Evidence for Cell Death in the Vascular Endothelium in Vivo *and* in Vitro

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Focal, spontaneous cell death in the rat aortic endothelium was demonstrated by cytochemistry. Cells with intracellular calcium deposits, indicating cell death with mitochondrial calcification, were identified by chlorotetracycline fluorescence. The same cells also contained cytoplasmic IgG, which binds to cytoskeletal components of the dead cell. The immunocytochemical detection of IgG in *en face* preparations was used as a quantitative method for detecting cell death in the aortic endothelium. The use of an indirect immunoperoxidase technique and "Häutchens" of paraformaldehyde-fixed tissue provided high sensitivity and cellular recovery with low background. A cell death frequency of 0.19% ± 0.04% was observed in 5-month-old Sprague–Dawley rats. When compared with the replication rate of aortic endothelium in these animals, the data suggest that dead cells remain in the endothelium for more than 24 hours. This conclusion was supported by *in vitro* studies. Confluent cultures of bovine aortic endothelium were pulsed with trypan blue, and the residence time of blue cells was 3.5–4 days in the nonflow culture system. Time-lapse video microscopy showed a prolonged cell death process with a phase of rapid intracellular movements, followed by undermining by surrounding cells and fragmentation of the dead cell. Migration of surrounding cells rapidly covered partial detachments of the dead cell, so that no holes could be detected in the monolayer when the dead cell finally detached. It is concluded that the normal turnover of cells in the aortic endothelium involves a prolonged phase of *in situ* cell death and finally detachment with very little or no exposure of subendothelial structures. (Am J Pathol 1983; 112:278–286)

ENDOTHELIAL CONTINUITY is vital to vascular homeostasis and to the prevention of thrombosis, atherosclerosis, and vascular inflammatory diseases.[1,2] There is, however, evidence of functional heterogeneity in the endothelium. Most of the data come from studies of the endothelial lining of the aorta and large arteries. Permeability to plasma proteins is increased in focal areas[3,4]; and while overall rates of cell turnover are very low, there are clusters of increased cell replication.[5] Gerrity et al have presented data indicating that these two phenomena are associated and have suggested that endothelial cell dysfunction and injury at sites of cell loss may be responsible for the elevated permeability.[4] Similarly, the labeling index increases in response to noxious stimuli such as endotoxemia,[6,7] again suggesting that endothelial cells die. Such cell death would stimulate replication of other cells in the population.

Very little is known about cell death in the endothelium of the intact animal. Transmission electron micrographs have shown cells with pyknotic nuclei, membrane discontinuities, disrupted organelles, and loss of cytoplasmic content after treatment with various agents.[2,8,9] The frequency of ultrastructural changes is, however, difficult to determine, and their interpretation is often rather subjective. Scanning electron microscopy provides larger samples and should therefore be better suited for quantitative evaluation of cell injury and death in the endotheli-

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um. The cell death process is, however, reflected in the surface ultrastructure only at a later stage, and the scanning electron micrographs are therefore difficult to interpret.[9] As an alternative approach, Björkerud et al used cytochemical stains such as the viability indicator, Evans blue.[10] Incubation of aortas with this dye demonstrated the presence of cells unable to exclude the dye, but the frequency of these cells *in vivo* was difficult to determine because of the risk of introducing artifacts during staining.

In this paper we describe an immunocytochemical technique for identifying and quantitating dead endothelial cells in the aorta *in vivo*. The observations confirm our earlier finding of IgG binding to endothelial cells with ultrastructural signs of injury[11,12] and extend the earlier study by establishing a quantitative technique. The correlation to cell death was established by the demonstration of calcium deposits in the IgG-containing cells and by the monitoring of the process of cell death in cultured endothelial cells showing similar IgG-binding properties.

Materials and Methods

Reagents

Rabbit anti-rat IgG, fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit IgG, tetramethyl rhodamine (TRITC)-conjugated rabbit anti-rat IgG, and horseradish peroxidase (HRP)-conjugated swine anti-rabbit IgG were purchased from Dako, Copenhagen, Denmark, and rabbit anti-bovine IgG from Cappel, Cochranville, Pennsylvania. These immunoglobulin fractions of the specific antiserums were used at optimal dilutions according to chessboard titrations, usually 1:100 for the indirect immunoperoxidase technique and 1:40 for the immunofluorescence technique. Dilutions were in phosphate-buffered saline (PBS, 150mM NaCl, 10mM phosphate buffer, pH 7.2) containing 1% ovalbumin (Sigma Chemical Company, St. Louis, Mo.).

Horseradish peroxidase (Type II), diaminobenzidine tetrahydrochloride (DAB), chlorotetracycline (CTC), and oxytetracycline (OTC) were all obtained from Sigma.

Animals

Twenty 5-month-old male albino rats of the Sprague–Dawley strain were used for the *in vivo* experiments. They were fed normal rat pelleted food and water *ad libitum*.

