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Glomerular mesangial cells and inflammatory macrophages ingest neutrophils undergoing apoptosis

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Glomerular mesangial cells and inflammatory macrophages ingest neutrophils undergoing apoptosis. Apoptosis or programmed cell death of senescent neutrophils leading to their uptake by phagocytes is a general mechanism by which neutrophils may be removed from inflamed sites in vivo, promoting resolution rather than persistence of inflammation. We now report morphological evidence of neutrophil apoptosis leading to uptake by glomerular cells in rats with experimental glomerulonephritis. In addition to confirming that inflammatory macrophages take up apoptotic neutrophils, these studies indicated that glomerular mesangial cells can also participate in this mode of neutrophil clearance. Furthermore, human neutrophils which had been "aged" in vitro so as to undergo apoptosis were ingested by 31.5 ± 1.3% (mean ± SE) of cultured human mesangial cells, but there was minimal recognition of freshly isolated neutrophils (2.2 ± 0.1%). Centrifugal elutriation of aged neutrophil populations yielded fractions with varying degrees of apoptosis (from 11.1 to 79.4%). Uptake of these farctions (by 8.2% to 59.8% of mesangial cells) was closely correlated with apoptosis ($r = 0.96$, $P < 0.0001$). This demonstrated that recognition was dependent upon apoptosis, as in previous reports of macrophage recognition of aged neutrophils. However, by contrast, a partial requirement for serum was observed. These data indicate a hitherto unexpected function for the mesangial cell in clearance of senescent neutrophils from the glomerulus which may supplement inflammatory macrophage uptake of leucocytes undergoing apoptosis.

Neutrophil polymorphonuclear granulocytes are a prominent component of the inflammatory infiltrate in many types of human glomerulonephritis. The neutrophil has evolved systems which generate reactive oxygen intermediates, powerful degradative granule enzymes and toxic cationic proteins. Although essential in defence against invading micro-organisms, there is growing evidence that neutrophils mediate injury to host tissue in a number of inflammatory diseases, including glomerulonephritis [1, 2]. Neutrophils and their contents can damage isolated glomerular components in vitro, and injure isolated perfused kidneys [3, 4]. Furthermore, a direct role in glomerular injury in vivo has been demonstrated by specific depletion and reconstitution of circulating neutrophils in animal models of nephritis [5, 6].

The safe disposal of neutrophils and their toxic contents may be expected to promote resolution of glomerular inflammation. However, neutrophil clearance from the inflamed glomerulus has received little attention, and the mechanisms responsible are obscure. Many have assumed that the inevitable fate of the extravasated neutrophil is to undergo necrosis and disintegration at the inflamed site, which would expose local tissues to toxic cell contents [7]. Nevertheless, it has been known since Metchnikoff's seminal work on the resolution of inflammation that senescent neutrophils can be ingested while still intact by macrophages, a disposal mechanism with potential to protect tissues from neutrophil contents [8]. Indeed, in models allowing frequent sampling of the cellular infiltrate, such as the experimentally-inflamed peritoneum, it appears that this is the dominant route of neutrophil disposal [9]. However, the mechanisms by which "self" neutrophils are recognized by macrophages have been obscure. Building on the earlier work of Newman, Henson and Henson [10], we showed that human neutrophils isolated from blood or inflamed sites and "aged" in culture for 24 hours underwent apoptosis [11]. This is a programmed form of cell death, first identified on the basis of clear morphological features such as condensation of nuclear chromatin and subsequently shown to be dependent upon endogenous endonuclease activation [12–14]. Apoptosis leads to recognition and swift uptake of intact but "unwanted" cells in a wide variety of important biological processes. In vitro we showed that apoptosis in aging neutrophils determined specific recognition of the intact senescent cell by macrophages [11, 15]. Furthermore, there was clear morphological evidence that neutrophil apoptosis leading to uptake by macrophages occurs in the inflamed joint and lung [11, 16]. The in vitro data indicate that this mode of neutrophil removal has been overlooked because apoptotic neutrophils are swiftly ingested and very rapidly degraded [10, 11], so that "free" apoptotic neutrophils at inflamed sites are uncommon, and most ingested neutrophil material is too degraded to be recognized as being derived from apoptotic cells. Because neutrophil apoptosis leads to macrophage ingestion of intact granulocytes which retain their toxic contents, it appears that this represents a potentially "injury-limiting" neutrophil clearance mechanism likely to promote resolution rather than persistence of inflammation [11].

However, to date there has been no direct evidence in glomerulonephritis of neutrophil apoptosis leading to clearance by phagocytes. Macrophage phagocytosis of apparently intact neutrophils in injured rat glomeruli was demonstrated by electron microscopy [17] but, in retrospect, the degree of cellular degeneration was too advanced to discern whether the neutrophils had undergone apoptosis prior to ingestion. Harrison's

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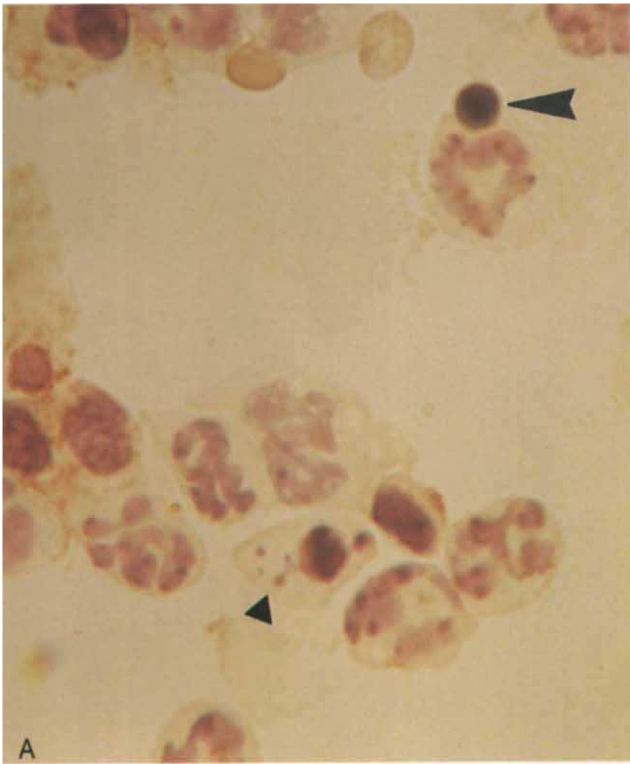

