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Macrophages kill capillary cells in G_1 phase of the cell cycle during programmed vascular regression

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SUMMARY

Programmed capillary regression occurs during normal development of the eye and serves as a useful model for assessing the forces that drive vascular involution. Using a combination of S-phase labeling and liposome-mediated macrophage elimination, we show that during regression, macrophages induce apoptosis of both pericytes and endothelial cells in a cell cycle stage-dependent manner. Target cells are signaled to die by macrophages approximately 15 hours after S-phase labeling and this corresponds to a point in mid-G_1 phase of the cell cycle. The tight correlation between the restriction point of the cell cycle and the point where the macrophage death signal is received suggests that the mitogen, matrix and cytoskeletal signals essential for cell-cycle progression may be inhibited by macrophages as a means of inducing cell death. Furthermore, these experiments show that cells from two distinct lineages are induced to die as a consequence of macrophage action, and this provides evidence that macrophage-induced cell death may be a general phenomenon during development and homeostasis.

Key words: Apoptosis, Macrophage, Vascular regression, Tissue remodeling, Tissue regression

INTRODUCTION

The ability of an organism to maintain homeostasis is dependent upon a balance between cell proliferation, cell death and differentiation. Several lines of evidence indicate that control of cell-cycle progression and regulation of programmed cell death (PCD) may coincide (Wyllie et al., 1980; Kerr and Harmon, 1991). In many culture systems (Lissy et al., 1998) and in vivo (Thomaidou et al., 1997) cell death can occur preferentially in G_1 phase of the cell cycle. Furthermore, there is evidence that transit through G_1 phase of the cell cycle is dependent upon external signals; this control interval is referred to as the restriction point (Pardee, 1974). Once cells pass this point in mid-G_1 phase, completion of the cell cycle is cell-autonomous (Pardee, 1974; Zetterberg et al., 1995). At the restriction point, cell-cycle progression requires mitogenic growth factors (Zetterberg et al., 1995), extracellular matrix signaling via integrins (Assoian, 1997) and intact cytoskeleton (Bohmer et al., 1996; Huang et al., 1998). Absence of restriction point signals for some cell types results in a cell-cycle arrest (Assoian, 1997; Huang et al., 1998) and in others, cell death (Meredith et al., 1993; Chen et al., 1997). Although the links between restriction point signals and intracellular regulators of the cell cycle are unclear, it is perhaps not surprising that G_1-phase mediators such as Rb (Macleod et al., 1996) and $p27^{kip1}$ (Park et al., 1997; Wang et al., 1997) can influence the decision between continued cycling and death.

There has been much recent progress in the understanding of vascular morphogenesis and maintenance (Hanahan, 1997), of angiogenesis (Risau, 1997), and of molecules that might be used to impose vascular regression (Folkman, 1997). During development, microvascular blood vessels originate as simple endothelial cell (EC) tubes that are subsequently covered by migratory cells which differentiate into pericytes. Based on the phenotype of platelet-derived growth factor B (PDGF-B) null mutant mice, it has been proposed that PDGF-B secreted by ECs is responsible for the recruitment of pericytes (Lindahl et al., 1997; Crosby et al., 1998) and that in turn, pericytes are required for the formation of normal capillary structures. A number of earlier studies also suggested that pericytes play an important role in capillary maintenance. In particular, pericyte degeneration has been proposed as a key event in the development of diabetic retinopathy (Addison et al., 1970), and consistent with this suggestion is the observation that pericyte association ends a period of plasticity in the development of retinal capillaries (Benjamin et al., 1998).

Macrophages are thought to have an important role in regulating angiogenesis (Sunderkotter et al., 1994). They can

produce a variety of molecules that might positively or negatively influence angiogenesis, including growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), inhibitors of cycling such as transforming growth factor-β (TGFβ) and proteases that can remodel matrix.

