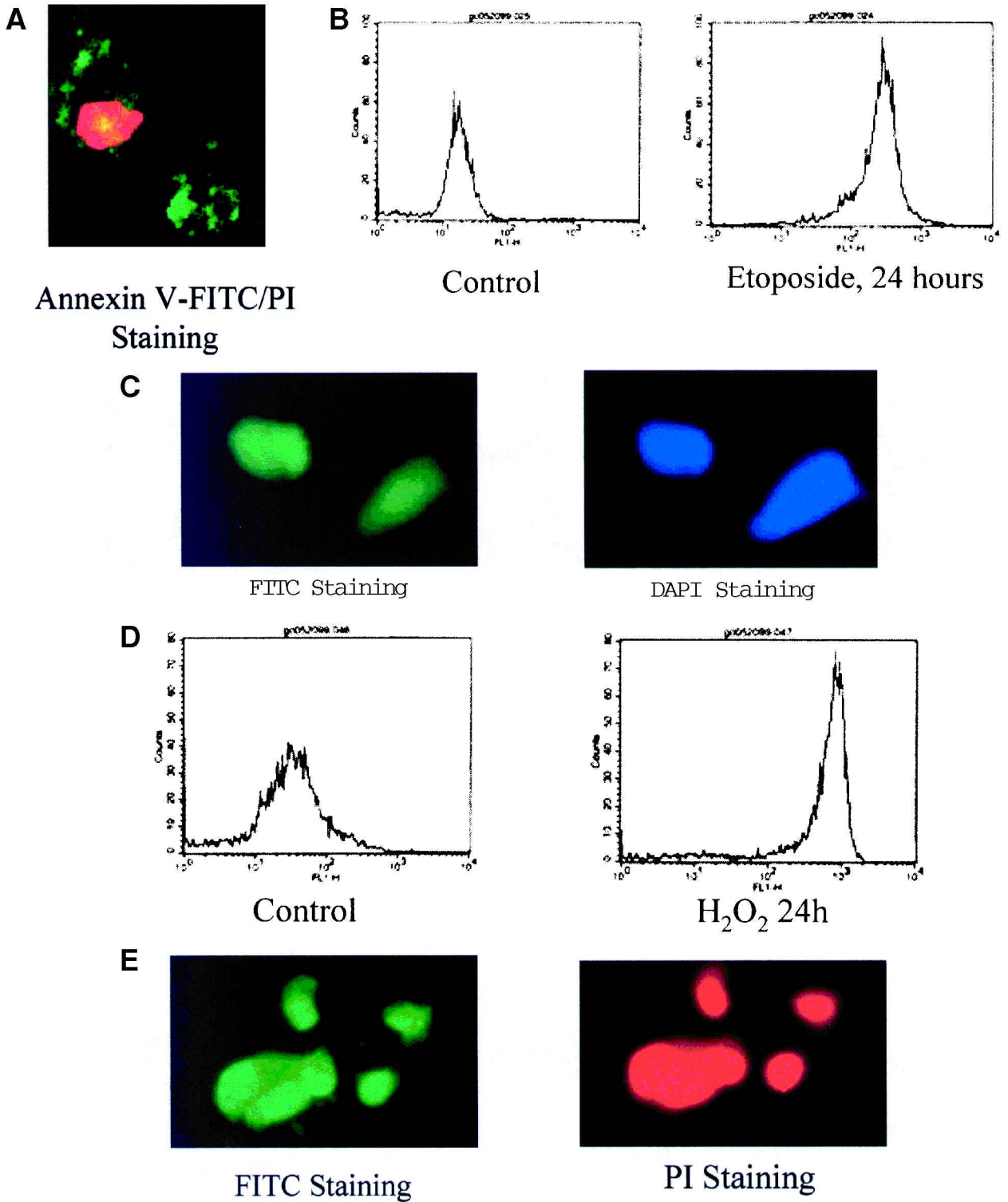


Fig. 1. Apoptotic profile of U-251 MG cells (A-C) and U-87 MG cells (D and E). Etoposide (25 µg/ml) was added to U-251 MG cells and H₂O₂ (1 mM) was added to U-87 MG cells for 24 h to induce apoptosis. Apoptosis was assessed by both annexin-V-fluorescein isothiocyanate (FITC) binding assay (A, B, and D) and terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay (C and E). A. Microscopy observation of U-251 MG cells displaying early (green annexin V-FITC staining) and late (green annexin V-FITC and red propidium iodide staining) stages of apoptosis. B. Flow cytometry analysis of annexin-V-FITC fluorescence in control U-251 MG cells (left panel) and in U-251 MG cells after etoposide treatment (right panel). C. TUNEL assay of etoposide-treated U-251 MG cells (green, left panel). 4',6-Diamidino-2-phenylindole(DAPI) was used as a control for nuclear staining (blue, right panel). D. Flow cytometry analysis of control U-87 MG cells (left panel) and U-87 MG cells treated with H₂O₂ (right panel). E. Microscopy observation of TUNEL staining (green, left panel) in H₂O₂-treated U-87 MG cells. Propidium iodide was used as a control for nuclear staining (red, right panel). PI, phagocytosis index.

