Macrophages induce apoptosis in normal cells in vivo

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SUMMARY

It is well established that macrophages have a function in scavenging apoptotic bodies from cells undergoing programmed cell death. Here we show that macrophages can also induce apoptosis of normal cells. Using injected toxic liposomes to eliminate macrophages in the anterior chamber of the rat eye, we provide direct evidence that, in vivo, macrophages induce apoptosis in normal vascular endothelial cells during programmed capillary regression. Macrophage elimination resulted in the survival of endothelial cells that normally would die and the persistence of functional capillaries. Furthermore, replacement of eliminated macrophages with bone-marrow-derived macrophages ‘rescued’ lack of capillary regression. Viability of the persistent target cells was demonstrated through their lack of apoptotic morphology, expression of intracellular esterases and synthesis of DNA. These results uncover a new function for macrophages in the remodeling of tissues through the induction of programmed cell death and provide direct evidence of a key role for macrophages in capillary regression.

Key words: apoptosis, macrophage, vascular regression, tissue remodeling, tissue regression, rat, eye

INTRODUCTION

Despite decades of interest in the function of macrophages, the in vivo significance of their ability to kill non-immune target cells in culture has remained obscure. Two disparate developmental systems have suggested that phagocytes might have activity in non-immune induction of target cell death. Laser ablation studies in C. elegans show that death of the gonadal linker cell (a component of the male sexual apparatus) requires the action of adjacent phagocytic epidermal cells (Sulston and Thompson, 1980), while cellular ablation transgenic mice (Lang and Bishop, 1993) have led to the proposal that, in mammals too, programmed cell death might sometimes be dependent on phagocytes.

In mammalian systems, mediators of macrophage cytotoxicity in vitro include TNFα (van de Loosdrecht et al., 1993) and nitric oxide (Cui et al., 1994). It has been presumed that these cytotoxic activities might be consistent with a role for macrophages in tissue maintenance (Vlassara et al., 1988) or in the case of a tumor cell target, with anti-neoplastic surveillance (Urban et al., 1986). However, in all cases characterized so far (Bernasconi et al., 1991), macrophage killing has required stimulation with inflammatory mediators and the relevance of this activity to normal physiology is unclear. In most cases, macrophage cytotoxicity results in the death of target cells through apoptosis (Aliprantis et al., 1996).

The pupillary membrane is a temporary capillary network in the anterior chamber of the eye (Matsuo and Smelser, 1971). It adheres to the anterior surface of the lens and iris diaphragm and regresses between weeks 1 and 3 after birth in the rat (Lang et al., 1994). Previous studies on the pattern of cell death in the pupillary membrane have suggested that capillary regression occurs in two stages. The first stage was proposed to be a macrophage-dependent induction of endothelial cell apoptosis (Lang and Bishop, 1993; Lang et al., 1994) and the second a coordinated apoptosis of capillary endothelial cells dependent on cessation of plasma flow (Meeson et al., 1996). The accessibility and relative simplicity of the pupillary membrane recommend it as a system in which to study vascular regression and, in particular, macrophage-endothelial cell interactions in an in vivo setting.

In the current study, we have used the liposome-mediated macrophage elimination technique (Van Rooijen, 1989) combined with macrophage-reconstitution to show that programmed capillary regression is dependent upon macrophage-induced apoptosis. Elimination of macrophages in the anterior chamber results in prevention of endothelial cell apoptosis and a dramatic persistence of the pupillary membrane in functional form. Macrophage reconstitution following macrophage elimination results in a ‘rescue’ of the persistent membrane. These experiments demonstrate that macrophages can induce apoptosis in normal cells in vivo and that they play a key role in programmed capillary regression.

