Apoptosis during macrophage-dependent ocular tissue remodelling

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SUMMARY

We have characterized the nature and pattern of cell death during regression of the pupillary membrane, a developmentally transient capillary network found in the anterior chamber of the eye. This analysis has revealed that the cellular components of the pupillary membrane include vascular endothelial cells in an intricate network of fine capillaries as well as attendant macrophages. The capillaries are situated on the anterior surface of the lens and held in relative position by a cobweb-like meshwork of extracellular matrix fibres that regress along with the cellular components of this structure. Cell death during regression of the pupillary membrane is characteristic of apoptosis. Specifically, apoptotic bodies containing condensed chromatin can be observed in vascular endothelial cells and genomic DNA isolated from the pupillary membrane shows the nucleosomal fragmentation pattern typical of apoptotic cells. Using a method for labelling fragmented DNA in tissue preparations (TUNEL), we have assessed the overall pattern of apoptotic cell death during pupillary membrane regression. We find that apoptosis occurs either in single cells in healthy vessels or synchronously along the entire length of a capillary segment. Both morphological and TUNEL analysis indicate that capillary regression occurs from junction to junction one segment at a time. We propose a model to explain the pattern of capillary regression observed and conclude from these and previous experiments (Lang and Bishop (1993) Cell 74, 453-462), that during regression of the pupillary membrane, the macrophage elicits target cell death by inducing apoptosis.

Key words: development, tissue remodelling, tissue regression, macrophage, apoptosis

INTRODUCTION

Tissue regression is central to the development of species distributed throughout the animal kingdom (Glucksman, 1951; Saunders, 1966; Saunders and Fallon, 1967; Ellis et al., 1991b; Ucker, 1991) but investigation of this phenomenon has largely been restricted to histological analyses. In insects, work on tissue regression has involved an examination of involution of larval organs during metamorphosis including hormonally induced cell death (Robinow et al., 1993). In vertebrates, the tadpole tail (Weber, 1964; Tata, 1966; Yoshizato, 1989), interdigital epithelium (Feinberg et al., 1986; Latker et al., 1986), the kidney precursor organs pronephros and mesonephros (Torrey, 1965; Maraud et al., 1979; Ellis and Youson, 1990), the central nervous system (CNS)(Lance-Jones, 1982; Young, 1984) and ocular capillary networks (Jack, 1972; Balazs et al., 1980; Latker and Kuwabara, 1981) have been favoured as tissues in which to study regression. However, only in the nematode Caenorhabditis has a miniature version of tissue regression been examined at the cellular and molecular levels in any detail. A significant proportion of cells die autonomously (Ellis et al., 1991b) or are killed (Sulston and Thompson, 1980) and eliminated during development. Genetic analysis has allowed the identification of genes that function autonomously in permitting or denying the occurrence of programmed cell death (Ellis and Horvitz, 1986; Hengartner et al., 1992) as well as genes that are essential for the engulfment of dead cell corpses (Ellis et al., 1991a).

One feature common to most, if not all examples of tissue regression is the involvement of phagocytes. Phagocytes can be observed engorged with the pyknotic nuclei of redundant neurons of the inner retina of the mouse (Young, 1984), with debris from dying cells in the posterior necrotic zone of the developing chick wing (Saunders, 1966) and with apoptotic bodies from the regressing intestinal epithelium of the tadpole (Weber, 1964). In Drosophila too, phagocytes engulf dead cell debris and appear to be stimulated to differentiate by dying cells (Tepass et al., 1994). Thus, it is clear that one function of the macrophage during tissue regression is the engulfment of cellular debris. In vitro studies have demonstrated that depending upon the macrophage population, engulfment of apoptosing cells can be mediated either by the vitronectin or phosphatidyl serine receptors (Fadok et al., 1992).