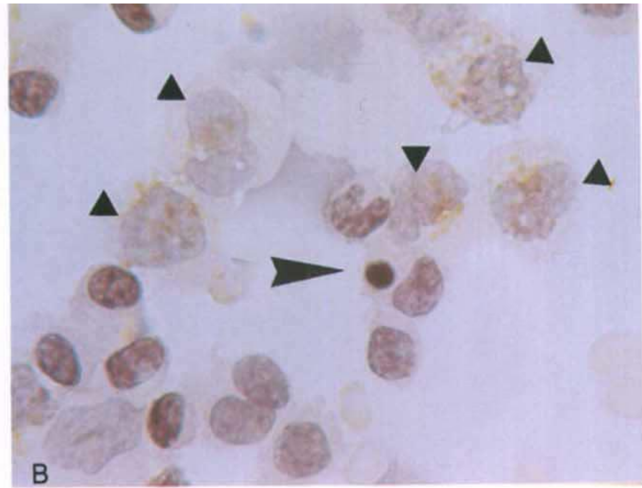

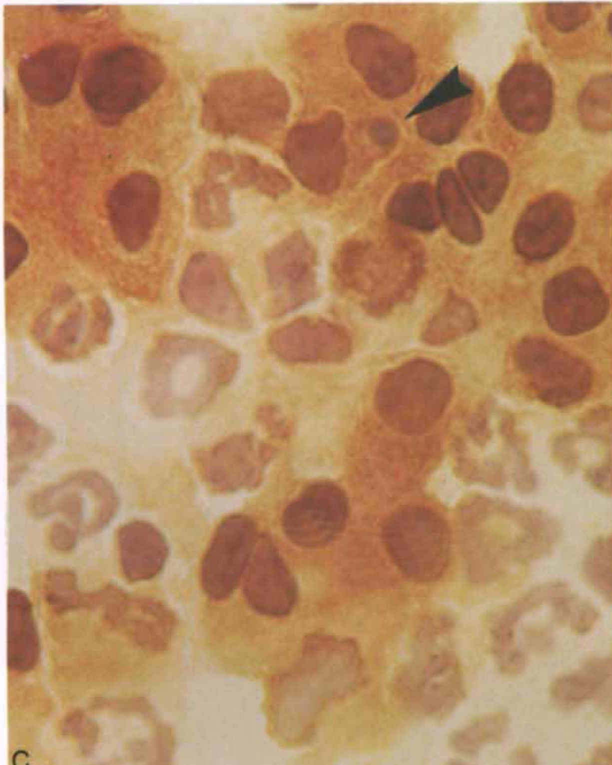

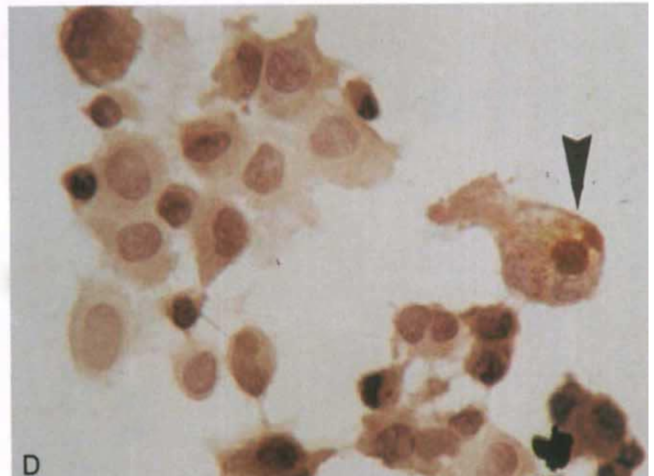

Fig. 1. *Evidence of uptake of apoptotic cells in vivo by rat mesangial cells.* Cytocentrifuge preparations of cell populations obtained from dissociated inflamed rat glomeruli. **(A)** Apoptotic cells in early (triangle) and late (arrow) stages of the process; note condensation of nuclear chromatin and cytoplasm (×680: Acetone-fixed preparation immunoperoxidase-stained for Thy1.1 and counterstained with haematoxylin). **(B)** Apoptotic body with densely staining chromatin within glomerular cell which does not express ED1 (arrow); note different morphology of surrounding macrophages (triangles) which are lightly immunoperoxidase-stained for the monocytic marker ED1 (×400: Acetone-fixed preparation counterstained with hematoxylin). **(C)** Apoptotic body within mesangial cell (arrow) identified by positive (orange-brown) immunoperoxidase staining for Thy 1.1 (×680: Acetone-fixed preparation counterstained with hematoxylin). **(D)** Glomerular cell population "enriched" for mesangial cells by positive FACS selection on the basis of Thy1.1 expression. The arrowed cell contains at least one discrete circular mass of orange/brown myeloperoxidase-positive material, compatible with a phagosome contained a neutrophil undergoing degradation (×400: glutaraldehyde-fixed preparation counterstained with Hamulum). Compare with appearances of mesangial cell ingestion of neutrophils in vitro (Figs. 5 and 6).

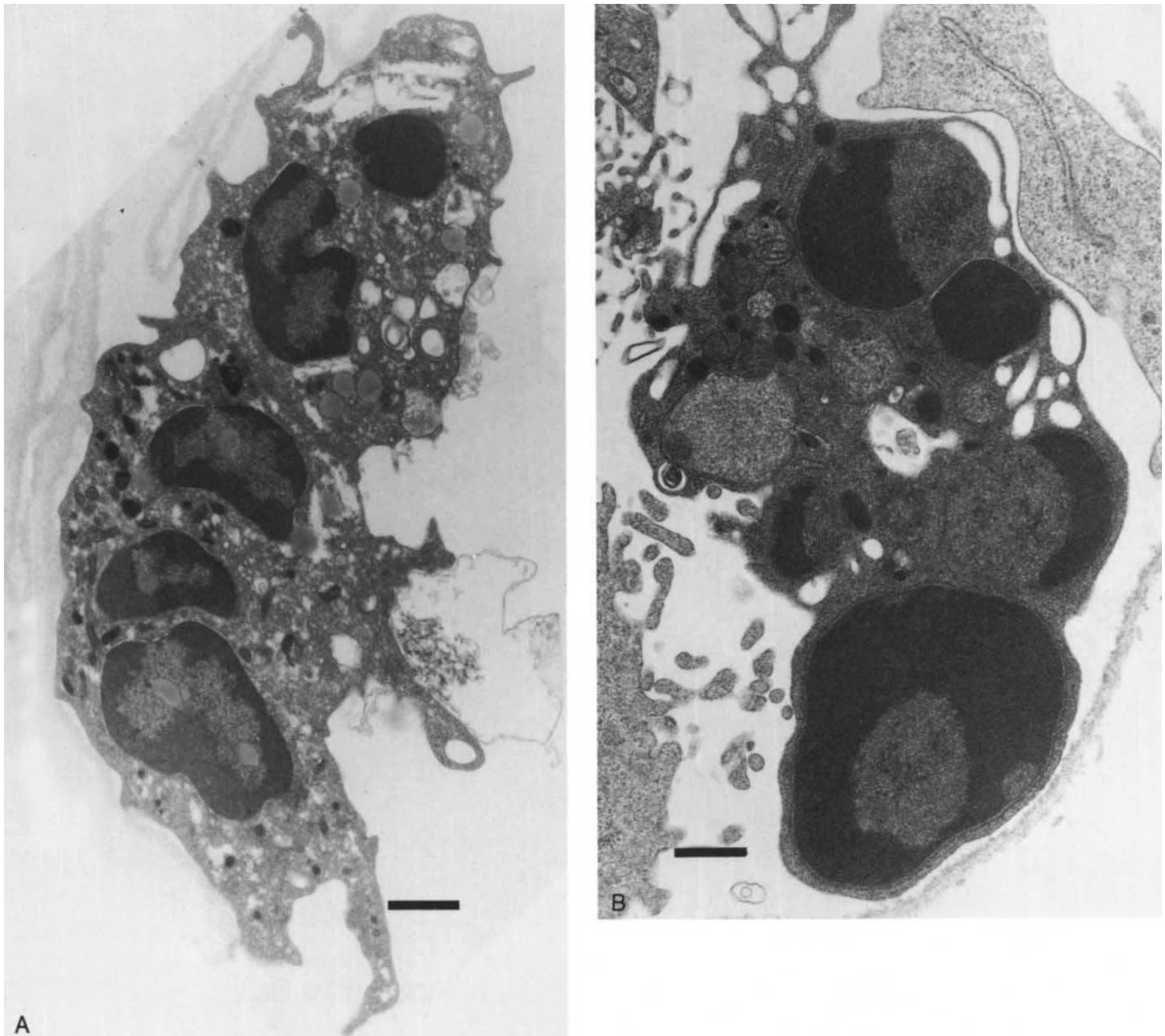

Fig. 2. *Neutrophil apoptosis in dissociated glomerular cell populations obtained from nephritic rats.* Transmission electron microscopy. **A.** Normal neutrophil (bar = 0.9 microns). **B.** Neutrophil in early stages of apoptosis. Note cytoplasmic condensation, retention of granules and condensation of heterochromatin to form "crescents" in the lobes of the nucleus. The cell also appears smaller (bar = 0.6 microns).

light microscopical studies demonstrated cells with features typical of apoptosis within the glomeruli of patients with nephritis [18]. However, although such cells were most frequent where neutrophil infiltration was evident, positive identification of scarce apoptotic cells as neutrophils was not possible in the tissue sections examined by electron microscopy. Nevertheless, in a single case of acute glomerular injury, apoptotic cells of unknown origin were identified by electron microscopy within mesangial cells [19], suggesting that this glomerular cell type may have the capacity to participate in clearance of neutrophils undergoing apoptosis, in keeping with previous reports of apoptotic cell uptake by other "semi-professional" phagocytes, such as epithelial and tumor cells [12, 13].