In order to investigate the function of macrophages in regulating vascular potential, we have developed the pupillary membrane (PM) as a model system for experimental analysis. The PM is a transient vascular network traversing the pupillary opening of the iris in the eye of mammals (Lang, 1997). In humans, the PM regresses in utero, while in rats (Lang et al., 1994) and mice (Ito and Yoshioka, 1999) involution occurs in the early postnatal period. Regression of the PM occurs as a result of macrophage-dependent programmed cell death (Lang and Bishop, 1993; Diez-Roux and Lang, 1997).

In the present study, we have employed the PM to further investigate the interaction between macrophages and their target cells during capillary regression. We show that the PM consists of microvessels with varying degrees of pericyte investment, and that both endothelial cells and pericytes are killed by macrophages. By following cohorts of synchronously dividing cells in this system, we show that macrophage-dependent programmed cell death is initiated in mid-G_1 phase of the cell cycle. This finding implies that macrophages kill by subverting the external signals that normally promote cell-cycle progression at the restriction point.

MATERIALS AND METHODS

Animal breeding

Timed pregnant Sprague-Dawley rats were obtained from Taconic Farms (German Town, NY). Ages of rats are noted as days post-partum (e.g. A8 is 8 days after birth) with the assumption that birth occurs at gestational day 22. Animal care was in accordance with institutional guidelines.

Liposomes

Control liposomes and toxic liposomes containing dichloromethylene bisphosphonate (clodronate) for macrophage ablation were generated according to established procedures (Van Rooijen, 1989). Clodronate was kindly provided by Boehringer Mannheim GMBH. For macrophage elimination in the anterior chamber, liposome suspensions were injected trans-corneally in a volume of 500 nl. Trans-corneal injection was performed as previously described (Diez-Roux and Lang, 1997).

Explantation procedure

PM explants were performed as previously described (Meeson et al., 1996). Staurosporine was used to supplement culture medium at a concentration of 1 μM.

Histological procedures

The PM/iris diaphragm complex was fixed by perfusion and dissected from the rat eye as previously described (Lang et al., 1994). For identifying apoptotic cells, nuclei were stained with Hoechst 33258 (Sigma, St Louis, MO) or Hematoxylin; apoptosis was recognized as the characteristic condensation of apoptotic bodies (Wyllie et al., 1980).

PMs for immunolabeling were fixed for 10 minutes with 4% paraformaldehyde in 0.1 M PBS (pH 7.4), washed in PBS, mounted onto glass slides and dried at room temperature to maintain adhesion to the slide during labeling. PMs were permeabilized with 1% Triton X-100 in 0.1 M PBS for 30 minutes, and chromatin denatured for BrdU detection by incubation in 2 N HCl for 30 minutes. PMs subsequently were incubated twice in 0.2 M sodium borate buffer for 15 minutes to neutralize acid, and washed and blocked 3 times in 0.1 M L-lysine in 0.1 M PBS (pH 7.4) for 5 minutes. Incubation with primary antibodies was overnight at 4°C, and with secondary antibodies was for 1 hour at room temperature. Cells which incorporated BrdU were detected using a monoclonal rat anti-BrdU antibody (clone BUI/75-1CR1, Accurate Chemical and Scientific Corporation, Westbury, NY) at a dilution of 1:50. This was followed either by a goat anti-rat antibody conjugated to Alexa 488 (Molecular Probes Eugene, OR) or a horseradish peroxidase-conjugated goat anti-rat antibody (Boehringer Mannheim). Both secondary antibodies were used at a dilution of 1:100 and established protocols used for immunoperoxidase labeling (Harlow and Lane, 1988). Pericytes were labeled using a monoclonal mouse anti-smooth muscle α-actin antibody (Clone 1A4, Sigma Chemical Co.) or a monoclonal mouse anti-desmin antibody (Clone DE-U-10, Sigma Chemical Co.) at a dilution of 1:100. These were followed by secondary goat anti-mouse antibody conjugated to Texas Red or FITC (Molecular Probes Eugene, OR) at 1:100 dilution. Nuclei were counterstained with OliGreen (Molecular Probes, Eugene, OR) or propidium iodide. Membranes were viewed on a Leica TCS 4D laser-scanning confocal microscope, and 512×512-pixel images of single optical sections or projections through the depth of the PM were captured and stored as digital images for quantitative analysis.