Preparation of Tissue for Immunocytochemistry

Rats were perfused at 100 mm Hg for 10 minutes with 4% paraformaldehyde in phosphate buffer via a catheter in the left carotid artery, and the aorta was excised and immersed in the same solution for 1 hour. The thoracic aorta was divided into four pieces, which were pinned onto Teflon and rinsed four times for 15 minutes each with PBS. They were then incubated with rabbit anti-rat IgG for 30 minutes, rinsed three times for 10 minutes each with PBS, incubated 30 minutes with HRP–swine anti-rabbit IgG, and rinsed three times for 10 minutes each. Peroxidase was visualized by a modification of the technique described by Graham and Karnovsky.[13] The specimens were first incubated for 15 minutes in 0.50 mg/ml DAB in 50 mM Tris buffer, pH 7.6, and then for 2 minutes in the same solution containing 0.01% hydrogen peroxide. After repeated rinsing, the specimens were immersed in 4% paraformaldehyde overnight. Häutchen preparations were made according to Schwartz and Benditt[14] and stained with Harris's hematoxylin.

Quantitation of IgG-Containing Cells

Immunoperoxidase-stained IgG-containing endothelial cells were counted in a brightfield microscope with a $\times 40$ objective and a $\times 10$ eyepiece. All peroxidase-positive cells on the slide were counted. The total number of endothelial cells on the slide was determined by multiplying the number of fields per slide with the average cell density in 40 randomly selected fields. The cell death frequency in each aorta was obtained from the mean of two samples, each containing approximately 50,000 cells. The data were analyzed by one-sided analysis of variance, and differences between pairs were calculated with the Student t test.

Calcium Cytochemistry

Six rats were injected subcutaneously with 30 mg chlorotetracycline. Four hours later, each rat was anesthetized with ether and perfused with PBS through the left ventricle of the heart. The thoracic aorta was excised and chilled in ice-cold PBS. It was cut into 10 $\times$ 10-mm pieces, which were used for preparation of frozen Häutchens.[15] The tissue pieces were placed, endothelial side down, on gelatin-coated slides that were chilled by placing them on a metal rod, which in turn was chilled in a bath with solid carbon dioxide and alcohol. As soon as the first sign of freezing appeared, the tissue was peeled off, leav-

ing the endothelium frozen onto the slide. All slides were kept at −20 C and used within 3 days.

The slides were thawed and incubated with TRITC-labeled rabbit anti-rat IgG for 30 minutes, rinsed three times for 10 minutes each with PBS, and mounted in Tris-buffered polyvinyl alcohol.[16] The slides were examined in a Zeiss Microscope III equipped with an HBO 50 mercury lamp, epi-illumination, and interference filters. CTC fluorescence was studied with excitation filters at 450–490 nm, a dichroic mirror at 510 nm, and a barrier filter at 560 nm. Rhodamine fluorescence was observed with a Zeiss 546/10 filter set.

Cell Culture

Bovine aortic endothelial (BAE) cells were obtained from the bovine aorta as described[17] and cloned by limiting dilution in conditioned medium for the elimination of smooth-muscle contamination.[18] The cells were fed Waymouth's medium with 10% fetal calf serum (FCS; K.C. Biologicals, Lenexa, Kansas). BAE cultures of the sixth to eighth passage after cloning were used at confluency, at which they formed a monolayer with a density of approximately 150,000 cells/sq cm.

Detection of IgG in Cultured Cells

BAE cells were grown on 22-mm glass coverslips in Petri dishes. Confluent cultures were incubated with 10% IgG-containing adult bovine serum (Gibco, Grand Island, NY) in Waymouth's medium for 1 hour at 37 C, rinsed three times with Dulbecco's PBS (DPBS), and incubated for 5 minutes with 0.1% trypan blue in DPBS. After repeated rinsing with DPBS, the cells were fixed in 1% paraformaldehyde in phosphate buffer, pH 7.2, for 30 minutes at +4 C. The cells were rinsed three times in PBS and incubated for 30 minutes at room temperature with rabbit anti-bovine IgG, diluted 1:50 in PBS with 1% bovine serum albumin. After rinsing, they were incubated for 30 minutes with FITC-conjugated swine anti-rabbit IgG at a 1:50 dilution, rinsed repeatedly, and mounted in Tris-buffered polyvinyl alcohol.[16] The coverslips were examined in a Zeiss fluorescence microscope with a ×25 objective. The number of IgG-positive cells was determined with the use of interference filters for FITC, trypan blue-positive cells with an H 546 filter set, and the total number of cells with phase-contrast optics. Four cultures were analyzed, and four fields were counted per coverslip. Controls were incubated with fetal bovine serum instead of adult serum, or did not receive rabbit anti-bovine IgG.

Residence Time of Dying Cells *in Vitro*

Bovine aortic endothelial cells in Falcon 0.5-sq cm multiwells were rinsed three times with DPBS, incubated for 5 minutes at 37 C in 0.1% trypan blue in DPBS, rinsed again three times in DPBS, and fed Waymouth's medium with 10% fetal calf serum (FCS). The frequency of stained cells attached to the monolayer was counted at a magnification of ×200 in four randomly chosen fields of each well. Detached cells accumulated in the center of the well, which was therefore never used for counting. Counts were made daily after staining. Some cultures were rinsed with DPBS before every cell count to eliminate cells very loosely attached to the monolayer.