In many instances where macrophages engulf dying cells, it is probable that target cell death is independent of the macrophage. This is likely to be true in the CNS for example, where neuronal regression will result from target innervation failure (Oppenheim, 1989) and in Drosophila melanogaster where developmental cell death occurs in the absence of macrophages (Tepass et al., 1994). By contrast, in other examples, there is strong evidence that macrophages actively elicit the death of target cells. In vitro, macrophages can
display cytoidal activity towards normal vascular endothelial cells (Peri et al., 1990; Joniec et al., 1992) as well as tumour cells (Esgro et al., 1990). Macrophage cytoidal activity may be analogous to events occurring during tissue remodelling. In ex vivo experiments studying the role of phagocytes in regression of the amphibian tail, catalase-mediated cleavage of hydrogen peroxide can reduce the rate of regression of tail slice cultures suggesting that the phagocyte may have an active role in regression (Little and Flores, 1993). In transgenic mice, macrophage ablation results in a lack of normal cell death and tissue remodelling in the eye giving persistent forms of both the hyaloid vasculature and the pupillary membrane (PM) (Lang and Bishop, 1993). Importantly, according to morphological criteria the PM persisted in viable form implying that DNA fragmentation and characteristic histological changes occur.

Another common feature in developmental tissue regression is the presence of dying cells with ultrastructural changes characteristic of programmed cell death or apoptosis. This defined series of morphological and molecular changes that occurs during physiological cell death (Wyllie et al., 1980) is a widespread phenomenon throughout normal development and homeostasis. Apoptosis is known to be the mechanism of cell death during elimination of self reactive T cells (Schwartz, 1989), when haemopoietic cells are denied growth and survival factors (Williams et al., 1990) and during redundant neuron elimination in the CNS (Raff, 1992).

In the current report, we investigate whether the macrophage might elicit cell death during PM regression by inducing apoptosis in the target cells. To this end we have determined whether DNA fragmentation and characteristic histological and ultrastructural changes occur. Secondly, we have determined whether the wider pattern of cell death in the PM is consistent with macrophage-dependent tissue regression. Our experiments allow us to conclude that apoptosis does take place in cells of the PM during its regression and that the pattern of cell death is consistent with macrophage cytoidal activity. In addition, we propose a model to explain the pattern of PM regression at the cellular level.

**MATERIALS AND METHODS**

**Animal breeding**

Sprague-Dawley rats were obtained from Taconic Farms (German Town, NY). The age of newborn rats was determined by the presence of sperm in a vaginal fluid smear in the breeding female and the time of conception assigned to the previous midnight. Ages of rats are noted as days post conception (e.g. PC 31.5).

**Visualization of rat pupillary membrane**

The technique for dissection of the rat PM is described in Fig. 1. The dissection begins with removal of the orbit, its immersion in phosphate-buffered saline (PBS) and piercing of the sclera and choroid with the tip of a discussion knife (Storz, St. Louis, MO) in the vicinity of the optic nerve. A longitudinal incision is then made with a 7 mm blade curved tip vannas (Storz, St. Louis, MO) forward as far as the cornea and then continued perpendicularly along the corneal-scleral junction (Fig. 1A,B). Vessels of the hyaloid system including the hyaloid artery are then severed to allow separation and disposal of PM regression at the cellular level. (A) The orbit is removed, the sclera punctured and longitudinal and circumferential incisions made as shown to expose the lens and retina. (B) The retina, choroid and sclera are then removed to produce the group of tissues including the lens, iris diaphragm and cornea (C). The lens is gently peeled away from the iris diaphragm to reveal the PM (D) at which point, the tissue is fixed. (E) After fixation, the joining edge of the cornea and iris diaphragm is removed, the cornea discarded and the remaining iris diaphragm and attached PM laid flat on a slide (F). C, cornea; ID, iris diaphragm; L, lens; PM, pupillary membrane; R, retina; S, sclera; SL, slide.

**Fig. 1.** Removal of the PM from the eye by dissection. (A) The orbit is removed, the sclera punctured and longitudinal and circumferential incisions made as shown to expose the lens and retina. (B) The retina, choroid and sclera are then removed to produce the group of tissues including the lens, iris diaphragm and cornea (C). The lens is gently peeled away from the iris diaphragm to reveal the PM (D) at which point, the tissue is fixed. (E) After fixation, the joining edge of the cornea and iris diaphragm is removed, the cornea discarded and the remaining iris diaphragm and attached PM laid flat on a slide (F). C, cornea; ID, iris diaphragm; L, lens; PM, pupillary membrane; R, retina; S, sclera; SL, slide.