In experiments performed to assess the rate of cell proliferation (Takahashi et al., 1993) animals were injected intraperitoneally (i.p.) with 5-bromo-2′-deoxyuridine (BrdU) (20 mg/ml in 0.007 M NaOH) (Sigma Chemical Co.) at a dose of 100 μg/g body mass. Loss of BrdU signal due to competing thymidine incorporation was blocked with 5-fluoro-2-deoxyuridine (1.4 mg/ml in 0.007 M NaOH) (Sigma Chemical Co.) at a dose of 6.7 μg/g body mass. Animals were killed and PMs harvested at various times. Quantitation to determine the BrdU labeling index of vascular cells of the PM was performed using the 40× objective of a Zeiss Axiophot epifluorescence microscope. With the photographic frame of the microscope camera serving to delimit the area of each field, five randomly selected fields were analyzed per PM and used to determine the average percentage of vascular cells incorporating BrdU.

In the experiment to define the M-G_1 boundary (Fig. 3B-D), an apparent 'pair' of BrdU-positive nuclei could arise from two situations. First, two S-phase cells could be in close proximity by chance, or second, a single S-phase cell could have undergone cytokinesis. Since any apparent pairs of labeled nuclei arising at 1 hour must have arisen by chance (since S-phase BrdU incorporation and cytokinesis could not have occurred within the time elapsed), we used this number as a correction factor and subtracted it from the 'pairs' values obtained at each time point. Conversely, the number of nuclei observed as apparent pairs at the 1 hour time point were counted as 'single' nuclei, and this number was added to the 'singles' values of all subsequent time points. Since the overall density of labeled cells did not change significantly over the course of the experiment, it was valid to apply this correction to every time point.

RESULTS

Cells of the PM are a cycling population

Since cells of the PM could be observed in mitosis (data not shown) and would label with BrdU (Diez-Roux and Lang, 1997), they were evidently a cycling population. To ask whether there was a correlation between rates of cycling and cell death, we quantitated both over the course of PM regression. The beginning of regression in normal rats was indicated by the first apoptotic cells appearing on the PM at 5

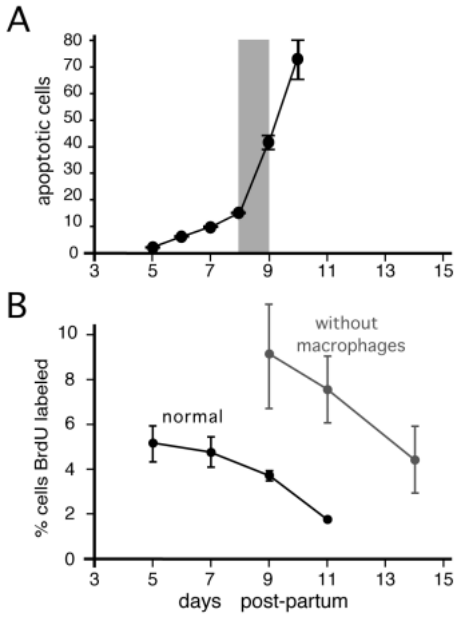

Fig. 1. (A) The total number of apoptotic cells observed on the PM during the initial phase of capillary regression. The gray bar shows the time interval used for experiments described in Fig. 2. (B) The percentage of cells that will incorporate BrdU at a given stage of regression for a normal PM (black line) or in the absence of macrophages (gray line). Developmental stage is indicated as days after birth.

days after birth (A5) (Fig. 1A). 3 days later at A8, the number of apoptoses increased dramatically. At the beginning of regression, the BrdU-labeling index was approximately 5%, but diminished as regression proceeded (Fig. 1B, black line). In the absence of macrophages and the cell-death signal they provide (Diez-Roux and Lang, 1997), the labeling index was increased compared with normal animals at each time point examined (Fig. 1B, gray line). Both diminishing numbers of cycling cells during normal regression and the increased numbers in the absence of cell death argued that during PM regression, the cycling cells might be the population destined for death.