Time-Lapse Video Microscopy

BAE cells in 25-sq cm Corning flasks were rinsed with DPBS, stained with trypan blue, and again rinsed with DPBS, as described above for the cell assay of residence time. The cells were put back into Waymouth's medium with 10% FCS and 10 mM HEPES buffer and placed under a Leitz inverted microscope with a ×32 objective, to which an MTI television camera was attached. The camera was connected to a Colorado video processor and a Panasonic time-lapse video tape recorder. The specimen stage of the microscope was kept at 37 C with a Sage air curtain. Optimal contrast was achieved with minimal transmitted light and without phase or interference optics by adjusting pedestal and amplifier gain controls of the video processor, as described by Inoue.[19] Fields containing at least one trypan blue-positive cell were followed by time-lapse recording for up to 3 days.

Results

As expected from our previous studies of tissue sections, we observed a significant number of IgG-positive endothelial cells in *en face* preparations of aortic endothelium. In order to estimate the frequency of these cells in the aortic endothelium, we adapted the immunocytochemical technique to *en face* preparations of the rat aorta. Paraformaldehyde-fixed thoracic aortas were incubated with rabbit anti-rat IgG followed by peroxidase-conjugated swine anti-rabbit IgG and stained for peroxidase. Häutchen preparations were then made as described.[14]

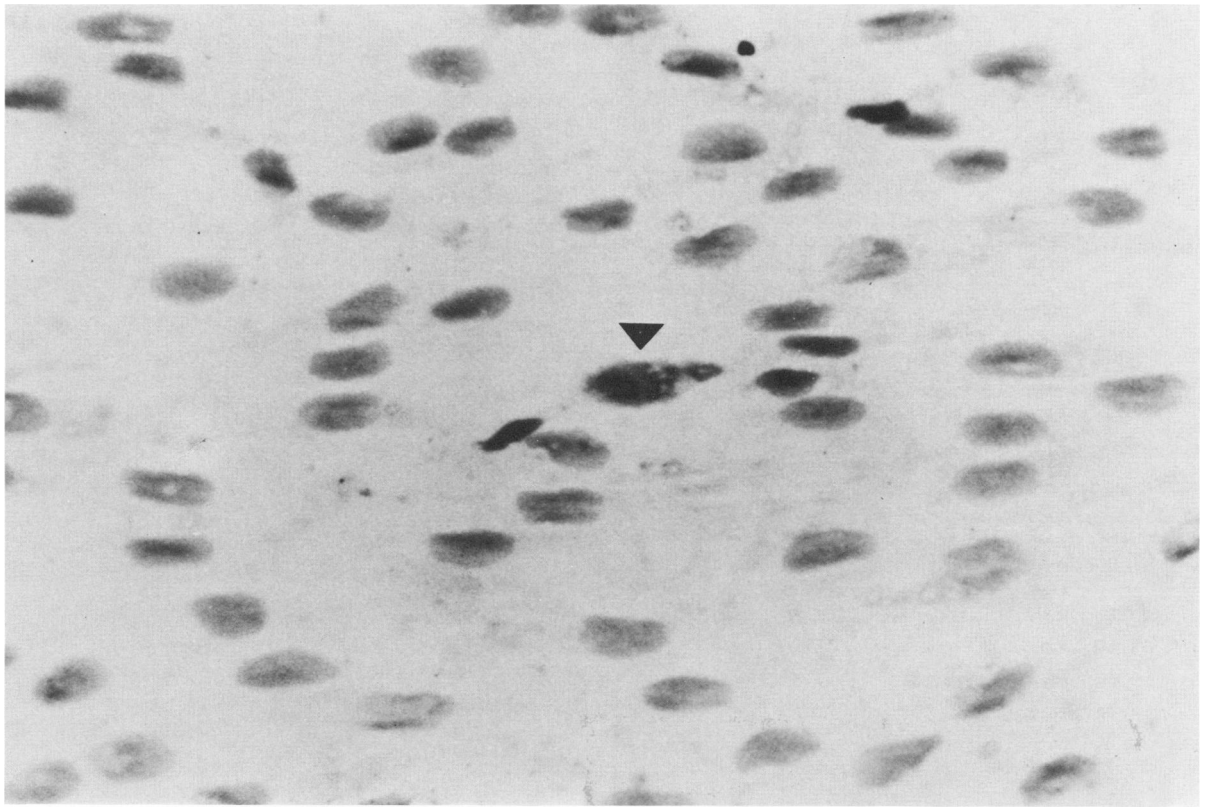

Figure 1 – *En face* preparation of aortic endothelium reacted for IgG with the indirect immunoperoxidase technique and stained with hematoxylin. An IgG-containing cell is indicated by the *arrow*. (×330)

A small fraction of the endothelial cells contained cytoplasmic IgG (Figure 1). In the 20 5-month-old rats studied, 0.19% ± 0.04% (SE) of the cells were positive. The variability of the quantitative technique was low, since no significant differences between experiments or between different aortic specimens taken from each rat could be detected by analysis of variance. There were, on the other hand, significant differences ($P < 0.001$) between different rats, suggesting that the frequency of dead cells may vary even between normal, unmanipulated animals.