By employing swift enzymatic dissociation of glomerular cells from rats with experimental glomerulonephritis we are now able to report direct morphological evidence of neutrophil apoptosis leading to uptake by glomerular phagocytes. The data indicate that in addition to inflammatory macrophages, neutrophil clearance is undertaken by glomerular mesangial cells. Furthermore, cultured human mesangial cells ingest human neutrophils undergoing apoptosis during "aging" in vitro. These findings indicate a hitherto unrecognized role for the

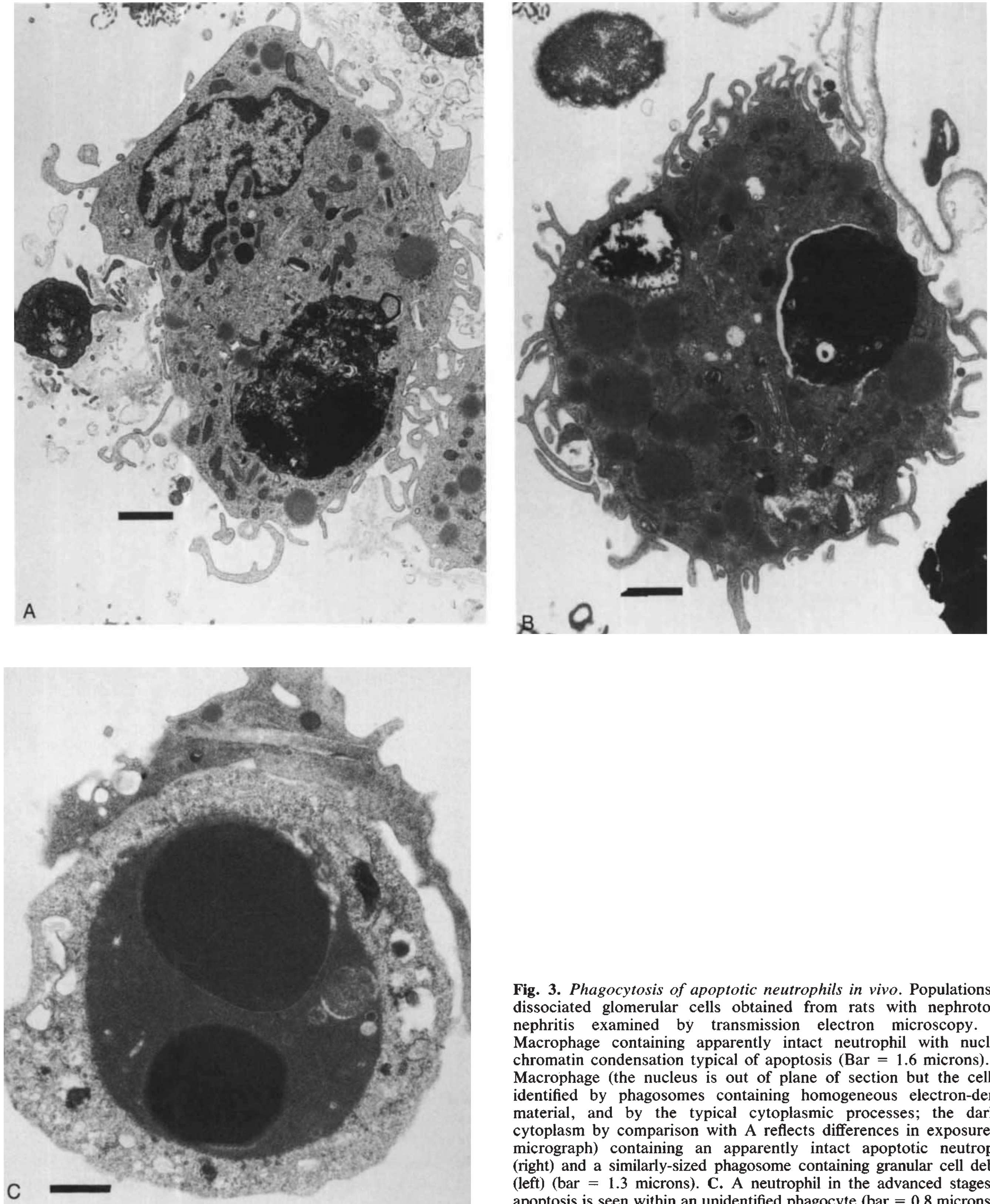

Fig. 3. *Phagocytosis of apoptotic neutrophils in vivo.* Populations of dissociated glomerular cells obtained from rats with nephrotoxic nephritis examined by transmission electron microscopy. **A.** Macrophage containing apparently intact neutrophil with nuclear chromatin condensation typical of apoptosis (Bar = 1.6 microns). **B.** Macrophage (the nucleus is out of plane of section but the cell is identified by phagosomes containing homogeneous electron-dense material, and by the typical cytoplasmic processes; the darker cytoplasm by comparison with A reflects differences in exposure of micrograph) containing an apparently intact apoptotic neutrophil (right) and a similarly-sized phagosome containing granular cell debris (left) (bar = 1.3 microns). **C.** A neutrophil in the advanced stages of apoptosis is seen within an unidentified phagocyte (bar = 0.8 microns).

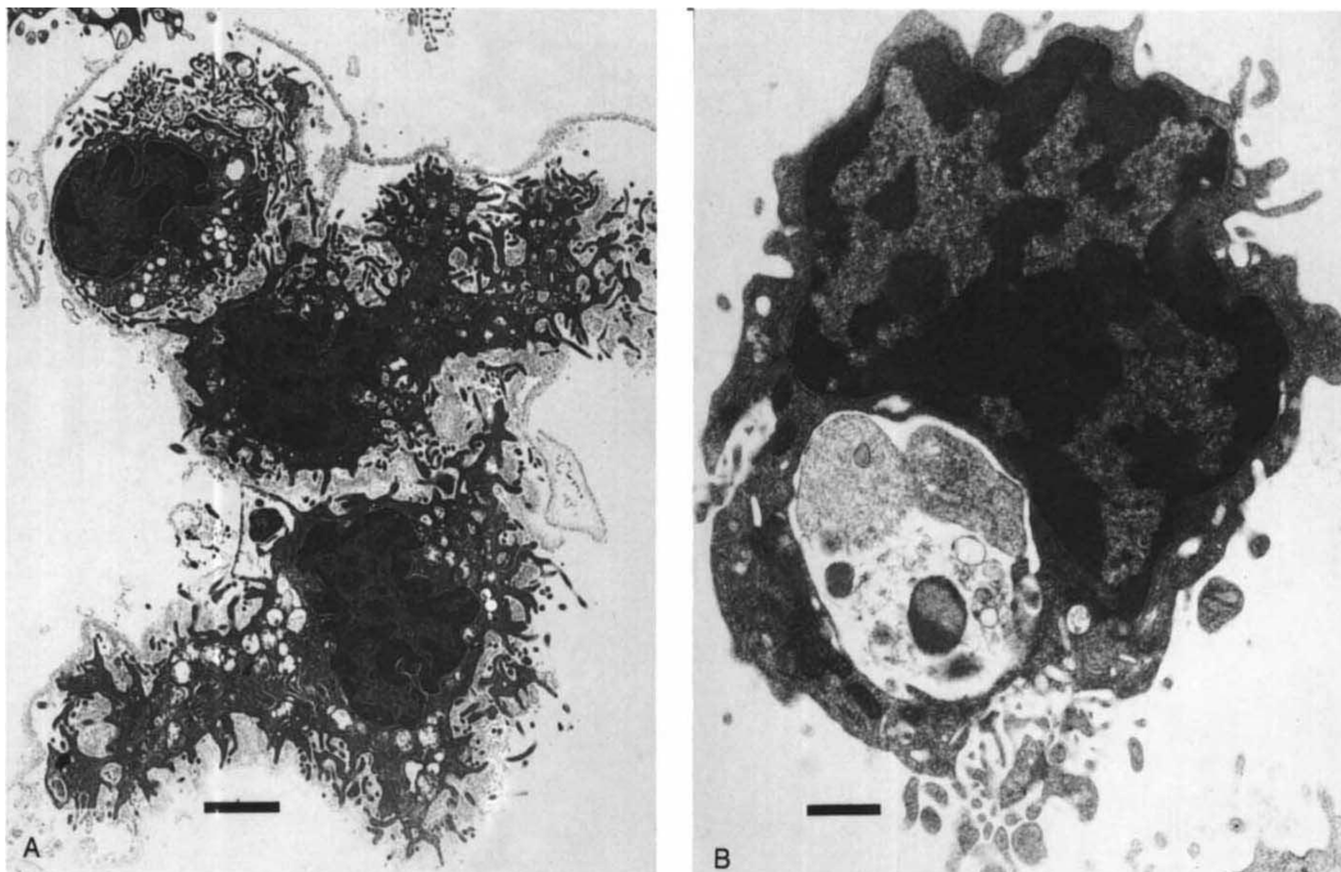

Fig. 4. *Mesangial cell phagocytosis in vivo.* **A.** Cluster of partially-dissociated mesangial cells within glomerular cells isolated from a rat with nephritis. Note by comparison with inflammatory macrophages (Fig. 3A), the different pattern of nuclear heterochromatin, relatively electron dense cytoplasm, paucity of organelles and phagosomes containing homogeneous electron-dense material, more complex cytoplasmic protrusions often localized to one pole of the cell, and in view of partial dissociation, presence of surrounding matrix (bar = 1.6 microns). **B.** Phagocytic mesangial cell in a dissociated preparation of nephritic rat glomerular cells. The phagosome contains cellular debris, apparently comprising granules and fragments of condensed chromatin, suggesting possible neutrophil origin (bar = 0.6 microns).

mesangial cell in clearance from the inflamed glomerulus of senescent neutrophils undergoing apoptosis.