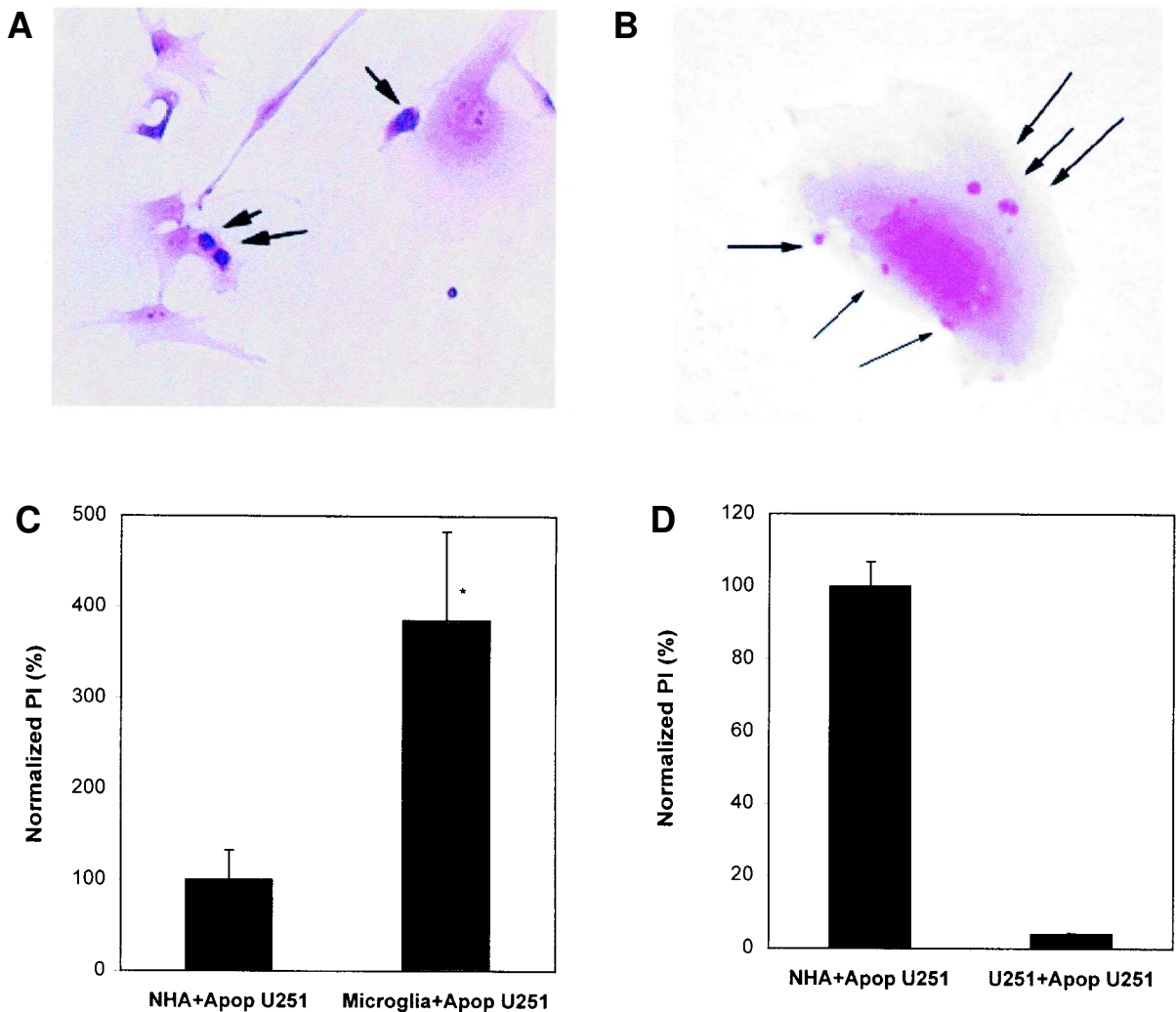


Fig. 2. Phagocytosis of apoptotic U-251 MG cells by normal human astrocytes (NHA), microglia, and normal U-251 MG cells. Apoptotic U-251 MG cells were added to various phagocytes for 3 h. Phagocytes tested were then washed, fixed, stained, and counted to determine the phagocytosis index (PI). The results are expressed as means \pm SD for at least 4 replicate determinations for each experiment. The statistical analysis was done using *t* test. A. Crystal violet staining of astrocytes engulfing dark purple apoptotic U-251 MG cells (arrows). B. Crystal violet staining of 1 microglial cell engulfing 6 apoptotic U-251 MG cells (arrows). C. Phagocytosis of apoptotic U-251 MG cells by NHA and microglia in vitro. *Significantly different ($P < 0.05$) from the NHA group. D. Phagocytosis of apoptotic U-251 MG cells by NHA and normal U-251 MG cells.

phagocytic ability of NHA and microglia is therefore not a generalized response to dead cells.

Effect of PS Exposure on Phagocytosis by NHA and Microglia Cells

Like most apoptotic cells, apoptotic glioma cells exhibit PS at the cell surface (Fig. 1A). If PS appearance at the apoptotic cell surface is required for phagocytic recognition in glial cells, then complexing PS with annexin-V should prevent the phagocytic recognition by NHA and microglia. To assess the role of PS exposure, apoptotic glioma cells were preincubated with recombinant annexin-V protein for 30 min before the in vitro phagocytosis assay. As shown in Fig. 6, the addition of annexin-V inhibited 90% of phagocytosis of apoptotic U-251 MG cells by both NHA and microglia. These results suggest