In order to exclude the possibility that adherent leukocytes, and not endothelial cells, exhibited IgG positivity, we excluded incubation with antibodies in the treatment of one Häutchen per rat to detect endogenous peroxidase and catalase. In some aortas, a small number of erythrocytes were seen, but less than one peroxidase-positive nucleated cell was detected per 100,000 endothelial cells (0.0008% ± 0.0008%, n = 8). Similar results were obtained by specific staining for monocytes with the α-naphthyl acetate-esterase reaction (data not shown).

A continuum of morphologic changes were observed among the IgG-positive endothelial cells, suggesting a sequence from plasma membrane damage to overt necrosis. Many IgG-positive cells were morphologically similar to IgG-negative cells, differing only in the presence of IgG in part of the cytoplasm of the former (Figure 2A). Of the positive cells 46% ± 4% belonged to this category; 38% ± 3% exhibited prominent shape changes and chromatin condensation in addition to the IgG-positive cytoplasm (Figure 2B). Finally, 16% ± 2% of IgG-positive cells showed characteristic features of necrosis, with a pyknotic nucleus surrounded by a thin rim of cytoplasm (Figure 2C). These cells were often undermined by surrounding IgG-negative cells.

In order to further explore the relationship between IgG uptake and cell death, we studied the correlation of IgG binding to intracellular calcification, a characteristic phenomenon in cell death.[20] The rats were given injected CTC, a fluorescent calcium probe.[21] *En face* preparations of the aortas were incubated with rhodamine-labeled anti-rat IgG. Of the IgG-positive endothelial cells 94.8% ± 0.6% also emitted CTC fluorescence, and 98.0% ± 0.8% of all CTC positive cells contained IgG. The difference between the two means is significant ($P < 0.005$),

