High-affinity binding sites for heparin generated on leukocytes during apoptosis arise from nuclear structures segregated during cell death

Milena A. Gebska, Ian Titley, Hugh F. Paterson, Ricardo M. Morilla, Derek C. Davies, Alicja M. Gruszka-Westwood, Vijay V. Kakkar, Suzanne Eccles, and Michael F. Scully

During cell death of human cultured leukocytes (Jurkat, HL-60, THP-1, U937) and freshly prepared leukocytes, we observed a greater than 100-fold increase in the affinity of apoptotic and necrotic cells for fluorescein isothiocyanate (FITC)-heparin in comparison with live cells. Binding of FITC-heparin was reversed in the presence of high ionic strength, unlabeled heparan sulfate, and heparin and pentosan polysulfate, but not in the presence of chondroitin and dermatan sulfates. During the course of cell death, the increase in the percentage of cells positive for annexin V binding correlated with the increase in the population positive for binding FITC-heparin. Confocal microscopy demonstrated that heparin binding to dead cells was restricted to 1 or 2 small domains on the surfaces of apoptotic cells and to larger, but still discrete, areas that did not localize with chromatin on ruptured necrotic cells. The heparin-binding domains originated from the nucleus and may correspond to the ribonucleoprotein-containing structures that have recently been shown to segregate within the nucleus of cells and to move onto the cell membrane. We observed that phagocytosis of dead Jurkat cells by monocyte-derived macrophages was blocked when the heparin-binding capacity of the dead cells was saturated by the addition of pentosan polysulfate. From this we concluded that the ability of dead cells to bind to heparan sulfate proteoglycans on the surfaces of macrophages may assist in phagocytic clearance.

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Materials and methods

Materials

Camptothecin and 7β-hydroxycholesterol were obtained from Sigma (Poole, United Kingdom); CD95 (Fas antibody, R and O Systems, United Kingdom); phycoerythrin (PE)-annexin V and viability dye 7-AAD (7-aminoactinomycin D, Via-Probe, CA) and other reagents for fluorescence-activated cell sorter (FACS) analysis were obtained from Becton Dickinson, CA; TAMRA 5,6-carboxytetramethyl-rhodamine-succinimidyl-ester was obtained from Molecular Probes (Eugene, OR); Vectashield mounting medium supported by a grant from the British Heart Foundation (PG/98/82). No commercial support has been received for this study.

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medium for fluorescence with DAPI was obtained from Vector Laboratories (Burlingame, CA), and Mowirol from Calbiochem (United Kingdom). Recombinant unlabelled annexin V was obtained from Dr T. R. Hawthorne (Novartis, Basel, Switzerland); FITC-labeled anti-human CD14 and CD45 and the respective isotype controls were obtained from Sigma.

Unfractionated porcine heparin (Mr, 15 kDa) was obtained from Pharmacia Upjohn (Sweden), and pharmaceutical preparations of low-molecular-weight heparin (LMWH) were obtained as follows: Certoparin (7 kDa), Novarit; Fraxiparin (6 kDa), Pharmacia Upjohn; Fraxiparin (4.5 kDa) and CY222 (3.5 kDa), Sanofi (France); and Benemiparin (3 kDa), Laboratorios Farmaceutivos ROCO S.A. (Spain). Heparin sulfate (30 kDa; Laboratorio Derivati Organici, Italy) and pentosan polysulfate (3.5 kDa, SP 54, Benechene, Germany) were kindly supplied by Dr B. Mulloy (NIHSC, Herts, United Kingdom). Chondroitin sulfate A and C were purchased from Sigma and dermatan sulfate was obtained from Mediolanum Farmaceutici (Italy). FITC-labeled unfractionated heparin and FITC-LMWH (labeled Benemiparin) were prepared by Dr F. Wüsteman (School of Molecular Medicine and Biosciences, University of Wales, Cardiff). In brief, free amino groups on the heparin were acetylated, and free reducing ends were coupled to dianmoethane using pyridine/borohydride as a reducing agent. This material was labeled with FITC and was purified by repeated purification and ion exchange chromatography on Dowex 1. No loss in anticoagulant activity was observed after the labeling. All other chemicals used were of analytical grade and were obtained from Sigma.