that the glial phagocytosis involves PS exposure at the membrane of apoptotic cells.

Scramblase Activity and Expression in Glioma Cells

Scramblase activity has been shown to be required for the PS appearance at the apoptotic cell surface, and its expression has been found in various human cell lines (Zhou et al., 1997). We assessed whether scramblase was also expressed in glioma cells and whether scramblase activity was increased during apoptosis. Western blotting of scramblase expression in U-87 MG, U-251 MG, and SF268 cells showed that U-251 MG cells had the highest scramblase protein expression among the glioma cells tested, whereas both U-87 MG and SF268 cells have very little scramblase expression (Fig. 7). Scramblase activity was assessed by measuring its ability to mediate the bidi-

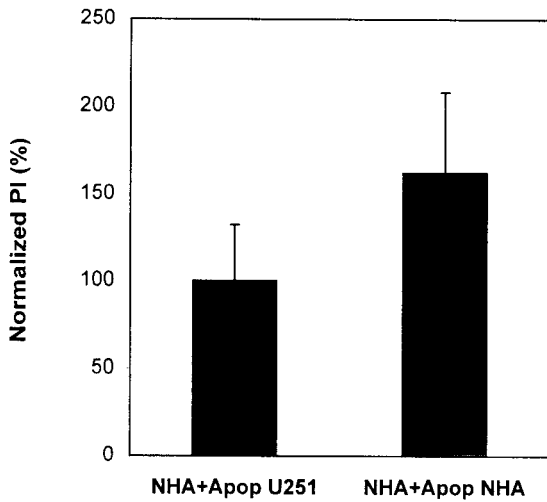


Fig. 3. Glial cells can phagocytose apoptotic cells of the same type. Both U-251 MG cells and normal human astrocytes (NHA) were made apoptotic and were then added to a monolayer of NHA. After 3 h, cells were washed to remove nonengulfed apoptotic cells, fixed, stained, and counted to determine the phagocytosis index (PI). Results are expressed as means \pm SD for at least 4 replicate determinations for each experiment.