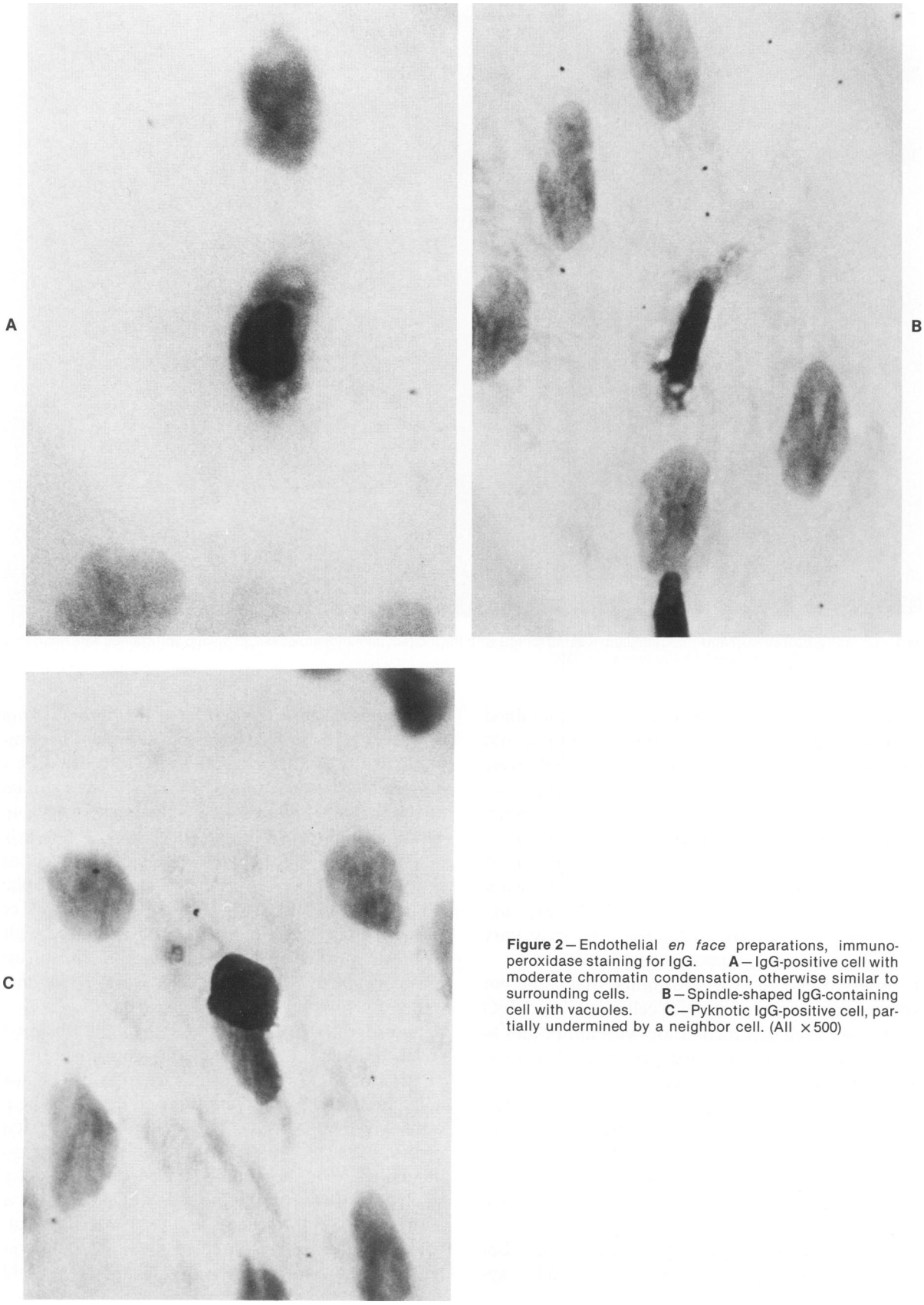

Figure 2—Endothelial *en face* preparations, immunoperoxidase staining for IgG. **A**—IgG-positive cell with moderate chromatin condensation, otherwise similar to surrounding cells. **B**—Spindle-shaped IgG-containing cell with vacuoles. **C**—Pyknotic IgG-positive cell, partially undermined by a neighbor cell. (All ×500)

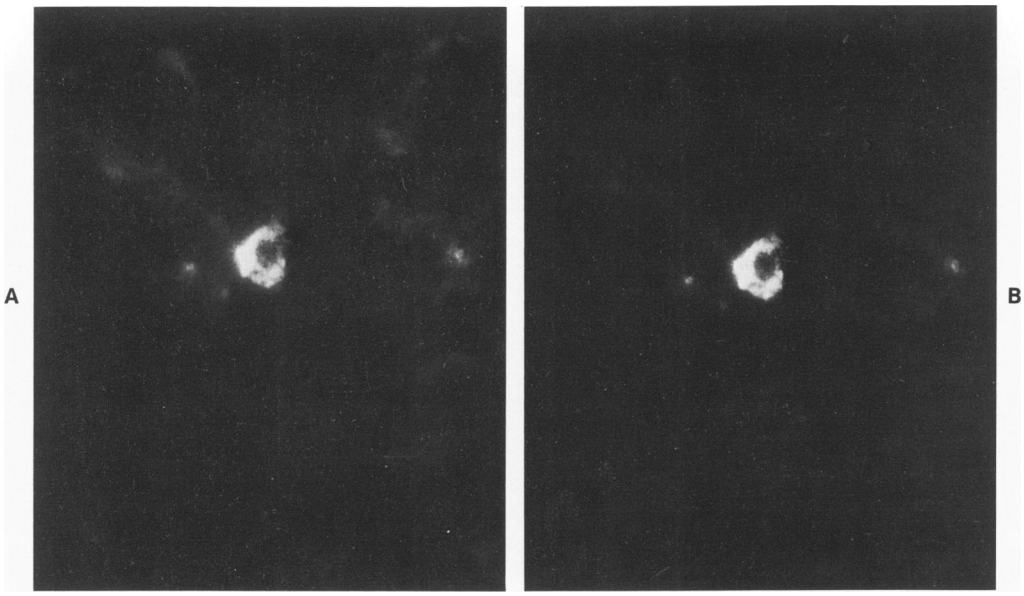

Figure 3—Fluorescent micrographs of the same *en face* preparation of endothelium of a rat given injected CTC intraperitoneally to demonstrate intracellular calcium deposits. **A**—Green fluorescence of CTC in one cell. **B**—The same cell also contains IgG, as demonstrated by rhodamine-labeled anti-IgG. (×460)

suggesting that IgG enters the cytoplasm before calcium accumulation can be detected. Both CTC and antibody fluorescence were confined to the cytoplasm (Figure 3). Control rats were injected with OTC, an analog of CTC that lacks the calcium-binding properties of the latter. Aortas from these animals were negative for tetracycline fluorescence.

We undertook *in vitro* experiments in order to follow the process of cell death in detail. BAE cells were grown to confluence and then incubated with a viability dye, trypan blue, for 5 minutes. The cells were then rinsed and put back into medium. Dead cells, identified by uptake of the dye, were counted under an inverted microscope, and the frequency of attached dead cells was determined daily (Figure 4). The average residence time of dead cells, as defined by the time when 50% of trypan blue-positive cells had detached from the monolayer, was 3.5 days if the cultures were rinsed daily with buffer before counting and 4 days if they were not rinsed. All trypan blue-positive cells detached within 8 days. The presence of trypan blue correlated well with staining of cells for IgG with fluorescent antibodies, since 91% ± 1.4% of trypan blue-positive cells contained IgG.

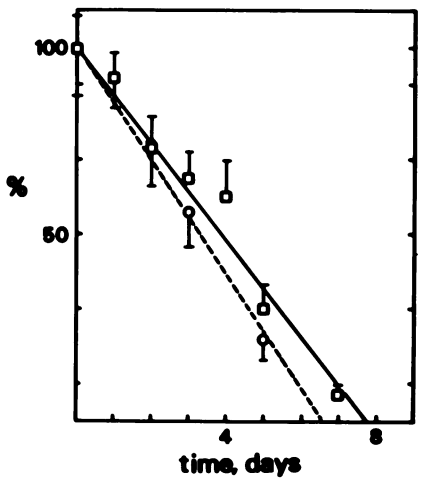

Figure 4—The frequency of attached trypan blue-positive BAE cells after a pulse of trypan blue at Day 0: O- - - -O, cultures rinsed daily with Dulbecco's PBS before counting; □——□, cultures without rinsing (mean ± SE). Positive cells were counted daily at ×400 magnification in four fields per dish and quadruplicate dishes; 100% represents the frequency of trypan blue-positive cells immediately after staining, which was 5.5% ± 1.2% of all cells in the culture.