Cell culture

Human leukemia cells Jurkat, HL-60, THP-1, and U937 were obtained from the American Type Culture Collection (ATCC; Rockville, MD). Cells were cultured in suspension in RPMI 1640 medium (Sigma) supplemented with 10% heat-inactivated fetal calf serum (Harlan Serata Laboratories, United Kingdom), 100 U/mL penicillin G, 100 U/mL streptomycin, and 2 mM L-glutamine. 2-mercaptoethanol was added to the culture medium of THP-1 cells according to ATCC recommendations. The culture was kept in a humidified incubator with 95% air and 5% CO₂ at 37°C and was split every 3 to 4 days to ensure logarithmic growth to a maximum density of 10⁶ cells/mL. After transfer to fresh medium containing 5% fetal calf serum, the cells were treated with either Camptothecin (stock prepared in dimethyl sulfoxide), 7-AAD and 7-AAD were added according to the manufacturer’s instructions, and samples were immediately analyzed using a Becton Dickinson FACSCalibur flow cytometer calibrated using fluorescent beads. Acquisition processing of data from 10,000 cells was carried out during each experiment. The Kᵢ for binding to dead cells was determined from triplicate values of relative fluorescence intensity (RFI) obtained at dilutions of FITC-heparin from 0.04 to 1.3 μM that were fit to a single-site ligand-binding equation using Enzfitter (Sigma).

Cell sorting

Cells were labeled with annexin V and 7-AAD, as described above, and were sorted into live, apoptotic, and necrotic fractions by a FACS Vantage SE cell sorter (Becton Dickinson). The minimum number of cells collected into RPMI 1640 medium was 40,000 in each confocal microscopy sample. Sorted cells were resuspended in HEPS buffer before the addition of FITC-heparin (1 μM). After 10 minutes on ice, cells were washed once in annexin V binding buffer and were relabeled with PE-annexin V and 7-AAD. The cell suspension was then mixed with Mowirol in a 1:5 (vol/vol) proportion before they were mounted on glass slides. Confocal laser scanning microscopy was performed using a Bio-Rad MRC 1024 equipped with a krypton-argon laser in conjunction with a Nikon Eclipse 600 microscope (objective ×60). Eight thin horizontal planes of sections were made, each 1 μm apart (zoom factor, 3.23). A standard FITC filter set combined with the 488-nm laser line was used to visualize FITC-labeled heparin-binding sites, whereas a Texas Red filter set combined with the 588-nm laser line enabled localization of areas that bound PE-annexin V (cell membrane) and 7-AAD (intracellular distribution).

Fluorescence microscopy

Cells were sorted as described above and were labeled with FITC-heparin at a final concentration of 1 μM for 10 minutes. After one wash with HEPS buffer, the cells were stained with DAPI, and glass slides were prepared. Fluorescence microscopy was performed using a Zeiss AxioShot, and images were captured using Quips-FISH application (Vysis, United Kingdom).

Heparin binding to isolated nuclei

Jurkat cells were treated with Camptothecin and were isolated according to the procedure of Martelli et al. In brief, cells were resuspended to 1.5 × 10⁶/mL.
in TM-2 buffer (10 mM Tris, pH 7.4, 2 mM MgCl$_2$, 0.5 mM phenylmethylsulfonyl fluoride, 1 µg/mL aprotinin, 1 µg/mL leupeptin) and were incubated for 5 minutes on ice. Triton X-100 was added at 0.5% (wt/vol), washed once in buffer containing 10 mM Tris-HCl, pH 7.4, 0.25 M sucrose, and g/mL aprotinin and leupeptin. A sample of nuclei in suspension was transferred to a glass slide, stained with DAPI, and examined by fluorescence microscopy as described above.