**Fig. 2.** Histological analysis of the rat PM during regression. (A) Capillaries of the PM at PC27.5. The iris diaphragm (ID) encircles the capillary network (×66 magnification). (B) Bright field of PC31.5 PM labelled with ED-1 antibody showing the capillary network and macrophages (×132). (C) Fluorescence illumination of B showing macrophages as red spots (×132). (D) ED-1-labelled macrophages on the PM at PC30.5 prior to any morphological signs of regression (×1663). (E) A PC33.5 PM showing a single, ED-1-labelled macrophage adjacent to an apoptosing cell (arrowed) (×1663). (F) A single apoptosing cell within a capillary segment at PC32.5 (arrow). The capillary has narrowed and macrophages are in close attendance (×1663). (G) Junction-to-junction capillary regression at PC32.5. The entire capillary segment has narrowed between junctions (small arrows) with two normal capillaries. The large arrow indicates a macrophage with an engulfed apoptotic body (×1663). (H) Junction-to-junction capillary regression in a PC32.5 PM. Note numerous apoptotic bodies along the capillary segment. The large arrows indicate macrophages associated with the regressing capillary and the small arrows the capillary junctions (×1056). (I) Fluorescence illumination of (H) showing ED-1-labelled macrophages (large arrows) (×1056). (J) A long PC33.5 capillary segment with apoptosing cells along its length. (K) A macrophage that has engulfed apoptotic bodies containing condensed chromatin (PC32.5, ×2640).
of the sclera, choroid and retina from the lens, cornea and iris diaphragm (Fig. 1C). The latter tissue group is then oriented cornea down, the lens capsule pierced and the lens partially collapsed. The lens is then peeled gently away from the iris diaphragm and cornea to expose the PM (Fig. 1D). At this stage, the tissue was fixed for 30 minutes in PBS containing 4% formaldehyde. The tissue was then re-immersed in PBS alone and the dissection completed. This required making tangential incisions around the periphery of the iris diaphragm and cornea so that the two structures could be separated (Fig. 1E). The PM and iris diaphragm were then laid flat on a slide (Fig. 1F) ready for processing.

ED-1 labeling of rat macrophages

Macrophages associated with the PM were labeled immunohisto-chemically with the antibody ED-1 (Harms et al., 1990) using an anti-rat IgG alkaline phosphatase (AP) labelling kit from Vector Laboratories (Burlingame, CA). PM/iris diaphragm preparations were fixed with acetone on a silane-treated histology slide. Steps in ED-1 labeling were essentially as described by the manufacturer. ED-1 was used at a 1:50 or 1:100 dilution and incubated with the PM preparations for a period of 1.5 hours. The endogenous AP inhibitor levamisole and avidin and biotin blocking steps were essential in eliminating non-specific background. Preparations were counterstained with hematoxylin for a period of 1 minute and Vector Red substrate visualized either in bright-field or fluorescence microscopy (with a rhodamine filter set) as a red reaction product.

Visualization of fragmented DNA in situ

We have used a modified version of the TUNEL technique described in Gavrieli et al. (1992) to identify cells undergoing apoptosis during the regression of the PM. The PM/iris diaphragm complex was dissected from the rat eye as described above and dried onto a silane-treated microscopy slide. The tissue sample was fixed for 30 minutes with 4% formyl-PBS, rinsed with distilled water then fixed in acetone for 10 minutes. After bringing the tissue sample to water through a graded alcohol series, they were treated with proteinase K (20 µg/ml) for 15 minutes at RT, then washed three times with water and once with PBS. Tissue samples were then exposed to blocking serum for 20 minutes, to avidin blocking solution for 10 minutes and to biotin blocking solution for 10 minutes (Vector Laboratories) with each step followed by a 2 minute distilled water wash. Terminal deoxynucleotidyl transferase (TdT) (Stratagene) at 80 U/ml and biotinylated dUTP at 1 mM were combined on the tissue sample in TdT buffer (30 mM Tris, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride) and incubated for 60 minutes. Preparations were then incubated with avidin-conjugated alkaline phosphatase (AP) (Vector Laboratories) for 45 minutes, rinsed in PBS for 10 minutes and exposed to the Vector Red AP substrate in the presence of the endogenous AP inhibitor levamisole for 15 minutes. Preparations were counterstained and the reaction product visualized as described above.

Transmission electron microscopy

For transmission electron microscopy, PM preparations were fixed in 2% glutaraldehyde (Sigma Chemicals), 0.1 M cacodylate pH 7.4. Samples were postfixed in 2% osmium tetroxide, 0.1 M cacodylate pH 7.4, en-bloc stained in 1% aqueous uranyl acetate and embedded in Epon (Hayat, 1989).