rectional transverse movement of plasma phospholipids across the membrane bilayer. Fluorescent NBD-PC was used as the substrate for scramblase and was added exogenously to drug-induced apoptotic cells. The appearance of NBD-PC at the inner leaflet of the plasma membrane within 24 h after administration of the apoptosis-inducing agents was measured by flow cytometry. The cell number (*y*-axis) was then plotted against the fluorescent intensity of NBD-PC inside the apoptotic cells (*x*-axis). As shown in Fig. 8, in U-251 MG cells and U-87 MG cells undergoing apoptosis, scramblase activity was elevated in a time-dependent manner, with an increase of scramblase activity seen at 16 h after drug treatment. These results suggest that the phagocytosis process induced by PS exposure in apoptotic glial cells is associated with increase in scramblase activity.

Discussion

In this study, microglia, NHA, and the glioma cells tested (except U-251 MG cells) were all able to phagocytose apoptotic cells. Moreover, both NHA and microglia selectively phagocytosed apoptotic but not nonapoptotic glioma cells. Among the cells tested, microglial cells were the most effective phagocyte, with an approximate 4-fold higher PI than NHA. In addition, phagocytosis by both NHA and microglia was dependent on the presence of PS at the surface of apoptotic glioma cells. The activity of scramblase, an enzyme crucial in PS exposure, was also elevated during the apoptotic process of glioma cells, implying its involvement in PS appearance. These results suggest that NHA, and to an even greater extent

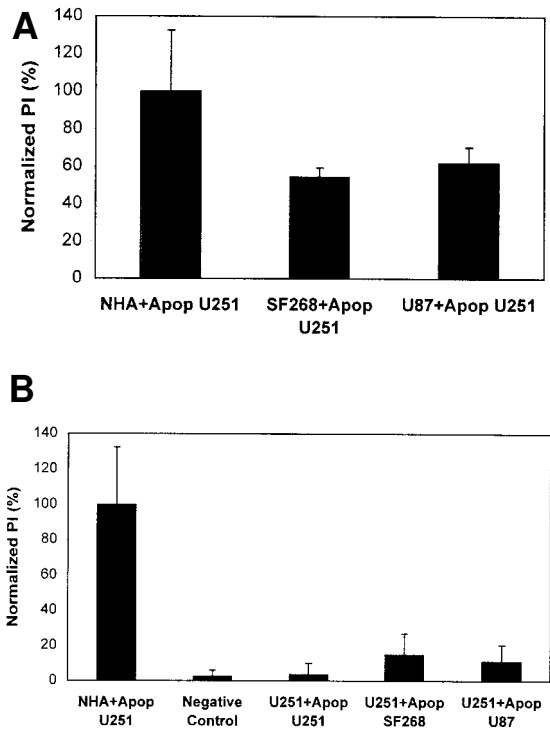


Fig. 4. Phagocytosis of apoptotic cells by U-87 MG cells, SF 268 cells, and U-251 MG cells. A. Apoptotic U-251 MG cells added to either normal U-87 MG cells or SF268 cells to test their phagocytic activity. After 3 h, phagocytes tested were washed, fixed, stained and counted to determine the phagocytosis index (PI) and compared with the PI of normal human astrocytes (NHA) phagocytosing apoptotic U-251 MG. B. U-251 MG cells, U-87 MG cells, and SF268 cells made apoptotic and presented to normal U-251 MG cells. The negative control was done by not adding any apoptotic cells to normal U-251 MG cells. After 3 h, the cells were then washed, fixed, stained, and counted to measure the PI. Results are expressed as means \pm SD for at least 4 replicate determinations for each experiment.

microglia, can remove neighboring apoptotic cells, including malignant glioma cells. The possibility exists that phagocytosis might be effective in removing glioma cells.