**Influence of the enhanced heparin affinity of dead cells on phagocytosis**

Monocyte-derived macrophages (MDMs) were prepared by differential centrifugation of human peripheral blood using Lymphoprep density gradient medium (Nycomed, Oslo, Norway) and were separated from human fresh blood using Lymphoprep density gradient medium (Nycomed, Oslo, Norway) and were cultured on Permanox chamber slides (Lab-Tek, United Kingdom) in the presence of 10% autologous serum. Live Jurkat cells were incubated with TAMRA as described previously.12 Half the Jurkat cells labeled with TAMRA (Jurkat) were treated with Camptothecin for 30 hours. Live and dead cell preparations were added (4 × 10$^5$ cells/mL) to the chambers culturing MDMs. After 60-minute incubation at 37°C, the supernatant was removed and MDM monolayers were washed with PBS containing 0.5% immunoglobulin G–free bovine serum albumin and 0.15% NaN$_3$ at 4°C. MDMs were then labeled with FITC-labeled anti-human CD14 at 4°C, and cell preparations were added (4 £ 10$^5$ cells/mL) to the chambers culturing MDMs.

**Results**

**Binding of heparin to live, apoptotic, and necrotic cells**

Heparin binding by leukocytic cells undergoing cell death induced by a number of agents was determined with each of 4 cell lines, and was cultured on Permanox chamber slides (Lab-Tek, United Kingdom) in the presence of 10% autologous serum.12 Live Jurkat cells were incubated with TAMRA as described previously.12 Half the Jurkat cells labeled with TAMRA (Jurkat) were treated with Camptothecin for 30 hours, and treated with Camptothecin for 24 hours before labeling with FITC-heparin at a range of concentrations (0.5 nM-5000 nM). Cells were washed and labeled with PE–annexin V and 7-AAD, as described in “Materials and methods.” Live, apoptotic, and necrotic cells derived from cultured Jurkat T cells.

**Table 1. FITC-heparin binding by live, apoptotic, and necrotic cells of different lineage and according to different treatment**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>RFI (± SEM)</th>
<th>RFI (± SEM)</th>
<th>RFI (± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jurkat</td>
<td>no treatment</td>
<td>2.6 (± 0.0)</td>
<td>—— (——)</td>
<td>—— (——)</td>
</tr>
<tr>
<td></td>
<td>Camptothecin 11 µM/6 h</td>
<td>—— (——)</td>
<td>6.3 (± 0.6)</td>
<td>510.0 (± 101.0)</td>
</tr>
<tr>
<td></td>
<td>7-ketocholesterol 110 µM/6 h</td>
<td>—— (——)</td>
<td>17.2 (± 2.6)</td>
<td>772.4 (± 69.0)</td>
</tr>
<tr>
<td></td>
<td>CD95 (Fas) antibody 5 µg/mL/6 h</td>
<td>—— (——)</td>
<td>7.5 (± 0.2)</td>
<td>656.0 (± 109.0)</td>
</tr>
<tr>
<td>THP-1</td>
<td>no treatment</td>
<td>5.9 (± 0.1)</td>
<td>—— (——)</td>
<td>—— (——)</td>
</tr>
<tr>
<td></td>
<td>Camptothecin 11 µM/6 h</td>
<td>—— (——)</td>
<td>18.0 (± 1.4)</td>
<td>686.0 (± 71.0)</td>
</tr>
<tr>
<td></td>
<td>7-ketocholesterol 110 µM/6 h</td>
<td>—— (——)</td>
<td>67.0 (± 1.8)</td>
<td>564.0 (± 65.0)</td>
</tr>
<tr>
<td>HL-60</td>
<td>no treatment</td>
<td>6.7 (± 0.1)</td>
<td>—— (——)</td>
<td>—— (——)</td>
</tr>
<tr>
<td></td>
<td>Camptothecin 11 µM/6 h</td>
<td>—— (——)</td>
<td>70.0 (± 7.1)</td>
<td>448.0 (± 51.0)</td>
</tr>
<tr>
<td></td>
<td>7-ketocholesterol 110 µM/6 h</td>
<td>—— (——)</td>
<td>107.0 (± 7.0)</td>
<td>868.0 (± 76.0)</td>
</tr>
<tr>
<td>U937</td>
<td>no treatment</td>
<td>3.6 (± 0.2)</td>
<td>—— (——)</td>
<td>—— (——)</td>
</tr>
<tr>
<td></td>
<td>Camptothecin 110 µM/6 h</td>
<td>—— (——)</td>
<td>1.6 (± 0.1)</td>
<td>173.0 (± 61.0)</td>
</tr>
<tr>
<td></td>
<td>7-ketocholesterol 110 µM/6 h</td>
<td>—— (——)</td>
<td>16.8 (± 3.0)</td>
<td>235.0 (± 17.0)</td>
</tr>
<tr>
<td>Fresh leukocytes</td>
<td>no treatment</td>
<td>2.2 (± 0.2)</td>
<td>—— (——)</td>
<td>—— (——)</td>
</tr>
<tr>
<td></td>
<td>Camptothecin 11 µM/6 h</td>
<td>—— (——)</td>
<td>17.2 (± 0.5)</td>
<td>92.0 (± 3.8)</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>4.2 (± 0.1)</td>
<td>32.9 (± 2.4)</td>
<td>500.4 (± 62.4)</td>
</tr>
</tbody>
</table>