Visualization of nucleosomal ladder

DNA was isolated from PMs that had been dissected away from other cellular material. Dissection was performed as described (Fig. 1) except that the iris diaphragm was removed from the PM with additional tangential incisions. PMs were then digested in 200 µl 50 mM Tris pH 8.0, 0.5% SDS, 100 mM EDTA, 200 µg proteinase K per ml, 1 mg RNase A per ml for 4 hours at 55°C. Purification was completed with phenol and chloroform extractions followed by precipitation with 0.3 M sodium acetate and ethanol. Radiolabelling of PM DNA was performed as previously described (Rosl, 1992) except that both α[32P]dCTP and α[32P]dATP were used in the reaction. The DNA from both PMs from a single young rat were used for a single lane of a 7% native acrylamide gel. DNA for a control nucleosomal ladder was isolated from granulocyte-macrophage colony stimulating factor dependent DA3 cells denied the growth factor for a period of 12 hours. Autoradiography was performed using Kodak XAR film and densitometry on a Molecular Dynamics Personal Densitometer.

RESULTS

Regression of the rat pupillary membrane

To improve on the rather limited utility of traditional histology for analysis of pupillary membrane (PM) regression, we have developed a PM dissection method using the larger eye of the rat (described in Materials and Methods; Fig. 1). Since the PM is essentially a two-dimensional array of capillaries that is at most a few cells thick, morphological details can be discerned with ease. Dissection of the rat PM revealed a network of capillaries with an imperfect tetra-radial symmetry resulting from four main capillaries extending from the iris diaphragm (Fig. 2A). Thus, the major cellular component of the PM is the vascular endothelial cell of the capillary wall. The capillaries are held in relative position by a cobweb-like meshwork of extracellular matrix fibres that cannot be visualized in the preparations shown.

Seen associated with the PM is a population of macrophages labelled with the macrophage-specific antibody ED-1 (Fig. 2B-E). Macrophages are evenly distributed across the capillary network at postconception day 31.5 (PC31.5) (Fig. 2B and C) and number on average about 400 per membrane over the region spanning the pupil. The number of macrophages associated with the membrane does not change significantly from PC27.5 to PC34.5±1 day. Thereafter, the number of macrophages diminishes as vessel regression is completed between PC35.5 and PC36.5. The number of vascular endothelial cells present prior to any signs of regression is estimated, by counts of nuclei in fractional areas of the PM, to be approximately 6000. This gives a target cell to macrophage ratio of approximately 15.

ED-1 labelled macrophages associated with the PM are found both between and in close contact with capillaries (Fig. 2D). Early in the phase of PM regression, isolated capillary cells undergoing apoptotic changes can be observed (Fig. 2E, 2F). Thus, the major cellular component of the PM is the vascular endothelial cell of the capillary wall. The capillaries are held in relative position by a cobweb-like meshwork of extracellular matrix fibres that cannot be visualized in the preparations shown.

Fig. 3. TUNEL labelling of PM cells. (A,B) Apoptosis, indicated by red TUNEL labelling in an isolated capillary cell in a PC34.5 PM preparation as in E, but illuminated in bright field showing many capillaries not TUNEL labelled (×118).
capillary cells are clearly delineated by the TUNEL labelling. In some examples, capillaries with single apoptosing cells (Fig. 2F, arrow) have narrowed over a portion of their length. This is a morphological change characteristic of regressing capillary segments throughout the phase of regression. Interestingly, the morphological features of capillary regression almost invariably occur between one capillary junction and the next. This is illustrated in Fig. 2G and H where capillary segments have narrowed and contain apoptosing cells. In both these examples, the morphological features of regression stop at the capillary segment junctions (small arrows). In both cases, macrophages can be seen closely associated with the capillary segments. In Fig. 2G, the macrophage (large arrow) has clearly engulfed an apoptotic body and, in Fig. 2H, macrophage identity is confirmed with ED-1 antibody (Fig. 2I). A high magnification of a single macrophage that has engulfed an apoptotic body is shown in Fig. 2K. Junction-to-junction regression is observed to occur in long as well as short capillary segments as illustrated in Fig. 2J where apoptotic bodies are observed along the entire length of the capillary. The phenomenon of junction-to-junction regression implies that there is a degree of synchrony to the cell death within a regressing capillary segment.