Methods

Tissue culture materials

All reagents were from Sigma (Poole, UK) unless otherwise indicated. Culture media (RPMI 1640, Iscove's DME) and supplements (100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 10% fetal calf serum, and insulin/selenium/transferrin growth supplement; see below) were from Gibco Ltd.(Paisley, UK). Tissue culture flasks and plates were from Flow Labs (Rickmansworth, UK). All cultures were carried out in humidified air with 5% CO_2 at 37°C.

Neutrophil preparation

Neutrophils (>98% pure on May-Giemsa stained cytopreps) were isolated from fresh, citrated normal human blood by dextran sedimentation and discontinuous plasma-Percoll (Pharmacia Fine Chemicals, Piscataway, New Jersey, USA) density gradient centrifugation, aged in tissue culture for ~24 hours in Iscove's DME with 10% autologous platelet-rich plasma-derived serum, and apoptosis quantified by oil-immersion microscopy of May-Giemsa stained cytopreps, exactly as described [11]. Viability was assessed by trypan blue dye exclusion; on the rare occasions that aged neutrophil viability was <98%, cells were not used.

In some experiments, fractions with varying degrees of apoptosis were prepared from individual aged neutrophil populations by centrifugal elutriation, as described [11]. Briefly, washed aged neutrophils were loaded into a J2-21 elutriation rotor (2500 rpm) and centrifuge system (Beckman Instruments Inc, Palo Alto, California, USA) at 14 ml/min, the pump brought to 28 ml/min and then fractions collected at 30, 32, 34, 37, 40 and 44 ml/min. Viability in excess of 98% by trypan blue exclusion was required for further use: apoptosis was quantified as above.

In further experiments designed to examine whether neutrophil disintegration was required before uptake by mesangial cells, freshly isolated and aged neutrophils were fixed in 2% paraformaldehyde in PBS and washed before use, and freshly

Fig. 5. *Interaction of human neutrophils with human glomerular mesangial cells in adherent culture.* **A.** Large-scale interaction of orange/brown staining, myeloperoxidase-positive aged neutrophils with mesangial cells (×200). **B.** Apparent phagocytosis of aged neutrophils seen at higher power (×500). **C.** Freshly isolated neutrophils appear merely to adhere to the mesangial cells (×500).

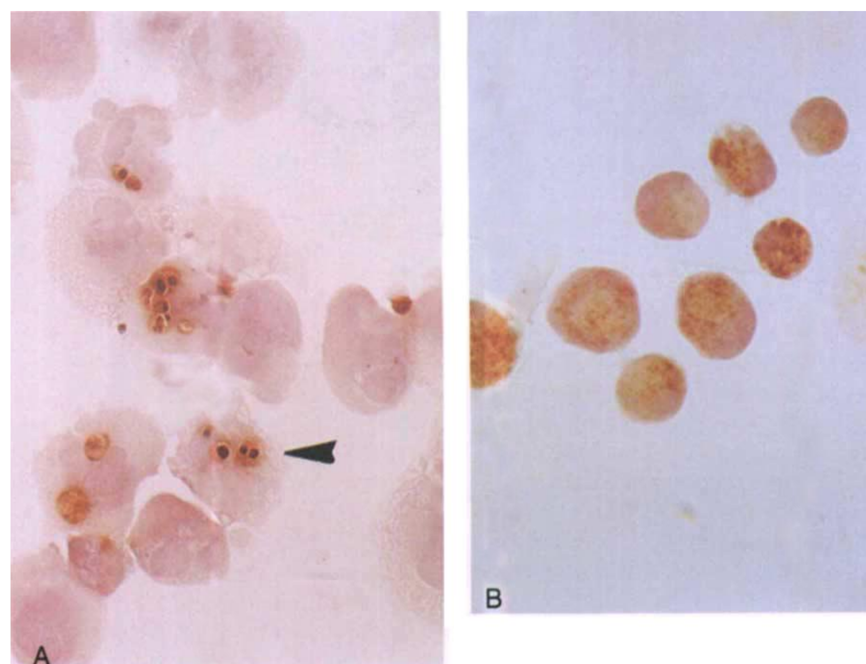

Fig. 6. *Comparison of mesangial cell phagocytosis of aged neutrophils with endocytosis of myeloperoxidase, demonstrated by trypsin-mediated dissociation of mesangial cells from adherent culture after interaction with neutrophils.* **A.** Light microscopy (×400) of cytoprep demonstrating many orange/brown, myeloperoxidase-positive neutrophils within rounded-up mesangial cells. Note some ingested neutrophils still retain identifiable nuclei (example arrowed), while in others chromatin is no longer identifiable. Compare with Fig. 1D. **B.** Light micrograph (×400) of cytoprep of mesangial cells taking up soluble purified myeloperoxidase despite prior incubation with 10^{-6} M colchicine. Identical appearances were observed in mesangial cells interacted with debris from deliberately disrupted neutrophils. Note fine granular pattern of staining different from that obtained after interaction with aged neutrophils.

isolated neutrophils were fragmented by freeze/thaw cycles on solid CO_2 followed by mechanical disruption by repeated aspiration through a 19G needle.

Culture of human mesangial cells

Mesangial cells were prepared by standard methods of serial culture/trypsinization in tissue culture flasks of adherent outgrowth cells from glomeruli obtained by sieving of normal cortex from kidneys removed because of renal cell carcinoma, as described [20, 21]. Cells were cultured in RPMI 1640 with 10% fetal calf serum and supplements as above, including 5 ml/500 ml growth supplement (25 mg/ml insulin from bovine pancreas, 25 mg human transferrin, and 25 μg/ml sodium selenite). The cells were used between passages 4 and 6 after subculture into 24-well plates.

Considerable care was taken to verify the phenotype of mesangial cells, as in our previous studies [20, 21]. Mesangial cells exhibited typical stellate morphology when subconfluent, while upon becoming confluent they adopted the well-recognized "elongated" conformation; if cultured beyond confluence typical "hillocks" were seen [22]. Upon electron microscopy (see below), mesangial cells exhibited characteristic features such as abundant microfilaments, and upon immunofluorescence were seen to contain organized α-smooth muscle actin (not shown; previously presented in [21]), excluding the remote possibility that these cells were fibroblasts. In particular, there was no evidence that macrophages had persisted to contaminate mesangial cell cultures; these cells did not exhibit macrophage markers such as LFA-1 integrin expression (assessed by immunofluorescence flow cytometry) or acetylated LDL uptake, nor did they exhibit macrophage functional properties such as phagocytosis of opsonized zymosan particles. Furthermore, in addition to lack of acetylated LDL uptake, lack of immunostaining for Factor VIII-related antigen and cytokeratin excluded contamination with glomerular endothelial or epithelial cells respectively (data not shown).