Leukocyte cells were treated as shown, and the binding of FITC-heparin at 500 nM to live, apoptotic, and necrotic cells was determined as described in the legend to Figure 2. Results shown are the mean of values for RFI (see “Materials and methods”) observed in 3 experiments. Untreated cells are those to which vehicle alone was added.

**Data analysis**

Data obtained during flow cytometry were analyzed using WinMDI version 2.8 computer software (Scripps Research Institute, La Jolla, CA), where RFI was calculated as a Gmean. Microsoft Excel was used to calculate mean values and standard error of the mean (SEM). Each experiment was repeated at least 3 times. Data for laser scanning cytometry were analyzed on a Hewlett Packard (Palo Alto, CA) computer using WinCyte 3.4 software (CompuCyte).
namely, Jurkat, THP-1, U937, and HL-60. Live, apoptotic, and secondary necrotic cells were discriminated according to the binding of PE-annexin V and the uptake of 7-AAD, as described in “Materials and methods.” When these parameters were used to sort the cells, the typical morphology of live, apoptotic, and necrotic cells was observed by light microscopy as shown in the inserted micrographs in Figure 1.

The binding of FITC-heparin to live, apoptotic, and necrotic Jurkat cells is shown in Figure 2. No binding was observed to live cells except for a slight increase in RFI at 5 μM FITC-heparin. In contrast, enhanced binding was observed to apoptotic and necrotic cells at all concentrations of FITC-heparin tested. The RFI reached a maximum at 5 μM FITC-heparin (tested up to 20 μM FITC-heparin, data not shown), and in comparison with live cells, it was an order of magnitude higher for apoptotic cells and more than 2 orders of magnitude higher for necrotic cells. The $K_d$ for binding of FITC-heparin to dead cells was determined as 259 nM (data not shown). Binding was rapid (complete within 1 minute), reversed by the inclusion of 0.8 M NaCl in the heparin binding buffer, and independent of calcium (data not shown).