The process of cell death and detachment was also followed by time-lapse video microscopy after the pulse-labeling of dead cells with trypan blue (Figure 5). Trypan blue-positive endothelial cells initially exhibited a phase of rapid intracellular movements, which lasted for 1–2 hours. The pattern of coarse rapid movements of these cells was easily distinguished from the finer, rapid movements seen in cells entering mitosis and in occasional cells undergoing a reversible morphologic change of as yet unknown significance. After this phase, the blue-stained cells often detached in part from the plastic surface, but this "denudation" was covered within 30 minutes by active migration of the surrounding, viable cells. These latter cells migrated in beneath the dead cell, which "rounded up" but remained attached to the

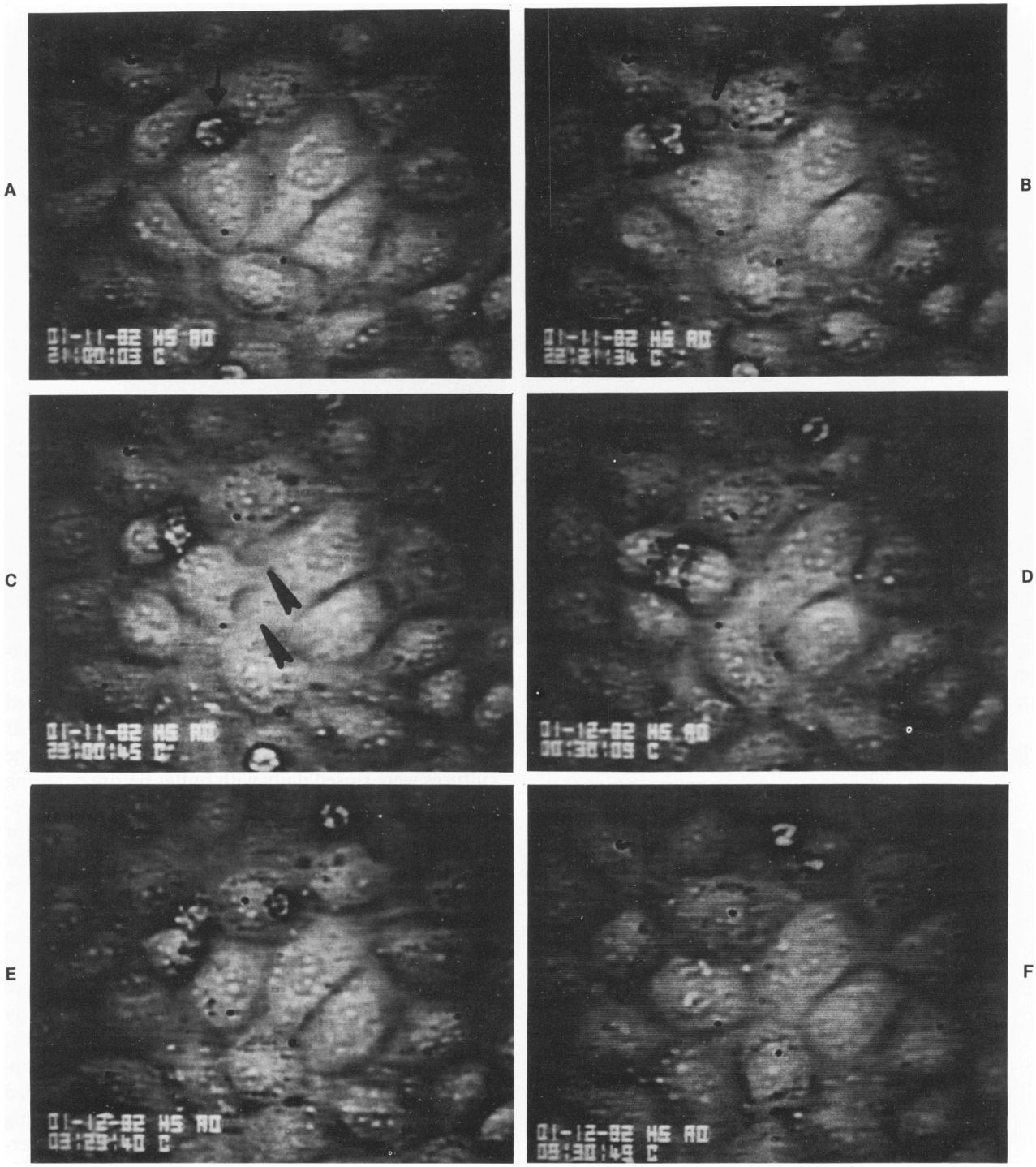

Figure 5—Frames from a time-lapse video film of a trypan blue-stained culture of BAE cells. **A**—At 9:00 PM a positive cell is identified (*arrow*). **B**—At 10:30 PM it has detached partly, leaving a hole in the monolayer (*arrowhead*). **C**—Migration of surrounding, viable cells has covered the hole at 11:00 PM, but new "secondary" holes have formed (*arrowheads*). **D**—Continuity has been restored completely at 12:30 AM, and surrounding cells have migrated in under the positive cell. **E**—At 3:30 AM parts of the positive cell have detached from it and drifted away, but the major part remains, although undermined by viable cells. **F**—At 9:30 AM the positive cell has detached, but continuity has been restored by a process parallel to cell death and detachment. (All ×450)