Assays of mesangial cell uptake of aged neutrophils

In all experiments cycling mesangial cells growing in culture medium with serum were used at passages 4 to 6. The culture medium was aspirated from mesangial cells subcultured in 24 × 1 cm^3 well plates, and replaced with 1 ml of a 2.5 × 10^6/ml suspension of neutrophils in RPMI 1640 with 10% FCS (in some experiments lower percentages of FCS or platelet-rich plasma-derived human serum autologous to the neutrophils were used). The neutrophils rapidly settled into a "carpet" in close apposition to the adherent mesangial cells and the two cell types were "interacted" by co-culture for three hours at 37°C and 5% CO_2. At the end of this period non-adherent neutrophils were dislodged by a standardized protocol of vigorous manual washing with a Pasteur pipette, adapted from that described for macrophage interaction with aged neutrophils [10].

In preliminary experiments the washed mesangial cell monolayer was fixed with 2% glutaraldehyde in PBS, and then stained for myeloperoxidase (MPO) using hydrogen peroxide and dimethoxybenzidine (*o*-diansidine) as previously described [10, 11, 23]. However, quantitation of neutrophil uptake was achieved by trypsinizing the washed (unfixed) mesangial cells at the end of the interaction, and preparing a separate cytocentrifuge preparation for each well. This was then fixed and stained for MPO as above, before being counterstained with Haemulum (BDH, Poole, UK). The proportion of mesangial cells containing MPO-positive globules wholly within the outline of the cell was then counted by light microscopy: a minimum of 500 cells per slide was examined and the results expressed as a percentage. In some experiments mesangial cells released from adherent culture after interaction with aged neutrophils were fixed in 2% glutaraldehyde in pH 7.2 phosphate buffer prior to preparation for electron microscopy (see below).

Effects of colchicine

In order to demonstrate that apparently ingested neutrophils had been taken up by active phagocytosis, mesangial cells were washed and then incubated in RPMI 1640 alone or with 10^{-6} M colchicine for one hour. Following loading with colchicine, mesangial cells were then washed twice and incubated with aged neutrophils (2.5 × 10^6 per well in RPMI 1640 with 10% FCS) for two hours, before washing, trypsinization, preparation of cytospins and staining for MPO as above. As a control, we studied the effects of the same protocol of colchicine treatment upon mesangial cell endocytosis over two hours of purified MPO (Calbiochem) at 2.5 μg/ml in RPMI 1640 with 10% FCS. Uptake of MPO was assessed by the same methods as neutrophil ingestion. As a further control to confirm that MPO was indeed being taken up by active endocytosis, mesangial cells treated with colchicine or medium alone for one hour were incubated with MPO for two hours at 4°C before preparation of cytospins.

Isolation of glomerular cells from rats with experimental nephritis

Nephrotoxic nephritis was induced in 200 g Sprague-Dawley rats by standard means: rats were preimmunized with 1 mg rabbit immunoglobulin in 50% complete Freund's adjuvant/50% saline divided between four subcutaneous sites, and then challenged at seven days with 1.5 ml i.v. of a rabbit anti-rat kidney serum. Two days later the rats were killed and glomerular cells isolated as described [24]. Briefly, glomeruli were prepared by sieving of crushed strips of cortex and a single cell suspension obtained by enzymatic digestion as follows: glomeruli were incubated at 37°C for 20 minutes in HBSS containing 0.5 mg/ml trypsin (Type III), 1 mg/ml collagenase (Type 1) and 0.1 mg/ml deoxyribonuclease (DNAase type III), washed in HBSS with no Ca^{++}/Mg^{++} and then incubated at 4°C in 2 mM disodium EDTA in the same buffer for 20 minutes. The supernatant containing isolated cells was removed and kept on ice, and the partially digested glomeruli were incubated with 1 mg/ml collagenase at 37°C in HBSS for a further 20 minutes before final dissociation by passage three times through a 23 gauge needle. The cells were then either used to prepare cytocentrifuge preparations or fixed in 2% glutaraldehyde in pH 7.2 phosphate buffer for two hours of preparation for electron microscopy (see below).

Cytocentrifuge preparations of glomerular cells were studied as follows. Cell morphology was assessed in methanol-fixed slides stained with May-Giemsa. Cells ingesting MPO-positive material were identified in glutaraldehyde-fixed slides by staining for MPO as above and counterstaining with Haemulum.

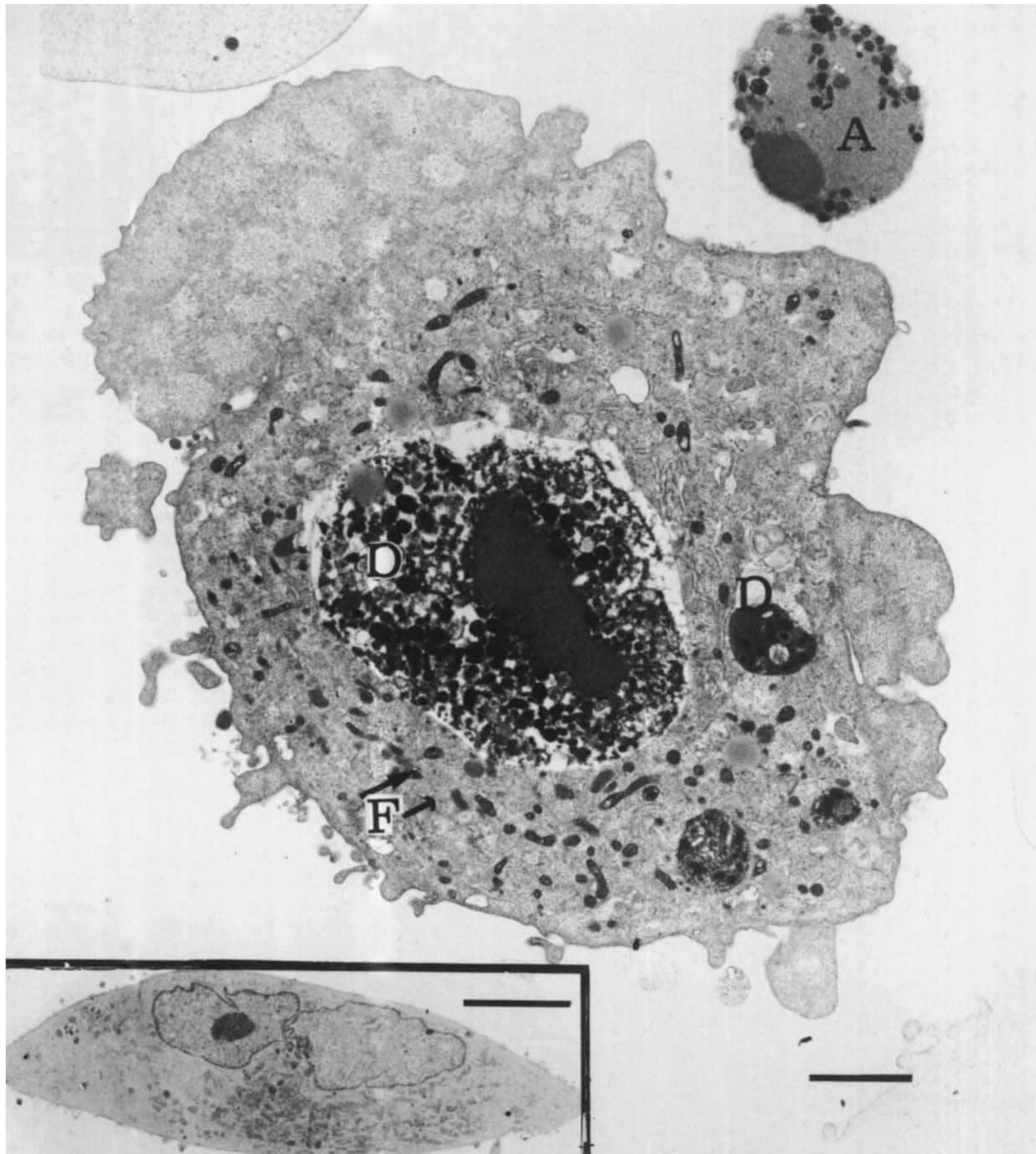

Fig. 7. *Cultured human mesangial cell phagocytosis of aged neutrophils.* Electron micrograph (bar = 2 microns) of mesangial cell enzymatically dissociated from adherent culture after interaction with aged neutrophils. Although the phagocyte nucleus is out of plane of section, this cell is identifiable as a mesangial cell by its abundant microfilaments (F). Ingested neutrophil material (D) takes the form of an apparently intact neutrophil with condensed nuclear chromatin characteristic of apoptosis, and smaller fragments of granular material: a free neutrophil-derived apoptotic body is also seen (A). The inset shows a section of a mesangial cell in adherent culture to demonstrate usual fusiform morphology (bar = 10 microns).