A summary of the results obtained with each of the cell lines is shown in Table 1. RFI values were determined for each of the populations at a single concentration of FITC-heparin (500 nM). The mean increase in RFI for apoptotic cells over live cells was 10-fold; for necrotic cells, it was more than 100-fold. A similar increase in RFI was observed during apoptosis of leukocytes prepared from fresh blood, as shown in Figure 3. The appearance of FITC-heparin-positive cells and PE-annexin V-positive cells was compared in timed samples taken during apoptosis of Jurkat cells (Figure 4). At later time points, good agreement was observed between the percentage of cells found to be PE-annexin V positive and the percentage of FITC-heparin-positive cells. Correlation was not as good in the early phase of the study, possibly because of a degree of overlap between the binding of FITC-heparin by apoptotic and live cells (Figure 2).

Figure 5 depicts the observations made on the ability of unlabeled heparan sulfate, pentosan polysulfate, and various molecular weights of heparin to block the binding of FITC-heparin to necrotic cells. FITC-LMWH was used for these experiments because it was observed in preliminary experiments that this has a weaker affinity for cells than FITC-heparin. As a block for the binding of FITC-LMWH, $IC_{50}$ decreased with increased molecular weight (Table 2). Affinity for dead cells was reduced as the molecular weight of heparin increased, with the exception of LMWH (average Mr, 15 kd), and pharmaceutical preparations of depolymerized heparin of various average molecular weights (as listed in “Materials and methods”). After 60 minutes, FITC-LMWH was added at 1 μM, and incubation continued for another 10 minutes.

Cells were then labeled with PE-annexin V and 7-AAD, and the RFI of the necrotic population was determined by FACS analysis as in Figure 2. The RFI at each concentration of competitive ligand was expressed as a percentage of the RFI of cells in its absence. 100 kDa heparan sulfate; 200 kDa heparan sulfate; 700 kDa, 6 kDa LMWH; 3.5 kDa LMWH; and 0.5 kDa LMWH.

![Figure 3. Flow cytometric analysis of the binding of FITC-heparin to live, apoptotic, and necrotic cells derived from freshly prepared leukocytes. Leukocytes were prepared from fresh blood and were treated with Camptothecin for 16 hours, as described in "Materials and methods." Cells were labeled with FITC-heparin at a range of concentrations (0.5 nM-5000 nM) and then with PE-annexin V and 7-AAD. Live, apoptotic, and necrotic cells were discriminated by FACS analysis. Histogram plots of the binding to each population at 5 μM FITC-heparin are shown as relative fluorescence intensity on the x-axis.](Image)

![Figure 4. Time course correlating the exposure of phosphatidylserine by cells during cell death with enhanced binding of FITC-heparin. Jurkat cells were treated with Camptothecin, and samples were taken at timed intervals for labeling with PE-annexin V and FITC-heparin (500 nM). The results show the correlation between the fraction of cells with exposed PS (enhanced PE-annexin V binding $\Delta$) to the fraction of cells binding FITC-heparin with an RFI that was 10-fold higher than untreated cells $\Box$. Counted cells are expressed as a percentage of PE-annexin V-positive or FITC-heparin-positive cells in the 24-hour sample.](Image)

![Figure 5. Inhibition of FITC-LMWH binding to dead cells by heparan sulfate, pentosan polysulfate, and heparin of various molecular weight forms. Necrotic Jurkat cells (induced by Camptothecin and discriminated by FACS analysis, as described in Figure 1) were preincubated with increasing concentrations of these heparin-like ligands—heparan sulfate, pentosan polysulfate, unfractionated heparin (average Mr, 15 kd), and pharmaceutical preparations of depolymerized heparin of various average molecular weights (as listed in "Materials and methods"). After 60 minutes, FITC-LMWH was added at 1 μM, and incubation continued for another 10 minutes. Cells were then labeled with PE-annexin V and 7-AAD, and the RFI of the necrotic population was determined by FACS analysis as in Figure 2. The RFI at each concentration of competitive ligand was expressed as a percentage of the RFI of cells in its absence. $\Box$: heparan sulfate; $\Delta$: unfractionated heparin; $\cdot$: 7 kd LMWH; $\cdot$: 6 kDa LMWH; $\cdot$: 4.5 kDa LMWH; $\cdot$: 3.5 kDa LMWH; and $\cdot$: 0.5 kDa LMWH.](Image)
Table 2. Inhibition of FITC-LMWH binding to dead Jurkat cells by heparan sulfate, pentosan polysulfate, and heparin of various molecular weights