If phagocytosis of tumor cells were to be a viable approach in tumor treatment, microglial cells would be a logical effector cell. Microglia have been shown to phagocytose apoptotic neuronal cells *in vitro* with high efficiency (Adayev et al., 1998), and in this report, we note that microglial cells were the most efficient phagocyte among a variety of cells tested. Because all experiments in the present report were performed in *in vitro* conditions, in which microglial cells are known to be activated and capable of phagocytosis, one can argue that microglial cells residing in gliomas *in vivo* may not be in an active state. Indeed, resting ramified microglia, characterized by the loss of macrophage-like properties, are the major type of microglial cells in normal adult brains (Murabe et al., 1982). However, under pathologic conditions such as ischemia, inflammation, and degenerating diseases, ramified microglia can rapidly transform into active amoeboid

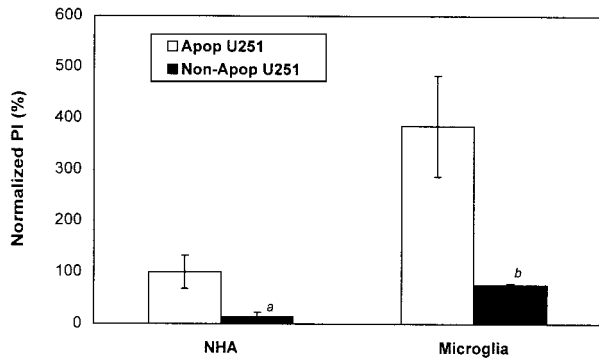


Fig. 5. Enhanced phagocytosis of apoptotic cells versus nonapoptotic cells by normal human astrocytes (NHA) and microglia. Nonapoptotic, dead, floating U-251 MG cells were added to either NHA or microglia. After 3 h, phagocytes were washed, fixed, stained, and counted to determine the phagocytosis index (PI). PI was then compared with the phagocytosis index PI of either NHA or microglia phagocytosing apoptotic U-251-MG cells. The results are expressed as means \pm SD for at least 4 replicate determinations for each experiment. *a*, significantly reduced ($P < 0.05$) from the group of NHA with apoptotic U-251 MG cells. *b*, significantly reduced ($P < 0.05$) from the group of microglia with apoptotic U-251 MG cells.

microglia capable of secreting various cytokines, expressing macrophage markers, and performing phagocytosis (Gehrmann et al., 1995). Because both the resting ramified type and active amoeboid type of microglial cells are present in high-grade gliomas (Roggendorf et al., 1996), it is likely that microglial cells infiltrating gliomas *in vivo* are active and able to phagocytose appropriate targets. The use of phagocytosis in the therapy of tumors has, in fact, been suggested (Ma et al., 1998), although in those studies drug-laden endothelial cells were used in hope of delivering cytotoxic agents to the tumor. An alternative phagocytosis approach might be to alter tumor cells so that they become better targets themselves for phagocytosis.

The present studies suggest that the creation of glioma targets for phagocytosis would involve PS exposure and an increase in scramblase activity. Previous studies suggested that PS externalization is achieved through the balance of activities of AT and scramblase (Bevers et al., 1999). However, recently it was reported that loss of AT activity alone was not enough to result in PS exposure, suggesting that scramblase activation is the driving force for PS appearance upon apoptotic stimulation (Bratton et

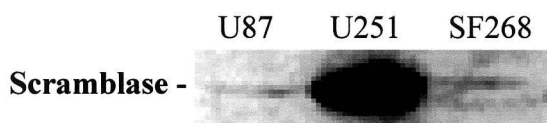


Fig. 7. Western blot of scramblase protein expression in various glioma cell lines. The expression of scramblase protein was analyzed by Western blotting with a rabbit antibody against human scramblase (2 μ g/ml). Each lane contains 25 μ g total protein extract, and equal loading was confirmed by Coomassie blue staining of the polyacrylamide gel and by Ponceau S staining of the blotted membrane.