To determine if both endothelial cells and pericytes were cycling, we double-labeled PMs for BrdU and smooth muscle α-actin, a pericyte marker (Diaz-Flores et al., 1991). Both smooth muscle α-actin-positive cells (Fig. 2A) and those without (not shown) would incorporate BrdU. This showed that both pericytes and ECs were actively cycling during PM regression.

Both vascular endothelial cells and pericytes are killed during PM regression

To determine whether both ECs and pericytes were killed during programmed regression, we labeled normal PMs for the alternative pericyte marker desmin (Diaz-Flores et al., 1991) (Fig. 2B, red label) and quantitated the relative proportion of normal and apoptotic ECs and pericytes. To resolve the closely apposed ECs and pericytes, this analysis was performed using optical sections generated with a confocal microscope. Pericytes were identified by the characteristic punctate desmin labeling pattern while apoptotic cells of either lineage were recognized by the distinctive condensation and nuclear margination of chromatin (Fig. 2C,D). Quantitation revealed that at A9, non-apoptotic cells made up 87.7% of the total (pericytes, 17.7%; ECs, 70%; Fig. 2E) while apoptotic cells comprised 12.3 % (pericytes, 2.7%; ECs, 9.6%; Fig. 2E). This indicated that approximately equal proportions of the pericyte and endothelial cell populations (13% and 12%, respectively) were dying at A9.

Cell death is cell cycle stage-dependent

If cycling were a requirement for PCD in cells of the PM, we reasoned that cell death might occur at a particular point in the cell cycle. To determine whether this was the case, we labeled a population of PM cells in S phase using BrdU and then followed the fate of these cells through cycle. Rat pups were injected with BrdU at 12 noon on day A8 (defined as t=0 and chosen because the increase in apoptosis from A8 to A9 is substantial; Fig. 1A, gray bar). They were then killed at different time points up to 24 hours after injection (Fig. 3) and the membranes dissected and stained for BrdU. Given the short half life of BrdU in vivo, this protocol would label a population of cells that was relatively synchronous with respect to cell-cycle stage.

Quantitation of the number of BrdU-positive, apoptotic PM cells over the 24 hour time course showed that the occurrence of apoptosis was not distributed evenly throughout the cell cycle but was observed primarily in a peak at 18 hours (Fig. 3A, black line). The existence of this peak indicated that the labeled population of cells was cycling synchronously and that PCD was cell cycle stage-dependent. Repetition of this experiment after macrophages had been ablated with toxic liposomes showed, as would be anticipated (Diez-Roux and Lang, 1997), that the cell cycle stage-dependent cell death was induced by macrophages (Fig. 3A, gray line). From these experiments, we concluded that cell death occurred approximately 18 hours after S phase.

Cell death occurs in G_1 phase of the cell cycle

To assist in determining when in cell-cycle capillary cells died, we quantitated the appearance of isolated BrdU-positive nuclei (Fig. 3B) as well as the pairs of labeled nuclei (Fig. 3C) that resulted from cell division (see Materials and methods). For every labeling event, the transition from one to two labeled nuclei marks cytokinesis at the M-G_1 boundary of the cell cycle.