Regression of the capillaries in the PM occurs on a segment-by-segment basis until it is complete at about two weeks after birth (between PC 35.5 and PC 37.5) just prior to the point at which young rats open their eyes. From PC 27.5 until PC 31.5 there is only an occasional regressing capillary segment. From this time on both the number and length of regressing capillary segments increased rapidly. Occasionally, a small group of capillaries would linger in an apparently normal rat. These capillaries often formed a single bundle, spanned the pupil chordally, and mimicked closely the persistent ‘white strands’ (Matsuo and Smelser, 1971) that can be observed in human partially persistent PM (data not shown).

Apoptosis in cells of the PM during regression

To examine further the notion that PM regression may involve an apoptotic cell death, we have subjected PM preparations to the TUNEL protocol (Gavrieli et al., 1992). TUNEL labelling of cells in PM preparations occurs in three circumstances and indicates that apoptosis occurs during regression. Macrophages that have engulfed material label strongly (Fig. 3A, large open arrow) demonstrating that macrophages have engulfed DNA fragments. The labelling observed in isolated macrophages is often associated with a phagosome and therefore consistent with macrophage engulfment of apoptotic bodies. The diffuse, cytoplasmic staining observed (Fig. 3D) is not a consistent feature of TUNEL labelling. Given that this technique requires steps for membrane dissolution and protease digestion, it is unlikely that the subcellular staining pattern can be assigned any significance.

The second type of TUNEL labelling that we observed was in isolated capillary cells in capillaries that otherwise looked normal (Fig. 3A-D, medium sized arrows). TUNEL labelled cells were almost invariably associated with macrophages (Fig. 3A-D, small arrows). In some cases, the boundaries of capillary cells are clearly delineated by the TUNEL labelling (Fig. 3D). Isolated apoptoses like these examples could be observed as early as PC27.5 (about 5 days after birth) and throughout regression until its completion.

In addition to isolated macrophage and endothelial cell labelling, TUNEL resulted in the labelling of cells along the entire length of capillary segments. Three different capillary segments are labelled in this manner in the PM preparation shown in Fig. 3E and F and are labelled 1 to 3 in both bright-field and fluorescence illumination. When fluorescently illuminated (Fig. 3E), this PM shows how even very long capillaries can display this labelling pattern. The TUNEL labelling reinforces the notion that apoptosis is occurring during regression, that capillaries regress one segment at a time from junction to junction and that there is a degree of synchrony to the apoptosis that occurs in the cells of any one capillary segment.

Particular ultrastructural changes in part define apoptosis (Wyllie et al., 1980). In vascular endothelial cells of the PM studied using transmission electron microscopy, we observe chromatin condensation typical of apoptotic nuclei. Crescent-shaped regions of condensed chromatin that lie at the nuclear margin can be observed in the nuclei of capillary cells (Fig. 4).

DNA isolated from dissected PMs showed a nucleosomal ladder pattern (Fig. 5A). The ladder was discernible at PC 34.5 in end-labelled DNA (Rosl, 1992) and in some experiments could be discerned in DNA from PC 33.5 and PC 35.5 PMs (data not shown). Given the small number of cells available in any one PM, this experiment is limited by our ability to detect nucleosomally fragmented DNA against a background of DNA fragmented non-specifically during purification. Densitometry tracings from the autoradiograph compare nucleosomal ladders of PM DNA and that from control apoptosing cells (Fig. 5B). This indicates that both have very similar peak patterns that correspond in size to the nucleosomal monomer and multimers.

The appearance of nucleosomal ladder DNA fragmentation in the PM correlates well with the time (PC 34.5) at which most vessels in the PM are regressing according to morphological criteria.

DISCUSSION

During development of the mouse eye, capillary networks called the hyaloid vessel system and the PM undergo regression. Recent work has implied that macrophages have a pivotal role in regression of the PM and actively elicit death of the vascular endothelial cells that constitute the target structure (Lang and Bishop, 1993). In the current study of rat PM regression, we have demonstrated that the cell death occurring has all the characteristics of apoptosis. Combined with previous studies, these results suggest that macrophage elicited cell death may occur through the induction of apoptosis.