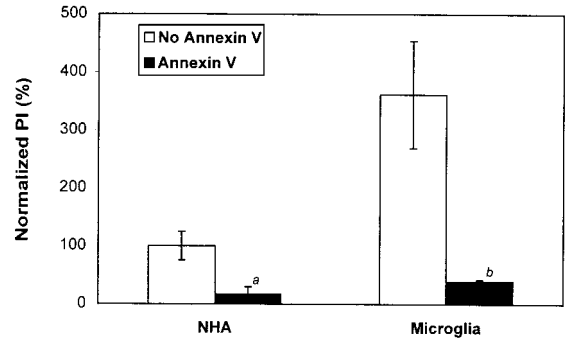


Fig. 6. Phagocytosis by glial cells is dependent on phosphatidylserine (PS) exposure on the apoptotic cells. Annexin-V (0.25 μ g/ 10^5 cells) was added to the apoptotic U-251 MG cells 30 minutes before the addition to the phagocyte monolayer. *In vitro* phagocytosis assay was then performed and phagocytosis index (PI) was measured. The results are expressed as means \pm SD for at least 4 replicate determinations for each experiment. *a*, Significantly reduced ($P < 0.05$) from the group of normal human astrocytes (NHA) without annexin-V incubation. *b*, Significantly reduced ($P < 0.05$) from the group of microglia without annexin-V incubation.

al., 1997). Scramblase is a 35-kDa protein found in a variety of cell lines and different tissues including spleen, thymus, colon, and prostate (Zhao et al., 1998). It mediates the bidirectional transbilayer movement of plasma phospholipids to cause PS exposure when activated. Scramblase activity can be induced by increasing intracellular Ca^{2+} concentration or by acidification of the cells to pH less than 6 (Stout et al., 1997). The level of scramblase expression has been shown to determine the extent of PS exposure upon Ca^{2+} stimulation (Zhao et al., 1998). This suggests that PS appearance at the cell surface could be manipulated by selectively increasing scramblase expression. However, because stimulation of scramblase activity by increasing Ca^{2+} concentration or lowering intracellular pH may be difficult to accomplish selectively under physiologic conditions, selective delivery and expression of a constitutively active scramblase in tumor cells might be a more reasonable approach. Our finding that scramblase was also expressed in glioma cells and activated during apoptosis indicates that phagocytosis can be triggered by manipulating scramblase activity to induce PS exposure at the glioma cell surface. A limited amount of structure/function information exists on scramblase, and a motif that mediates the Ca^{2+} -accelerated phospholipid transbilayer movement has been identified (Zhou et al., 1998). More information about the scramblase protein might allow the creation of an enzyme which, rather than being activated by increasing Ca^{2+} concentration or lowering intracellular pH, is constitutively active. This constitutively active enzyme, if delivered appropriately, could then induce PS externalization and trigger phagocytosis by either the infiltrating microglial cells or the surrounding NHA.

Current therapies against gliomas include surgical removal, irradiation, and chemotherapy. However, the prognosis of glioma patients is still poor, with a survival period less than 2 years for high-grade glioma patients