After BrdU injection the number of isolated BrdU-labeled nuclei first decreases and then reaches a plateau at about 8 hours (Fig. 3D, singles). In a reciprocal pattern, the number of paired labeled nuclei increases and then also reaches a plateau (Fig. 3D, pairs). The position of each plateau indicates that only about 25% of the cells initially labeled in S phase complete cell division and may argue that some enter a G_2 arrest (Kosaka et al., 1996). Despite this, the experiment indicates that the M-G_1 boundary falls 6-8 hours after labeling in S phase (Fig. 3D, green bar). When combined with the data in Fig. 3A, this analysis indicates that the morphological signs of cell death follow the M-G_1 boundary by 10-12 hours (Fig. 3A,D, red line). While the length of G_1 phase of the cell cycle can vary, these data suggest that the cell death signal is likely to be received by capillary cells in G_1 phase.

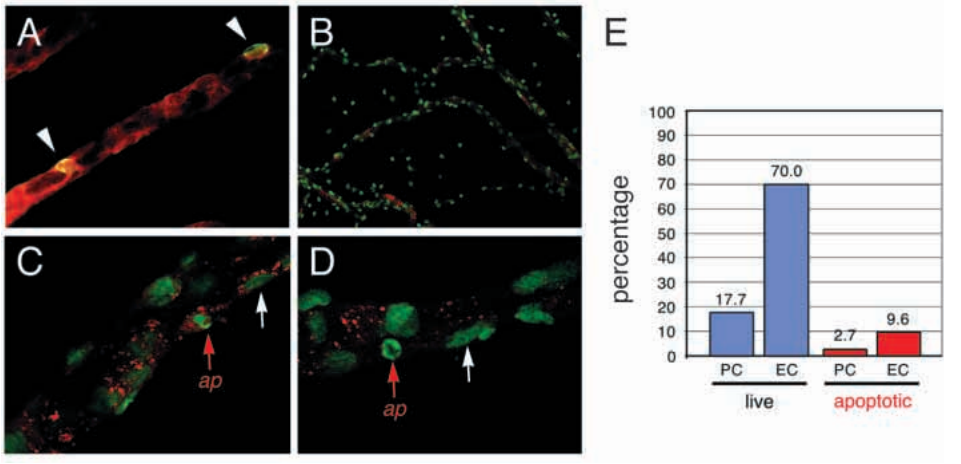

Fig. 2. (A) BrdU-labeled pericytes (white arrowheads) show smooth muscle α-actin labeling. (B) Desmin labeling (red) of a normal PM at A9. At this magnification (200×) pericytes are seen labeled with distinctive patches of red fluorescence. An OliGreen counterstain identifies chromatin in B-D. (C) An apoptotic pericyte is revealed by the characteristic chromatin condensation and margination in a cell surrounded by desmin labeling (red arrow). A normal pericyte (white arrow) is observed close by. (D) Apoptotic (red arrow) and normal (white arrow) ECs in a capillary where desmin labeling is apparent elsewhere in the segment. (E) The proportions of live and apoptotic pericytes (PC) and ECs in the pupillary membrane at A9.

Since we used apoptotic morphology as a read-out in these experiments, the time delay between commitment to cell death and the appearance of apoptotic morphology was an important consideration. Importantly, subtracting this interval from the time of cell death defined in previous experiments would identify the point in the cell cycle when a cell would receive a death signal. To determine the length of this interval, we explanted PMs in the presence of staurosporine, a potent inducer of apoptosis (Knox et al., 1992). A time course experiment showed that, on average, PM cells developed apoptotic morphology within 3 hours of exposure (Fig. 4). Subtracting this time from the 18 hour time-point where cell death is at a peak (Fig. 3A), we can suggest that cells of the PM are responding to a cell-death signal from macrophages at about 15 hours of the defined time course.

Another argument in favor of the assertion that PCD occurs in G_1 phase is the pairing of 90% (n=20) of BrdU-labeled apoptotic cells with BrdU-labeled normal nuclei (Fig. 3E,F). This implied that the paired BrdU-labeled apoptotic and normal nuclei arose as the products of cell division and that one daughter cell died after cytokinesis. Combined with the observation that essentially no macrophage-dependent cell death occurred at time points corresponding to S, G_2 or M phases (Fig. 3A-D), these data indicate that PCD is signaled after cytokinesis and thus in G_1 phase.