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Mr (kd)</th>
<th>IC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfractionated heparin</td>
<td>15.0</td>
<td>0.005</td>
</tr>
<tr>
<td>LMWH</td>
<td>7.0</td>
<td>0.3</td>
</tr>
<tr>
<td>LMWH</td>
<td>6.0</td>
<td>0.6</td>
</tr>
<tr>
<td>LMWH</td>
<td>4.5</td>
<td>2.0</td>
</tr>
<tr>
<td>LMWH</td>
<td>3.5</td>
<td>5.0</td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td>30.0</td>
<td>0.3</td>
</tr>
</tbody>
</table>

IC50 values for the blocking of the binding of FITC-LMWH to dead Jurkat cells were obtained from the results shown in Figure 5.

molecular weight of heparin was decreased, consistent with findings of previous reports regarding the binding of heparin to proteins, such as platelet factor 4. The IC50 of pentosan polysulfate (3.5 kd) was similar to that of unfractionated heparin. The ability of pentosan polysulfate to bind with high affinity despite its low molecular weight is attributed to the high-charge density of pentosan polysulfate (3.5 sulfate groups per disaccharide as opposed to 2.4-2.8 sulfate groups per disaccharide for heparin). Heparan sulfate, which has a lower degree of sulfation than heparin (0.8-1.8 sulfate groups per disaccharide) gave an IC50 value of 0.3 µM. Chondroitin sulfates A and C and dermatan sulfate had no effect on the binding of FITC-LMWH when tested up to 15 µM. These experiments demonstrate the specificity of heparin binding and rule out a contribution to the binding of the single FITC reporter moiety.

Localization of binding sites for FITC-heparin by confocal and fluorescence microscopy

Confocal microscopy of the FITC–heparin-labeled live, apoptotic, and necrotic Jurkat cells prepared by sorting confirmed that live cells do not bind heparin (Figure 6 [1A]). On apoptotic cells, 1 or 2 discrete domains of heparin binding were observed at the cell surface (Figure 6 [1B]). In cells that had membranes that were damaged but unruptured, a broader region of heparin binding was seen, though this was still discrete (Figure 6 [1C]). Heparin binding to ruptured necrotic cells was to discrete domains that exhibited high fluorescence intensity that did not coincide with the redness of chromatin present as lobules (Figure 6 [1D]) or as a residue (Figure 6 [1E]). Further histologic investigation by fluorescence microscopy confirmed that the sites responsible for heparin binding arose as discrete domains that were not present in live cells (Figure 6 [2A]) but that developed within the nucleus (stained with DAPI) during apoptosis. (Figure 6 [2B-C]). During the course of cell death, these domains became separated from the chromatin (Figure 6 [2C]) and eventually were attached to the cell membrane (Figure 6 [2D]) or appeared alongside the residual chromatin on the membrane of necrotic cells (Figure 6 [2E]). These domains were also observed in nuclei from cells harvested at earlier time points. Staining of the nuclei with FITC-heparin and DAPI medium showed no binding to nuclei from live cells (Figure 6 [3A]). In the first sample taken at 30 minutes, 1 or 2 larger areas of relatively less intense binding of heparin were observed (Figure 6 [3B]). In later samples at 1 and 2 hours, several discrete smaller domains of higher intensity were more common (Figure 6 [3C-D]). Nuclear material isolated at 4 and 6 hours showed lobulated nuclei that exhibited high-intensity binding to large domains that were separated from the DAPI-positive material (Figure 6 [3E-F]). During this series of experiments, the addition of excess unlabeled heparin blocked the fluorescence signal.