MATERIALS AND METHODS

Animal breeding

Timed pregnant Sprague-Dawley and inbred Fisher rats were obtained from Taconic Farms (German Town, NY). Ages of rats are noted as days after birth (e.g. A14). Animal care was in accordance with institutional guidelines.
Liposomes
Control and toxic liposomes were generated according to established procedures (Van Rooijen, 1989). The liposome-dependent drug dichloromethylene biphosphate (clodronate) was kindly provided by Boehringer Mannheim GMBH. For macrophage elimination in the anterior chamber, liposome suspensions were injected trans-corneally in volumes ranging from 50 to 650 nl.

Trans-corneal injection
Trans-corneal injection was performed using a modification of previously described vital observation technique (Fig. 1; Meeson et al., 1996). Beveled glass injection needles were used to deliver defined volumes of liposomes to the anterior chamber using a Nanoject Variable injector (Drummond, PA). Injection was observed with the 5x air objective of a Zeiss Axioshot microscope (Zeiss, Thornwood, NY). A custom-designed stage plate allowed mounting of micromanipulators for injector and the optic fibre channel that provided illumination. Anesthesia of rat pups was performed as previously described except that Ketamine and Xylazine were delivered intraperitoneally and the local anesthetic tetracaine hydrochloride (0.5%) applied prior to parting of the eyelids. Injection was performed into the right anterior chamber with the rat pups positioned in a silastic body mold.

Culture of bone-marrow-derived macrophages
Bone-marrow-derived macrophages were prepared according to the following protocol (I. Riviere, personal communication). Bone marrow cells were harvested from the femurs of 1- to 2-week-old Fisher rats. 10^7 cells were plated in 10 ml of RPMI medium containing 10% fetal calf serum, 5x10^{-5} M β-mercaptoethanol, 20% L929 supernatant, 10% horse serum (Gibco BRL). Immature macrophages were grown for 5-6 days and during this period fed with fresh medium every other day. Prior to trans-corneal injection, macrophages were removed from the culture dish with a cell dissociation solution, washed with serum-free medium and resuspended at a concentration of 2x10^6/ml. 1 µl of this cell suspension was injected for macrophage reconstitution experiments.

Histological procedures
The pupillary membrane/iris diaphragm complex was dissected from the rat eye as previously described (Lang et al., 1994) and nuclei stained with Hoechst 33258 (Sigma, St Louis, MO) or with hematoxylin. Apoptosis was recognized as the characteristic condensation of apoptotic bodies (Wyllie et al., 1980). Macrophages were identified using indirect immunofluorescence with the primary antibody OX42 (Robinson et al., 1986)/(Cedarlane Laboratories Ltd., Hornby, Ontario) and rhodamine-conjugated goat anti-mouse IgG as a secondary antibody. An assessment of cellular proliferation through S-phase labeling of cells with 5-bromo-2'-deoxyuridine was performed according to standard procedures (Takahashi et al., 1993). Calcein AM (Papadopoulos et al., 1994; Molecular Probes, Eugene, OR) was used to stain dissected pupillary membranes at 50 µM in PBS for 30 minutes at 37°C prior to fluorescence visualization.

Timing of trans-corneal injections
The following diagram (Fig. 2) summarizes the times, relative to normal pupillary membrane regression, when trans-corneal injection of liposomes and macrophages was performed for the different experiments described.

RESULTS
Toxic liposomes containing PBS-clodronate and control liposomes containing only PBS were injected into the anterior chamber of young rat eyes at A7 and the pupillary membranes