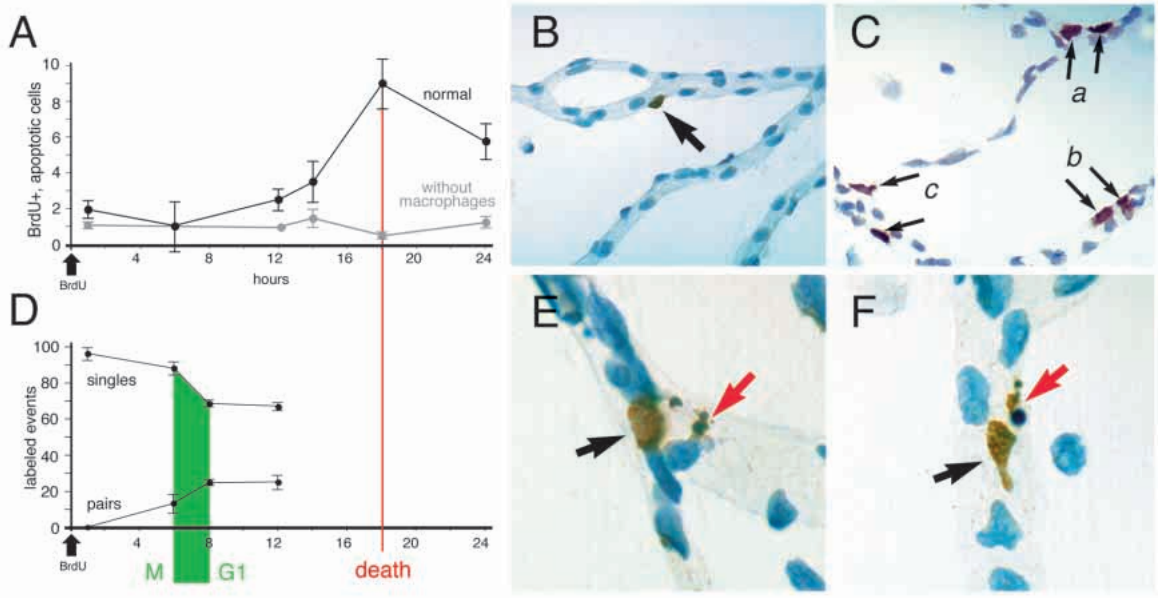

Fig. 3. (A) The total number of BrdU-positive, apoptotic cells appearing at various time points after an injection of BrdU at A8. The peak of cell death at the 18 hour time point in normal membranes (black line) is marked with the red vertical line. The gray line represents the consequences of macrophage elimination and shows that the peak of cell death at 18 hours is absent. The low level of PCD scored throughout the time course in either normal or macrophage-ablated membranes probably represents experimental background. (B) An example of an isolated BrdU-positive PM cell. (C) Pairs of BrdU-positive nuclei arising as a consequence of cell division (arrows labeled *a* and *b*) and two single labeled nuclei in close proximity (arrows labeled *c*). (D) The relationship between the number of single and paired BrdU-labeled nuclei (*y* axis) over developmental time (*x* axis) after S phase has been defined by BrdU-injection at A8. (E,F) BrdU-labeled apoptotic cells (red arrow) are observed paired with a BrdU-labeled nucleus that is normal in appearance (black arrow).