That macrophages can induce target cell apoptosis is also demonstrated by macrophage anti-tumour cell cytoidal activity (Greenberg, 1991). Furthermore, LPS and interferon-γ stimulated macrophages are known to display cytoidal activity towards primary vascular endothelial cells in vitro (Peri et al., 1990; Jonjic et al., 1992). This interaction may represent an in vitro equivalent of macrophage activity during PM regression. Whether macrophage anti-vascular endothelial cell activity in vitro involves the induction of apoptosis is
unclear but T- (Don et al., 1977; Duke et al., 1983), K- (Sanderson and Thomas, 1977; Stacey et al., 1985) and NK-cells (Bishop and Whiting, 1983) appear to kill target cells by this means. Developmental studies in the frog also suggest that macrophages induce cell death during tissue regression. Regression of tadpole tail slice cultures in vitro is inhibited by catalase implicating the macrophage product hydrogen peroxide in regression (Little and Flores, 1993). In addition to these examples of vertebrate tissue regression requiring active killer cells, the phylogenetically more distant Caenorhabditis has at least one cell, the gonadal linker cell, that requires the active participation of an epidermal cell for its programmed death (Sulston et al., 1980).

Developmental cell death is clearly not always the result of active macrophage intervention, however, as demonstrated by experiments in vertebrates (Oppenheim, 1989) in Caenorhabditis (Ellis et al., 1991b) and Drosophila (Tepass et al., 1994). Thus, it appears that the problem of killing and removing superfluous cells during development has been solved in a variety of ways. In some species and tissues, macrophage cytoidal activity may be essential in the induction of cell death. Our data are consistent with this notion and more specifically imply that developmentally programmed capillary regression can be driven by macrophage cytoidal activity and that this is equivalent to macrophage induction of target cell apoptosis.

**The cellular mechanism of pupillary membrane regression**

This study suggests a cellular mechanism by which PM regression might take place. Several considerations are important for the development of a model for the cellular interactions. First, we assume that macrophages drive the regression and actively elicit the death of cells making up the capillaries of the PM (Lang and Bishop, 1993). Second, based on a large body of data, we assume that when macrophages kill target cells, close cell-to-cell contact is required. Consistent with these assumptions is the observation that macrophages and apoptotic endothelial cells are co-localised. While other phenomena (such as secretion of chemottractants by apoptotic cells) could explain this observation in isolation, this would not be consistent with ablation experiments suggesting that endothelial cells die only in the presence of functional macrophages (Lang and Bishop, 1993).

The presumed requirement for close cell-to-cell contact raises an interesting question. Since there are approximately 15 target cells for each macrophage, one might suggest that macrophages must be very mobile if they are to contact and kill every cell within the structure. High macrophage mobility...
is a possibility in the current system and has been suggested in others, notably when macrophages clear redundant neurons from the developing retina (Young, 1984). We are currently examining macrophage mobility during PM regression using vital cell imaging techniques.

However, there is no strict necessity for highly mobile macrophages during regression. Capillary segment regression occurs from junction to junction, and according to TUNEL analysis, ultimately apoptosis occurs synchronously along the length of a capillary segment. These observations imply that there is a simultaneous signal for the induction of apoptosis in any one segment. While gap junctional communication could mediate synchronous apoptosis, cessation of blood flow is a more likely mechanism. The latter possibility is appealing for its simplicity, and because it is consistent with the general observation that withdrawal of growth factors can result in apoptosis (Wyllie et al., 1980), a condition that may well exist when capillary cells are denied a continual flow of serum. In addition, it is evident from the dynamics of fluid flow that blood need only cease flowing in one capillary segment at a time, a principle that is consistent with our observations of junction-to-junction regression and synchronous apoptosis in capillary segments. Thus, based on our observation, we suggest that capillary cells of the PM die either as a direct result of macrophage cytocidal action or as a consequence of diminished growth factor exposure when serum flow ceases.

The remaining issue in this working model (Fig. 6) is to explain how macrophages can bring about a cessation in blood flow in a capillary segment since, based on previous experiments (Lang and Bishop, 1993), they apparently drive all aspects of PM regression. We suggest, based on our observation of isolated capillary cell apoptosis, that macrophages sporadically kill target cells throughout the phase of regression. For any one capillary segment, there will then be a critical point where cell killing results in a lumen too narrow to support blood flow. We suggest that, at this point, synchronous apoptosis occurs along the capillary segment. Our continued analyses of PM regression at the cellular and molecular levels are designed to resolve some of the questions that arise from the current study.

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