Acetone-fixed preparations were also immunostained as follows. Monocyte/macrophages were identified with the specific murine monoclonal antibody ED1 (Serotec, Banbury, Oxon, UK). Slides were incubated for 60 minutes at 21°C in ED1 at 1:2000 dilution in PBS/0.2%BSA, washed three times and blocked with hydrogen peroxide (0.1% in methanol). After a

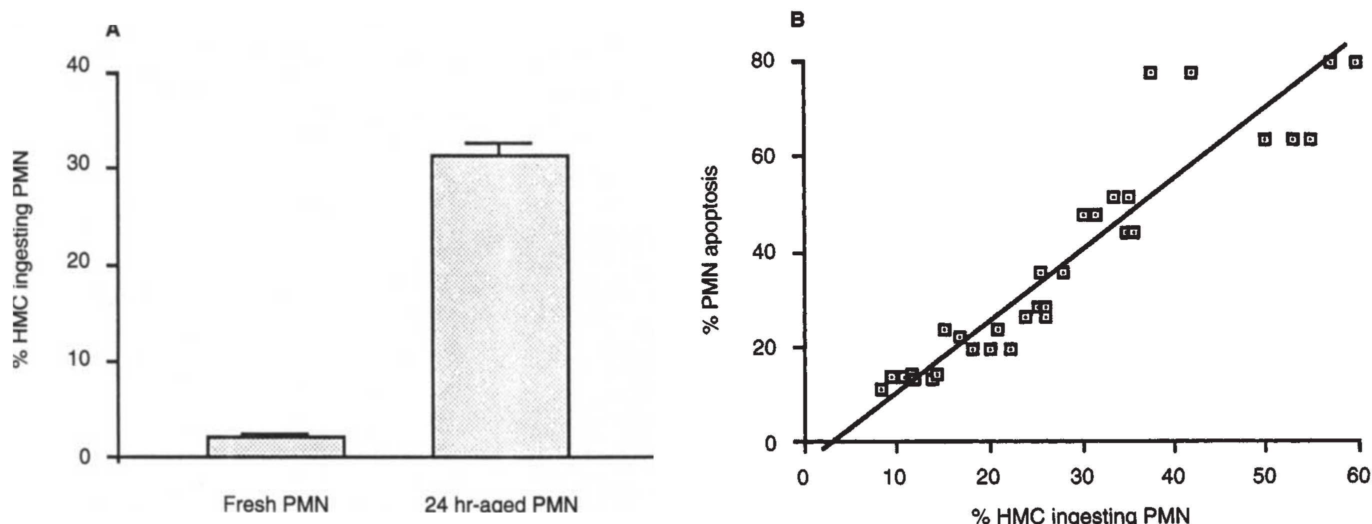

Fig. 8. *Dependence of mesangial cell phagocytosis of aged neutrophils upon apoptosis.* **A.** Preferential interaction with aged neutrophils (56 ± 4.5% apoptotic by light microscopy) 31.5 ± 1.3% (mean ± SE, $N = 33$) of mesangial cells took up aged neutrophils, but there was minimal recognition of freshly isolated neutrophils (2.2 ± 0.1%). **B.** Close correlation between percentage of mesangial cells taking up aged neutrophils within any one fraction of aged neutrophils prepared from single populations by centrifugal elutriation, and percentage of aged neutrophils appearing apoptotic by light microscopy within that fraction (from 11.1 to 79.4%); data from three experiments; $r = 0.96$, $P < 0.0001$.

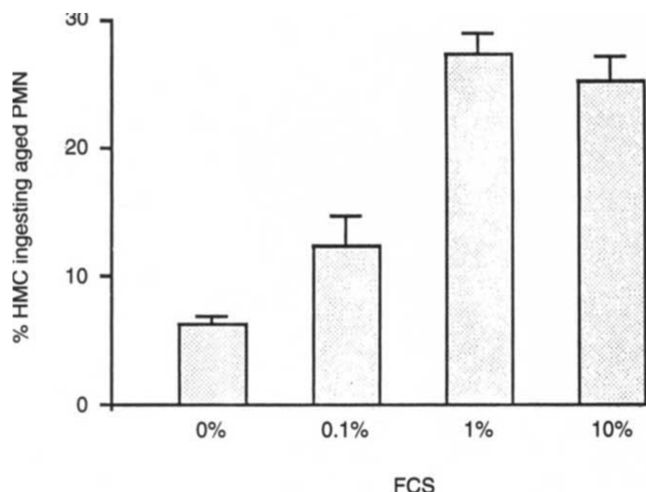

Fig. 9. *Partial serum dependence of mesangial cell uptake of aged neutrophils.*

further three washes bound antibody was detected with peroxidase-labeled anti-mouse immunoglobulin antibody (DAKO) at 1:40 dilution in PBS/0.2% BSA/1% normal rat serum, washed three times, developed for two to five minutes with diaminobenzidine hydrochloride (Sigma: 0.5% in PBS/0.1% hydrogen peroxide), washed and then counterstained with hematoxylin. Mesangial cells were identified with OX7, a monoclonal antibody specific for Thy 1.1 antigen (Serotec-used at 1:100) by identical methods.

In some experiments, glomerular cell populations were washed in PBS and immunolabelled by standard methods at 4°C with OX7 mouse monoclonal antibody to rat Thy1.1; cells were incubated with OX7 at 1 in 30 dilution in PBS-0.1% BSA for 30 minutes, washed three times and then incubated with FITC-conjugated $F(ab')_2$ sheep anti-mouse immunoglobulin antibody (Sigma) at 1 in 30 dilution in PBS-0.1% BSA for 30 minutes. Following three washes in PBS, cells were suspended at 1 × 106/ml in PBS at 4°C and then sorted on the basis of positive fluorescence (determined by comparison with cells labeled with an irrelevant isotype control monoclonal antibody) by FACS (EPICS CS, Coulter, Hialeah, Florida, USA). On re-analysis of fluorescence of "Thy1.1-positive" cells, over 90% expressed Thy1.1, indicating that while not entirely pure, the sorted population was "enriched" for mesangial cells.

Electron microscopy

Following fixation in 2% glutaraldehyde, cell suspensions were washed in phosphate buffer, osmicated and dehydrated in a series of graded alcohols. Between all solution changes cells were spun at 100 g, the supernatant removed and the pellet resuspended in the next solution. The final pellet was suspended in Taab resin and polymerized at 60°C overnight. Sections of 1 μm were cut and stained with toludine blue for observation at light microscopy level. Ultrathin sections of approximately 100 nM were then cut, collected on nickel grids and stained with uranyl acetate and lead citrate, for observation on a Philips CM10 electron microscope.

Statistical analysis

The Macintosh Statworks program was used for calculations.

Results

Neutrophil apoptosis and uptake by glomerular cells in vivo

Cytocentrifuge preparations of dissociated glomerular cells obtained from rats with nephrotoxic nephritis were examined by light microscopy. In keeping with our studies of inflammation at other sites, free apoptotic cells were identifiable by well-established criteria of chromatin condensation and loss of

Table 1. Effect of one hour preincubation with colchicine upon percentage of human mesangial cells taking up myeloperoxidase-positive material: Comparison of phagocytosis of aged neutrophils with temperature-dependent endocytosis of soluble myeloperoxidase

	Mesangial cells co-incubated with aged neutrophils	Mesangial cells co-incubated with myeloperoxidase
	(% MPO-positive: mean ± SE)	
Control 2 hr co-incubation at 37°C	32.1 ± 1.0	90.4 ± 3.6
Colchicine 10^{-6} M pre-treatment 2 hr co-incubation at 37°C	0.6 ± 0.1	91.4 ± 1.8
Colchicine 10^{-6} M pre-treatment 2 hr co-incubation at 4°C	not done	1.2 ± 0.2

cell volume (Fig. 1a). Identical cells could also be seen within phagocytes, many of which were identified as being of monocyte/macrophage origin by positive immunostaining for ED1. However, some phagocytes containing apoptotic cells were ED1-negative (Fig. 1b) and in separate preparations comparable cells containing apoptotic bodies were seen to be Thy1.1-positive mesangial cells (Fig. 1c), indicating that these cells are also capable of taking up apoptotic bodies.