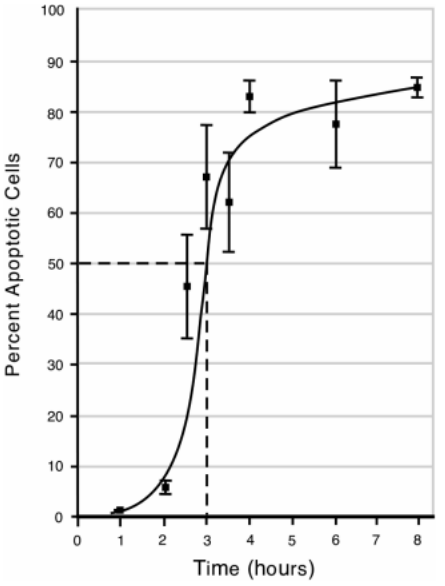

Fig. 4. Capillary cells were exposed to the proapoptotic agent staurosporine and the proportion of cells with apoptotic morphology was assessed over a subsequent time course.

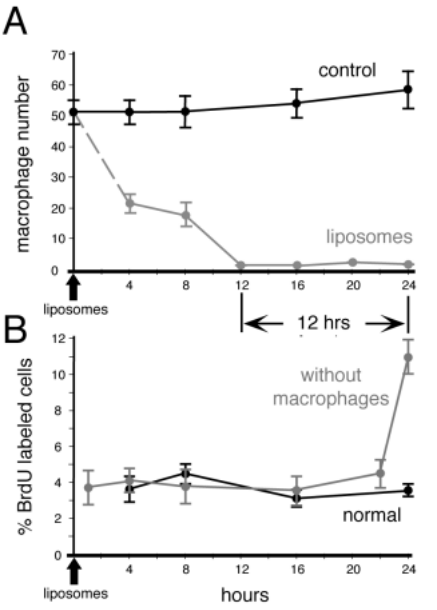

Fig. 5. (A) The relationship between total macrophage number on the PM and time (hours) after a single injection of toxic liposomes at A8. (B) The relationship between the percentage of BrdU-positive cells and developmental time (hours) after a single injection of toxic liposomes at A8.

Fig. 6. Summary of the timing of the macrophage cell-death signal relative to the cell-cycle phase of target cells. The 18 hour interval between the morphological appearance of apoptosis and S phase is defined by the data in Fig. 3A. Likewise, the 6-8 hour interval between S phase and cytokinesis (cyt) is defined in Fig. 3C. Making an allowance of 3 hours for the time between commitment to cell death and the appearance of apoptotic morphology (Fig. 4) suggests that cells of the PM receive a cell death signal at 15 hours of the defined time-course. From the data presented in Fig. 5, we can suggest that the *minimum* time from the point where a death signal would have been received to the following S phase is approximately 12 hours. Combining these data indicates that PM cells receive the cell-death signal from macrophages in mid-G_1 phase of the cell cycle.

Target cells receive a cell-death signal in mid-G_1 phase of the cell cycle

We next performed an experiment designed to provide corroborating evidence that cycling cells were destined to die, and to ask when cell death was signaled relative to S phase of the cell cycle. Thus, we asked how long it took for PM cells to reach S phase after the cell-death signal was blocked through macrophage elimination. Macrophages were ablated through liposome injection at 12 noon on A8 and PMs harvested subsequently at various time points over a 24 hour period. To determine when macrophage ablation was complete, the number of macrophages associated with PMs was quantitated (Fig. 5A). In a parallel experiment, the number of S-phase cells was determined by injecting BrdU 1 hour prior to the scheduled PM harvest (Fig. 5B).

Macrophages were completely eliminated by 12 hours (Fig. 5A). Following macrophage ablation, there was a significant increase in the percentage of BrdU-positive cells at 24 hours (Fig. 5B). The simplest interpretation of this observation is that cells that were not killed by macrophages have continued through G_1 phase into S phase and as a result, incorporated BrdU. These data showed that the *minimum* time from the lack of a cell-death signal (complete macrophage ablation) to the following S phase was approximately 12 hours. In addition, this experiment provides direct evidence that it is the cycling capillary cells that die. Using all the data presented, we can provide a good approximation of the time at which the cell death signal and the appearance of apoptosis occur relative to different stages of the cell cycle (Fig. 6). Combined, these data argue that the minimum length of G_1 is approximately 18 hours and that the signal for cell death is received in mid-G_1 phase.