![Fig. 2. The timing of normal pupillary membrane regression in rats relative to experimental manipulations. Morphological signs of regression occur between A7 and A14 as indicated in the three representations of the eye at the top of the figure. Experiments shown in Figs 3 and 4 were performed with a liposome injection at A7 and analysis at A11. Experiments shown in Figs 5 and 7 were performed with two injections of liposomes, the first at A3 and the second at A7. Analysis after dissection was performed at A14 at a time when a normal pupillary membrane has all but completely regressed. Macrophage reconstitution experiments (Fig. 6) were performed with a liposome injection at A7, macrophage injection at A12 and dissection for analysis at A14.](image-url)
assessed histologically after removal by dissection at A11
(Lang et al., 1994). Control liposomes encapsulating PBS and
tagged with the lipophilic fluorescent dye DiO effectively
labeled macrophages associated with the pupillary membrane
(Fig. 3A,C). In contrast, liposomes encapsulating the cytotoxic
agent clodronate (Van Rooijen, 1989) and carrying DiO did not
label macrophages implying that these
cells were rapidly eliminated; this was
apparent from the absence of labeled
nuclei lying between capillaries (Fig.
3B) and the lack of DiO-positive cells
on pupillary membranes (Fig. 3D).
Macrophage elimination was
confirmed through the labeling of
pupillary membrane preparations with
the macrophage-specific anti-CR3
antibody (Fig. 3E-H).

To test whether macrophages might
be required for the apoptosis occurring
during programmed capillary regression,
we eliminated macrophages associ-
ated with the pupillary membrane and
determined the proportion of putative
target cells undergoing apoptosis.
These experiments were performed
using different volumes of toxic
liposomes to determine if there was a
dose response for macrophage elimi-
nation and for endothelial cell death.
Toxic liposomes were injected at A7
and the numbers of macrophages and
apoptotic endothelial cells quantitated 4
days later. Macrophage numbers
decreased in a dose-dependent manner
in response to liposome injections (Fig.
4A-C, dashed line). Importantly, in six
independent experiments (Fig. 4A-D,
solid line), the number of apoptotic
endothelial cells also decreased in a
dose-dependent manner.

As a further test of the impact of
macrophage elimination on pro-
gr ammed regression, we examined the
morphology of pupillary membranes at
A14 after liposome injections at A3 and
A7. This liposome delivery schedule
resulted in an almost complete persis-
tence of the pupillary membrane as
assessed at A14 (Fig. 5A). Since only
one eye of each animal received
liposomes, the contralateral eye was
used as an internal control; this invari-
ably showed a pupillary membrane that
was almost completely regressed at
A14 (Fig. 5B). Comparison of an A7
normal pupillary membrane (Fig. 5C)
with the toxic-liposome-treated
membrane from A14 (Fig. 5A)
indicated that liposomes prevented
regression to a large degree. Pupillary
membranes treated with control

Fig 3. Toxic liposomes clear macrophages from the anterior chamber. (A-D) Pupillary
membranes at A11 after liposome injection at A7. (A,B) chromatin labelling with Hoechst
33258. (C,D) The same pupillary membrane preparations as in A and B, respectively,
illuminated for DiO tagged control (C) and toxic (D) liposomes. (E-H) Micrographs of A8
pupillary membranes treated with liposomes at A7. (E,F) DIC illumination; (G,H) fluorescence
illumination for CR3 receptor staining. (E,G) Control liposomes; (F,H) toxic liposomes.
not (Fig. 6B). However, given that programmed cell death was assessed by morphological criteria, it was possible that, while the persisting cells did not appear to undergo apoptosis, they were actually non-viable.

To address this issue, we used several methods for assessing cell viability. First, we determined whether persistent cells were able to synthesize DNA and thus incorporate bromodeoxyuridine (BrdU). Normal A9 membranes labeled with two injections of BrdU over a 3 hour period showed that 3.8±0.3% (n=3) of endothelial cells were in S-phase (Fig. 6C). Target cells persisting at A14 due to macrophage elimination incorporated BrdU at a similar level (4.4±1.1%, n=2) using the same labeling protocol; a 24 hour labelling period with 4 injections of BrdU resulted in 14.6±4.3% (n=3) BrdU-positive endothelial cells (Fig. 6D).