At the light microscopical level the appearances of cells undergoing apoptosis are stereotyped, so that it may be difficult to determine the cell type from which the apoptotic body has arisen [12, 13]. Possible myeloid (that is, neutrophil or monocyte) origin of ingested apoptotic cells was examined in glutaraldehyde-fixed preparations by seeking phagocytes containing discrete globules of myeloperoxidase (MPO)-positive material. This method has the advantage of identifying myeloid cell-containing phagosomes in which chromatin has been degraded, an event known to be rapid [10, 11, 16, 23]. Phagosomes containing MPO-positive material were seen within glomerular cells, in many cases appearing identical to those reported in previous studies of neutrophil uptake [10, 11, 16, 23]. Although some material may have been derived from monocytes, this supported the possibility that phagocytic clearance of neutrophils undergoing apoptosis was occurring in vivo. Indeed, a role for mesangial cells in this process was supported by the demonstration of cells containing MPO-positive globules amongst glomerular cell populations enriched for mesangial cells (Fig. 1d). This was done by FACS sorting on the basis of expression of the rat mesangial cell marker Thy 1.1 [25], yielding populations greater than 90% Thy 1.1-positive. Unfortunately, the number of such "mesangial-enriched" cells obtained by FACS sorting proved to be insufficient for electron microscopy, which would have been necessary to confirm both the neutrophil origin of ingested material and the mesangial phenotype of the sorted cells.

However, electron microscopy of freshly-dissociated glomeruli from nephritic rats confirmed this preliminary evidence of neutrophil apoptosis leading to phagocytosis by glomerular cells. As in previous studies [11, 16] occasional examples of free apoptotic neutrophils were observed, the illustrated cell (Fig. 2b) being in an early stage of the process, exhibiting the typical condensation of nuclear chromatin into crescents within each lobe of the nucleus [12, 13]. Apparently intact neutrophils exhibiting the cytoplasmic condensation and coalescence of nuclear lobes typical of the later stages of apoptosis were also observed within phagocytes (Fig. 3a,b,c). Nevertheless, in keeping with previous studies, the majority of ingested neutrophils within inflammatory macrophages were in variously advanced stages of degradation (Fig. 3b).

Within the preparation glomerular mesangial cells were easily identified by their characteristic pattern of nuclear heterochromatin, relatively electron dense cytoplasm, paucity of organelles by comparison with monocyte-macrophages, complex cytoplasmic protrusions often localized to one pole of the cell, and occasional presence of surrounding matrix (Fig. 4a) [17, 24]. Furthermore, mesangial cells did not exhibit phagolysosomes containing electron-dense "grey" material, which were abundant in macrophages (compare Figs. 3a and 3b with Fig. 4a). Many mesangial cells exhibited phagosomes containing cellular debris consisting of dense chromatin fragments and granule remnants which, on the basis of previous studies of macrophages [10, 16], appeared compatible with apoptotic neutrophils in the process of degradation (Fig. 4b). However, no definite example of a recently ingested intact apoptotic neutrophil could be demonstrated. Because this evidence was suggestive but not conclusive, we turned to in vitro experiments to confirm that mesangial cells can phagocytose apoptotic neutrophils.

Cultured mesangial cells take up and degrade intact "aged" neutrophils

Cycling human mesangial cells at passages 4 to 6 cultured in 24-well plates were interacted for three hours (in the presence of 10% fetal calf serum) with 24-hour-aged human neutrophils, which were >98% pure by cytology and >98% viable by trypan blue exclusion. Upon staining and light microscopic examination of the washed and fixed monolayers, aged neutrophils appeared to have been taken up by mesangial cells (Fig. 5a and 5b), whereas freshly isolated neutrophils appeared to be merely adherent (Fig. 5c).

To confirm phagocytosis, the monolayer was trypsinized at the end of the interaction to detach mesangial cells from the plate, and to dissociate attached but non-ingested neutrophils. Cytopreps (Fig. 6a) of such mesangial cell suspensions demonstrated that apparently intact neutrophils had indeed been ingested during the interaction. Nevertheless, some ingested material appeared by light microscopy to be in the form of discrete myeloperoxidase (MPO)-positive globules in which neutrophil nuclei could not be identified (Fig. 6a), as observed in previous in vitro and in vivo studies of the rapid uptake and degradation of apoptotic neutrophils by macrophages [10, 11, 16]. Mesangial cells did take up both MPO-positive material from deliberately fragmented neutrophils and purified MPO. However, in each case the MPO-positive material was distributed in fine granules throughout the mesangial cell cytoplasm (Fig. 6b), the appearances being totally different from those obtained with intact aged neutrophils (Fig. 6a). Furthermore, uptake of MPO-positive material by mesangial cells co-incubated with aged neutrophils was almost completely inhibited by pre-incubation of mesangial cells with colchicine, which inhibits microtubular assembly required for phagocytosis (Table 1). By contrast, mesangial cell endocytosis (verified by demonstration of temperature dependence) of purified MPO was not affected

by colchicine (Table 1, Fig. 6b). Taken together with uptake of paraformaldehyde-fixed aged neutrophils (data not shown), this indicated that MPO-positive material within mesangial cells was derived from active phagocytosis of intact neutrophils, not from possible invagination of mesangial cells by aged neutrophils, or from uptake of MPO which might have been released outside the mesangial cell as a consequence of undetected neutrophil necrosis and disintegration. Finally, while electron micrographs of mesangial cells trypsinized from adherent culture after interaction with aged neutrophils showed ingested but partially degraded neutrophils, apparently intact apoptotic cells were also seen (Fig. 7), particularly if the co-incubation time was reduced from three hours to one hour.

Dependence upon apoptosis in the aged neutrophil and the presence of serum

Neutrophils aged in culture undergo apoptosis which leads to recognition by macrophages; freshly isolated neutrophils are not recognized [10, 11]. Similarly, we observed minimal mesangial cell uptake of freshly isolated neutrophils compared with aged neutrophils (Fig. 8a). Furthermore, there was minimal uptake of paraformaldehyde-fixed fresh neutrophils, although MPO-positive material from disrupted freshly isolated neutrophils was avidly taken up and distributed throughout the cytoplasm (appearances were identical to Fig. 6b). These data indicated that mesangial cell uptake of aged neutrophils occurs by mechanisms specific for changes undergone by the *intact* neutrophil during "aging" in culture.

In previous studies of macrophage uptake of aged neutrophils we demonstrated dependence upon apoptosis by employing centrifugal elutriation of individual populations of aged neutrophils to prepare fractions of the same in vitro "age" but with varying proportions of apoptosis. Recognition of a particular fraction was closely correlated with the proportion of neutrophils within that fraction showing light microscopical evidence of apoptosis [11]. Directly comparable results were obtained for mesangial cell uptake of aged neutrophils (Fig. 8b), indicating that apoptotic cells were those recognized within a population of aged neutrophils. This tallied with electron microscopy of the interaction—in those ingested neutrophils where nuclear morphology could be assessed with reasonable confidence (that is, presumably recently ingested neutrophils in the earliest stages of degradation), the nucleus demonstrated apopotic appearances in every case (such as Fig. 7).

However, by contrast with macrophage recognition of aged neutrophils, mesangial cell uptake of these cells was partially dependent upon the presence of serum during the three hours of the interaction (Fig. 9). Data shown are for heat-inactivated fetal calf serum: similar results were obtained with human serum (not shown).

Discussion

In this paper we report in vivo and in vitro data which taken together indicate that apoptosis in intact senescent neutrophils leads to recognition and phagocytosis by both glomerular mesangial cells and glomerular inflammatory macrophages. Apoptosis is a "programmed" form of cell death which accounts for physiological deletion of "unwanted" cells in situations such as embryological remodelling, metamorphosis, endocrine-dependent organ atrophy, tumor regression and in the turnover of normal tissues [12, 13]. The biochemical hallmark of apoptosis/programmed cell death is internucleosomal DNA cleavage indicative of endogenous endonuclease activation [14]. Cells undergoing apoptosis also exhibit a typical series of structural changes, the most prominent of which is condensation of nuclear chromatin. They remain intact, do not spontaneously release cell contents (which in the case of the neutrophil include potentially injurious granule contents; [11]), and are swiftly recognized, ingested and degraded by local phagocytes. However, as a consequence of such rapid clearance, identifiably apoptotic cells may be scarce, even in tissue where large numbers of cells are being deleted in this manner [11–13, 26].