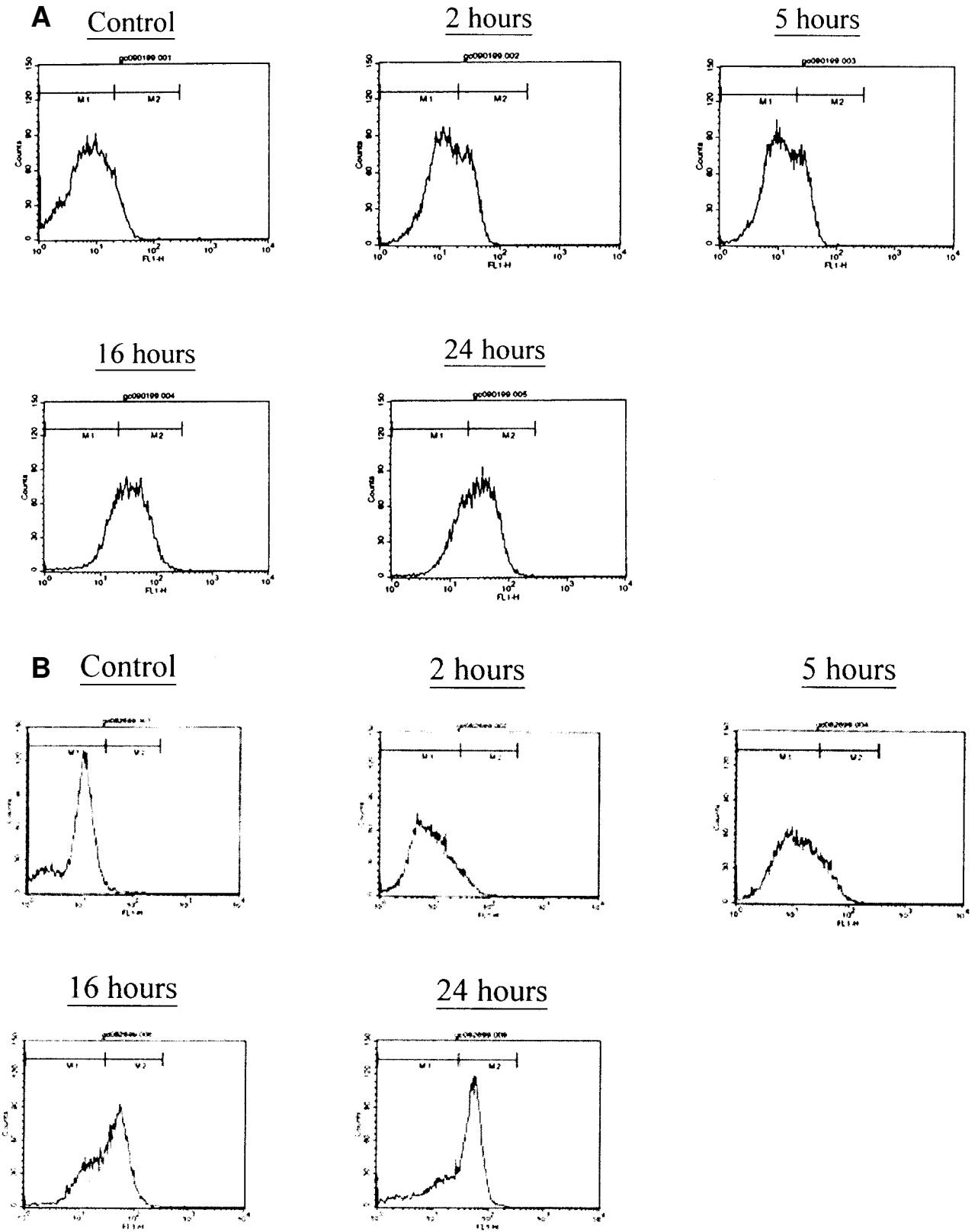


Fig. 8. Scramblase activity in apoptotic U-251 MG (A) and U-87 MG (B) cells. Representative histograms demonstrate the time course of the appearance of NBD-PC inside the cells. The x-axis represents the fluorescence of NBD-PC and the y-axis represents the number of cells. U-251 MG cells and U-87 MG cells were treated with apoptosis-inducing agents for various periods of time (0, 2, 5, 16, and 24 h) and were washed and collected. NBD-PC was then added to the cell suspension for 10 min to allow nonspecific phospholipid flip-flop after which cells were extracted to remove fluorescent NBD-PC that had not entered the cells. The cell suspension was then analyzed by flow cytometry.

after diagnosis. This is mainly due to the infiltrative behavior of malignant gliomas and the intrinsic resistance to both irradiation and cytotoxic drugs. Our findings that a variety of cells present in and near glioma cells

in vivo can eliminate glioma cells by PS-dependent phagocytosis suggest that manipulation of the phagocytosis process may be a promising direction in the treatment of gliomas.

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