DISCUSSION

We have examined the relationship between cell death and cell cycle in an example of tissue regression where cell death is dependent on macrophage function. Since the regressing structure is a capillary network, the targets of macrophage action are both ECs and pericytes. We have shown that both cell types cycle and that both undergo apoptosis. Combined with the observation that all macrophage-dependent cell death is cell cycle stage-dependent, these data imply that both ECs and pericytes are killed in G_1 phase.

External regulation of cell-cycle progression occurs at the so-called ‘restriction point’ in mid-G_1 phase. At the restriction

point, signals provided by extracellular matrix, an intact cytoskeleton and mitogens are required if the cell is to proceed through cycle. These signals are non-redundant in that if any one is absent, the cell cycle ceases (Zhu et al., 1996; Assoian, 1997; Huang et al., 1998) and in some cases (Howlett et al., 1995; Day et al., 1997), including endothelial cells (Meredith et al., 1993; Chen et al., 1997), the cells die. Given that the killing action of macrophages in the current system manifests in mid-G_1 phase, one obvious model to explain our observations is that to induce cell death, macrophages may inhibit restriction point signaling. Consistent with this model is the proposal that during post-lactation involution of the mammary gland, epithelial cell apoptosis is a consequence of the degradation of extracellular matrix with accompanying modulation of the expression of cell-cycle regulators (Boudreau et al., 1996).

The current analysis shows that in this system, cell cycling is required for cell death and provides a strong foundation for understanding the molecular mechanism of macrophage-mediated regression. However, one unresolved question is how there can be a net reduction in cell number (tissue regression) if a cell must divide before one daughter cell is killed. While we do not have a complete understanding at present, there are several possible explanations. Previous analysis argues that there is a second step during regression in which capillary cells die as a consequence of blood flow stasis (termed secondary apoptosis; Lang et al., 1994; Meeson et al., 1996; Lang, 1997). Vital analysis has revealed that in some capillary segments, flow stasis results when dying cells project into the capillary lumen and trap white or red blood cells (Meeson et al., 1996). Thus, since a small number of apoptotic cells can lead to flow stasis and cell death in an entire capillary segment, a lack of reduction in cell number as a direct consequence of macrophage killing may be inconsequential. Alternatively, it may be that both daughter cells resulting from division might be killed in G_1 phase. That we have not observed such events to date may be the result of the relatively early stage of regression in which we have concentrated our analysis.

The observation that both pericytes and ECs die as a result of macrophage action is significant for one of two reasons. First, these cells are of distinct developmental lineages and a direct induction of apoptosis in both cell types would suggest that macrophage-induced cell death may be a generally applicable mechanism for regulating cell number during development and homeostasis. Alternatively, it is possible that the macrophages, positioned outside the basal lamina, may directly induce apoptosis only in pericytes. This would be consistent with capillary topology where pericytes are positioned external to ECs. In such a model, one can suggest that macrophages might effect capillary regression by killing pericytes, thereby disrupting pericyte-EC cross-talk (Antonelli-Orlidge et al., 1989) and as a secondary consequence, inducing EC apoptosis. This possibility is currently under investigation.

The suggestion that macrophages have a central role in regulating capillary growth potential (Sunderkotter et al., 1994) is strongly supported by the results presented. Macrophages are known to produce a variety of factors that can regulate vascular potential (Sunderkotter et al., 1994) and the induction of EC and pericyte apoptosis by macrophages is one mechanism by which macrophages can exert control. The present results define a novel cell-cell interaction that culminates in EC and pericyte cell death and importantly, is required for vascular regression (Diez-Roux and Lang, 1997). It will be of great interest to determine whether macrophages have anti-angiogenic activity in other systems.

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