Influence of the heparin affinity of dead cells on phagocytosis

Phagocytosis was determined by the incorporation of TAMRA-labeled Jurkat cells into monolayers of MDM using laser scanning cytometry. When incubated with dead Jurkat cells, 31.9% of MDM were positive for TAMRA (Figure 7C) compared with only 2.1% in the presence of live cells (Figure 7B). When the dead cells were preincubated with annexin V (400 µg/mL for 15 minutes), the incorporation was reduced to 8.5% in agreement with the known importance of PS exposure as a ligand for the phagocytosis of dead cells (Figure 7D). The incorporation was reduced to 4.2% when cells were preincubated with pentosan polysulfate at a concentration that would block available heparin binding sites (2 µM) (Figure 7E). The observed reduction shows that the heparin-binding sites play a significant role in the uptake of dead cells, presumably through the interaction with macrophage HPSGs. The level of incorporation was reduced to 1% when pentosan polysulfate and annexin V were combined (Figure 7F).

Discussion

We have observed that in comparison with healthy leukocytes, apoptotic and necrotic leukocytes have a high affinity for heparin—defined as
TAMRA (red). (lower 6 panels) Series of sections taken through MDM after pentosan polysulfate (F). (lower panels) Phagocytosis of Jurkat T under these unlabeled annexin V (D), pentosan polysulfate (E), or a combination of annexin V and tations, 2000) and the pancreatic carcinoma cell line, MIA PaCa2. Our conclusion that live cells are relatively weak in binding heparin is in accord with the report of Harenberg et al, who investigated heparin binding to leukocytes in fresh blood. The high $K_d$ of live cells would also agree with the affinity of heparin for L-selectin, which is in the micromolar range. The lack of dependency on Ca$^{++}$ for high-affinity binding to dead cells rules out any contribution of L-selectin.

When we investigated the binding of heparin by confocal microscopy, we observed that binding occurred to discrete areas on the membrane of apoptotic cells (Figure 6 [1B]) or were separated from chromatin in necrotic cells. The development of focused, highly intense regions of heparin binding was confirmed by conventional fluorescence microscopy. The source of the heparin binding material appears to be the nucleus because the nuclei of live cells do not bind heparin. Early in the apoptotic process, however, a large binding domain was formed within the nucleus (Figure 6 [3B]). The intensity of heparin binding was considerably enhanced when it occurred outside the nucleus, indicating a lack of accessibility to binding proteins when located within the nucleus, perhaps because of the presence of other ligands.

This finding of discrete domains of nuclear material segregating during apoptosis corresponds closely to structures recently described as clusters of ribonucleoproteins, for which the term HERDS (heterogeneous ectopic ribonucleoprotein-derived structures) has been coined. These proteins are normally distributed throughout the nucleus in the nuclear matrix, but during apoptosis they form into small fibrillogranular structures that pass into the cytoplasm and are eventually extruded at the cell surface in membrane-bound blebs. The ribonucleoproteins are obvious candidates as heparin-binding proteins because many are highly basic proteins and contain sequences that confer high affinity for heparin.

Our findings demonstrate that the heparan sulfate–binding property of apoptotic cells may be of physiological significance in assisting phagocytic clearance. The exposure of PS is well established as a signal for the phagocytosis of apoptotic cells, a mechanism that can be blocked by the presence of annexin V. Macrophages are known to express HSPGs that have been observed to play a role in the phagocytosis of latex beads. We have now shown that the phagocytosis of dead cells can be effectively blocked by saturation of the heparin-binding sites with pentosan polysulfate, a highly potent inhibitor of heparin binding to nonviable cells. Moreover, because the exposure of areas with high affinity for heparin occurs early in apoptosis, coincidentally with the exposure of PS (Figure 4), HSPG binding may make a significant contribution to the clearance mechanism. If this is the case, then heparin treatment may reduce the rate of removal of dying cells.

Acknowledgments

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References


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