dish. At this stage, the dead cell underwent a fragmentation resulting in the shedding of cellular debris into the medium. The major part of the cell, however, remained attached to the surface for an additional period of several hours. The final detachment of the remaining part of the dead cell was always associated with active movements of the surrounding, viable cells.

Discussion

This study demonstrates the occurrence of dead cells in the aortic endothelium of rats. Dead cells were identified by immunocytochemistry in combination with calcium cytochemistry. Calcium is deposited in mitochondria and other organelles of dead cells,[20] and the detection of such calcium deposits is therefore considered to be a reliable criterion of cell death.[21] Calcium-containing cells were detected in *en face* preparations of the rat aorta after intraperitoneal injections of the fluorescent calcium probe, CTC. These CTC-positive cells also contained IgG, as demonstrated by rhodamine-labeled antibodies, confirming our previous conclusion that IgG accumulates intracellularly in damaged endothelial cells.[11,12] Finally, immunoelectron microscopy has shown that IgG is deposited in the cytoplasmic matrix of cells with organelle swelling, chromatin changes, plasma membrane damage, and other signs of irreversible injury.[12] In summary, it seems probable that IgG penetrates into the leaking cell and binds to intracellular binding sites.

We have explored these binding sites in a separate study on cultured bovine aortic endothelial cells[22,23] and found that IgG binds to intermediate filaments via specific binding sites for the Fc part of IgG. This observation explains the generalized occurrence of the phenomenon in all animals and species studied.

The frequency of dead endothelial cells was counted in *en face* preparations of rat aortic endothelium after staining for IgG with the indirect immunoperoxidase technique. Of the endothelial cells, 0.19% were IgG-positive, indicating that a small but significant proportion of the cells are dead. If the frequency of dead cells is compared with the cell replication rate as determined by uptake of ^{3}H-thymidine after a 24-hour exposure, the average residence time of a dead cell can be estimated with the formula RT = DF/RR, where RT is the residence time (days), DF is the cell death frequency (percentage), and RR is the replication rate (percentage per day). The cell replication rate in 5-month-old male Sprague–Dawley rats is 0.15% every 24 hours,[24] and the cell death frequency in these animals is 0.19%, as shown in the present study. The estimated residence time of a dead cell in the thoracic aorta is therefore more than 24 hours. This may be a surprisingly long time. Earlier studies have assumed that a damaged endothelial cell rapidly detaches and is removed by the bloodstream, leaving a denuded area to become covered with platelets. We therefore considered it important to study the process of cell death and detachment in detail. For this purpose, we turned to an *in vitro* model utilizing cultivated bovine aortic endothelial cells. In this system, the average residence time was 3.5–4 days. The differences in subendothelial matrix and in flow conditions make the correlation of data *in vivo* and *in vitro* less convincing. However, the long process of active cell movement seen in dying cells at least suggests that cells may undergo a similar series of agonal events before detaching *in vivo*. In cell cultures, the remodeling period with rapid movements, the fragmentation phase, and presumably also the initial phase of membrane damage all took several hours. By analogy, the low frequency of small, pyknotic, IgG-positive cells in the rat aorta could suggest that dead cells detach at an earlier stage *in vivo*.

Our observations on the capacity of the endothelium to rapidly reestablish continuity during focal cell death correlate well with *in vivo* studies of endothelial regeneration of small wounds. Reidy and Schwartz[25] showed that a denudation 1–2 cells wide in the rat aortic endothelium is closed within 8 hours by migration of surrounding cells. Both *in vivo* and *in vitro* studies therefore demonstrate that the vascular endothelium has a high capacity for maintaining continuity.

Finally, we would like to speculate on the role of cell death in the endothelium. The present data, combined with earlier studies from our laboratories,[11,25] suggest that while persistent denudation is not common in the aorta of normal rats, dying endothelial cells may be an important focus for pathophysiologic reactions. For example, the intracellular binding of IgG to the cytoskeleton might activate complement, which would lead to formation of potent serine proteases and to release of chemotactic factors that could attract monocytes and polymorphonuclear cells to the dead cell. Monocytes are frequent in areas with endothelial cell injury on atherosclerotic lesions,[26] and we have shown that this is related to the binding of IgG by the injured cells.[27] Monocytes release a growth factor that stimulates the proliferation of smooth-muscle cells[28] and this could be important for the development of atherosclerosis. They also produce proteolytic enzymes,[29] which could participate in the tissue damage seen in inflam-

matory vascular diseases. In contrast to the normal situation, nothing is yet known about the frequency of cell death under experimental conditions such as exposure to risk factors for atherosclerosis. It is now important to apply the technique for quantitation of cell death described in this paper, as well as other methods, to clarifying the role of the endothelium under such pathologic conditions.