The fate of neutrophils accumulating within the inflamed glomerulus has been obscure. However, there is evidence that neutrophil apoptosis leading to removal by phagocytes is likely to be a generally important mode of neutrophil "disposal" operating at inflamed sites in humans: apoptotic neutrophils, and macrophages which have recently ingested such cells, can be observed in inflammatory exudates from joints [11] and the respiratory tract [16]. Furthermore, previous studies provide preliminary evidence that these mechanisms may operate in the inflamed glomerulus [17–19]. The ultrastructural studies presented here now provide clear evidence that neutrophil apoptosis leading to uptake by inflammatory macrophages does indeed occur in glomerulonephritis. Although it is possible that the observed "free" apoptotic neutrophils might have undergone this process ex vivo during the short period that glomerular cells were being dissociated with enzymes at 37°C, apparently intact neutrophils with morphology typical of the later stages of apoptosis were observed within phagocytes (Fig. 3). Since the trypsin treatment used in the first stage of cell preparation is known to abolish the ability of cultured macrophages to ingest up apoptotic cells in vitro [27], these observations provide very strong evidence that neutrophil apoptosis and uptake by phagocytes do indeed occur in the inflamed glomerulus.

The current work also provides evidence that glomerular mesangial cells have a previously unrecognized ability to participate in clearance of neutrophils undergoing apoptosis. In previous studies such a role for the mesangial cell had been suggested by an ultrastructural report of intact apoptotic bodies of unknown cellular origin within a glomerular mesangial cell [19]. This was in a patient with intense but short-lived glomerular injury due to hemolytic-uremic syndrome, where an unusually high proportion of cells could be seen to be undergoing this type of programmed death. In the current study dissociated glomerular cells from rats with nephrotoxic nephritis were examined by light microscopy. Apoptotic cells were evident by their dense nuclear chromatin, and in addition to being found "free" in the cell suspension, apparently identical bodies were seen within ED1-negative glomerular cells with morphology different from macrophages. Such phagocytes were identified as mesangial cells as they expressed the marker Thy 1.1 [25]. Possible myeloid (that is, neutrophil or monocyte) origin of ingested cells was suggested by the presence of discrete globules of MPO-positive material within Thy1.1-enriched glomerular cells prepared by FACS, appearances similar to those obtained in previous studies of swift macrophage ingestion and

degradation of apoptotic neutrophils [10, 11, 16, 23]. In dissociated glomerular cells examined by transmission electron microscopy mesangial cells could be identified by well-established morphological criteria [17, 24]. Although no clear example of an intact apoptotic neutrophil within a mesangial cell could be identified, many mesangial cells exhibited phagosomes containing cellular debris apparently consisting of dense chromatin fragments and granule remnants, similar to phagosome contents seen in previous in vitro and in vivo studies of macrophage ingestion of apoptotic neutrophils [10, 11, 16, 23]. Although not conclusive, taken together with the ultrastructural demonstration of apoptotic neutrophils within the glomerular cell preparation, this evidence strongly supports the notion that mesangial cell ingestion of neutrophils undergoing apoptosis occurs in the inflamed glomerulus.

Nevertheless, because this evidence was suggestive rather than conclusive, it was necessary to adopt a complementary strategy to confirm that mesangial cells are capable of ingesting intact apoptotic neutrophils. This was done in vitro using methods previously employed to demonstrate that macrophages recognize apoptotic neutrophils. We found that cultured human mesangial cells ingested apparently intact human neutrophils which had been "aged" for 24 hours in culture, but freshly isolated neutrophils were not ingested. The specific inhibitory effects of colchicine pretreatment of mesangial cells confirmed active phagocytosis of aged neutrophils. The apoptotic subpopulation of aging neutrophils was that recognized by mesangial cells, as demonstrated by the recognition of fractions with varying degrees of apoptosis prepared from the same populations by centrifugal elutriation. Furthermore, within the three hour duration of the interaction many ingested neutrophils were apparently degraded, exhibiting morphology similar to that observed in mesangial cells from nephritic animals.

Obviously, it is possible that the capacity to ingest apoptotic neutrophils could reflect a change in the properties of the mesangial cell consequent upon culture. However, if this is the case than it is not a general response to in vitro conditions, since we have observed that cultures of human glomerular epithelial cells, human umbilical vein endothelial cells, bovine vascular smooth muscle cells and a range of tumor cell lines do not ingest apoptotic neutrophils under the conditions employed in the current study (S. Hall, J.S. Savill, P.M. Henson and C. Haslett, manuscript in preparation). Furthermore, in addition to ingestion of apoptotic neutrophils by mesangial cells purified by repeated passage in culture, we have observed in seven-day primary cultures that cells with similar morphology growing out of adherent glomeruli can also ingest aged neutrophils (data not shown), confirming that this property is not consequent upon cellular alterations due to long-term culture. Finally, it seems inherently unlikely that the ability to undertake the complex function of specific recognition and phagocytosis of apoptotic (but not "aged" non-apoptotic) neutrophils should represent an in vitro artifact of no in vivo relevance. Therefore, taken together with evidence from previous studies, the findings reported here constitute persuasive evidence that, in addition to uptake by macrophages, apoptotic neutrophils may also be phagocytosed by mesangial cells in vivo.

Nevertheless, the mesangial cell is not generally considered to be a "professional" phagocyte. Moreover, early reports of phagocytic potential [28] were later attributed to contamination of glomerular cell preparations by bone marrow-derived monocyte-macrophages [29]. However, despite the fact that an average of just over 30% of cultured cells took up apoptotic neutrophils, no such contamination could be demonstrated in our cultures. Furthermore, Baud et al took great pains to exclude macrophage contamination in their cultures of rat mesangial cells, which could be induced to phagocytose zymosan particles by prior dissociation from adherent culture [30]. Finally, various cell types which are not generally recognized as phagocytes can take up apoptotic cells in vivo. Such "semi-professional" phagocytes include epithelial and tumor cells [12, 13]. Therefore, it is not unexpected that the mesangial cell should be able to phagocytose neutrophils undergoing apoptosis. Indeed, a role for the mesangial cell in clearance of particulate debris from glomeruli has been recognized for many years [31]. The capacity to clear apoptotic cells does not appear incompatible with this property, serving only to emphasize a possible "cleansing" function for the mesangial cell.

The findings described here raise a number of questions which will need to be addressed in future studies. Firstly, the cell surface mechanisms by which mesangial cells recognize and ingest apoptotic neutrophils need to be defined. The dependence upon serum of this event suggests that these mechanisms may differ from those employed by the macrophage [11, 15, 23]. Secondly, although the proportion of mesangial cells taking up aged neutrophils was shown by elutriation to be dependent upon the proportion of aged neutrophils which had undergone apoptosis, it may prove possible to define phenotypic markers of a (large) subpopulation of cultured mesangial cells capable of phagocytosis of aged neutrophils. Finally, the in vivo significance of these mechanisms is not indicated by the present data. For example, the relative load of apoptotic neutrophils borne by mesangial cells by comparison with glomerular inflammatory macrophages is unknown. Monocytes must mature into macrophages before they can take up apoptotic neutrophils, a process which is likely to take at least 48 hours, whereas in vitro neutrophil apoptosis occurs more rapidly. Consequently, it is possible that the mesangial cell may represent an important route of neutrophil disposal early in the inflammatory response before monocytes have matured. Additionally, mesangial cells may come into play if macrophages have been overwhelmed by large numbers or repeated influx of neutrophils. Further studies will be needed to examine these possibilities.

In conclusion, we report that in glomerulonephritis infiltrating neutrophils may be eliminated by apoptosis, which leads to recognition and uptake of intact senescent neutrophils by both glomerular mesangial cells and glomerular inflammatory macrophages. These processes have potential to limit neutrophil-mediated glomerular injury and may play a role in determining whether there is resolution of glomerular inflammation: failure of these mechanisms might lead to disintegration of neutrophils within the inflamed glomerulus, direct exacerbation of tissue injury by toxic neutrophil contents, amplification of leucocyte infiltration by enzymatic generation of chemotactic factors, and the development of persistent inflammation leading to scarring [11, 32].

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