References

1. Schwartz SM, Gajdusek CM, Selden SC: Vascular growth control: the role of the endothelium. Arteriosclerosis 1981, 1:107–161
2. Majno G, Joris I: Endothelium 1977: A review. Adv Exp Med Biol 1978, 104:169–225
3. Caplan BA, Schwartz CJ: Increased endothelial cell turnover in areas of in vivo Evans blue uptake in the pig aorta. Atherosclerosis 1973, 17:401–417
4. Gerrity RG, Richardson M, Somer JB, Bell FP, Schwartz CJ: Endothelial cell morphology in areas of in vivo Evans blue uptake in the aorta of young pigs. Am J Pathol 1977, 89:313–334
5. Schwartz SM, Benditt EP: Clustering of replicating cells in aortic endothelium. Proc Natl Acad Sci USA 1976, 73:651–653
6. Gaynor E: Increased mitotic activity in rabbit endothelium after endotoxin: An autoradiographic study. Lab Invest 1971, 24:318–320
7. Reidy MA, Schwartz SM: Endothelial injury and regeneration: IV. Endotoxin—a non-denuding injury to aortic endothelium. Lab Invest 1983, 48:25–34
8. Constantinides P, Robinson M: Ultrastructural injury of arterial endothelium: I. Effects of pH, osmolarity, anoxia, and temperature. Arch Pathol 1969, 88:99–110
9. Hansson GK, Schwartz SM: Endothelial cell dysfunction without cell loss. In: Biochemical Interactions at the Endothelium, Edited by A Cryer. Amsterdam, Elsevier, 1983
10. Björkerud S, Bondjers G: Endothelial integrity and viability in the aorta of the normal rabbit and rat as evaluated with dye exclusion tests and interference contrast microscopy. Atherosclerosis 1972, 15:285–300
11. Hansson GK, Bondjers G, Nilsson L-Å: Plasma protein accumulation in injured endothelial cells. Immunofluorescent localization of IgG and fibrinogen in the rabbit aortic endothelium. Exp Mol Pathol 1979, 30: 12–26
12. Hansson GK, Bondjers G, Bylock A, Hjalmarsson L: Ultrastructural studies on the localization of IgG in the aortic endothelium and subendothelial intima of atherosclerotic and non-atherosclerotic rabbits. Exp Mol Pathol 1980, 33:302–315
13. Grahan RC, Karnovsky MJ: The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: Ultrastructural cytochemistry by a new technique. J Histochem Cytochem 1966, 14:291–302
14. Schwartz SM, Benditt EP: Cell replication in the aortic endothelium: A new method for study of the problem. Lab Invest 1973, 28:699–707
15. Warren BA: A method for the production of "en face" preparations one cell in thickness. J R Microsc Soc 1965, 84:407–413
16. Lennette DA: An improved mounting medium for immunofluorescence microscopy. Am J Clin Pathol 1978, 69:647–648
17. Schwartz SM: Selection and characterization of bovine aortic endothelial cells. In Vitro 1978, 14:966–980
18. Gajdusek CM, Schwartz SM: Clonal endothelial cell growth. Fed Proc 1982, 41:493
19. Inoue S: Video image processing greatly enhances contrast, quality, and speed in polarization-based microscopy. J Cell Biol 1981, 89:346–356
20. Farber JL, Chien KR, Mittnacht S: The pathogenesis of irreversible cell injury in ischemia. Am J Pathol 1981, 102:271–281
21. Tapp E, Carroll R, Kovacs K: Tetracycline fluorescence in experimental renal lesions. Arch Pathol 1965, 79:629–634
22. Hansson GK, Schwartz SM: Reactions between immunoglobulins, complement, and cytoskeletal intermediate filaments in vascular endothelial cells during cell injury and death. J Cell Biol 1982, 95:227a
23. Hansson GK, Starkebaum GA, Schwartz SM: Fc-mediated binding of IgG to vimentin-type intermediate filaments in vascular endothelial cells (Manuscript submitted)
24. Schwartz SM, Benditt EP: Aortic endothelial cell replication: I. Effects of age and hypertension in the rat. Circ Res 1977, 41:248–255
25. Reidy MA, Schwartz SM: Endothelial regeneration: III. Time course of intimal changes after small defined injury to rat aortic endothelium. Lab Invest 1981, 44: 301–308
26. Hansson GK, Bondjers G: Endothelial proliferation and atherogenesis in rabbits with moderate hypercholesterolemia. Artery 1980, 7:316–329
27. Hansson GK, Björnheden T, Bylock A, Bondjers G: Fc-dependent binding of monocytes to areas with endothelial injury in the rabbit aorta. Exp Mol Pathol 1981, 34:264–280
28. Glenn KC, Ross R: Human monocyte-derived growth factor(s) for mesenchymal cells: Activation of secretion by endotoxin and concanavalin A. Cell 1981, 25: 603–615
29. Werb Z, Gordon S: Secretion of a specific collagenase by stimulated macrophages. J Exp Med 1975, 142:346–360

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