As a stringent test of viability for the entire persistent population, we labeled both normal and persistent cells with the vital dye and esterase substrate calcein AM (Papadopoulos et al., 1994). Characteristic yellow-green fluorescence was observed in the cells of normal pupillary membranes at A7 (Fig. 6E) as well as in persistent membranes at A16 (Fig. 6F). A calcein AM-labeled A7 membrane, killed through fixation, showed only low-level autofluorescence of endothelial cells and red blood cells (Fig. 6G). Capillaries from A16 persistent membranes also showed a distinctive lumen with erythrocytes present immediately after dissection (Fig. 6H). This suggested that persistent capillaries maintained blood flow, and this was confirmed in the live animal using a vital cell imaging system (Meeson et al., 1996; data not shown). These observations show that persistent cells of the pupillary membrane are cycling, are active in metabolism and maintain capillary patency. Together, these observations indicate that persistent cells are viable.

To test whether a purified population of macrophages could re-establish regression, we performed a reconstitution experiment. Toxic liposomes were first injected into the anterior chamber at A7 to effect macrophage elimination. At A12, 2×10^3 bone-marrow-derived macrophages in serum-free medium were injected and the consequences for pupillary membrane regression assessed. Controls included untreated membranes (from the contralateral eye) and membranes that received liposomes at A7 but medium only at A12. Membranes receiving liposomes but no macrophages were persistent (Fig. 7A) by comparison with untreated membranes from contralateral eyes (Fig. 7B). Liposome-treated membranes that also received macrophages were further regressed (Fig. 7C) than those that received liposomes only (Fig. 7A). Injected macrophages were labelled with fluorescent latex spheres and could be detected adjacent to capillaries of the pupillary membrane after injection into the anterior chamber (Fig. 7C,D). As a measure of the relative complexity of experimental and control membranes, we counted capillary junctions and showed that pupillary membranes receiving macrophages were significantly more regressed than those that did not (Fig. 7E). These experiments provide additional evidence that the macrophage is responsible for induction of programmed cell death and regression.
Fig. 6. In the absence of macrophages, target cells in the pupillary membrane remain viable. (A) Hoechst 33258-labeled endothelial cell nuclei in a control A11 pupillary membrane have non-apoptotic morphology. (B) Nearly all the cells in an A14 persistent membrane have normal nuclear morphology. (C) BrdU-labeled A7 control pupillary membrane showing brown-labeled positive nuclei. (D) Persistent (A14) pupillary membrane after 4 BrdU injections over a 24 hour period. (E) Calcein AM-labeled normal A11 pupillary membrane shows bright green fluorescence as does (F), a calcein AM-labeled A15 persistent pupillary membrane. (G) Normal A7 pupillary membrane stained with calcein AM after being killed by fixation. (H) Persistent A14 pupillary membrane in DIC illumination showing capillary lumen containing erythrocytes.

Fig. 7. Macrophage injection reestablishes pupillary membrane regression. (A) Persistent pupillary membrane at A14 after liposome-mediated macrophage elimination at A7. (B) Contralateral eye of animal shown in A to indicate the normal degree of regression by this stage. (C) Pupillary membrane at A14 after treatment with liposomes at A7 and macrophages at A12. Exogenous macrophages are labeled with yellow-green fluorescent latex spheres. (D) Higher magnification of a liposome- and macrophage-treated pupillary membrane at A14 showing the association between injected macrophages and capillaries. (E) Quantification of the number of capillary junctions in pupillary membranes that are treated only with liposomes, those that are treated with liposomes followed by macrophage reconstitution and those that are untreated. The number of capillary junctions in a pupillary membrane is a measure of the degree of regression. Error bars represent standard deviations.
DISCUSSION

Our assertion that macrophages actively induce apoptosis in normal cells during programmed tissue regression rests on a number of elements. Specific killing of macrophages by liposomes is indicated by the restricted distribution of the fluorescent tracer in control liposomes and the dose response for macrophage number. Liposomes clearly do not kill endothelial cells; if this were so, the number of dying endothelial cells would be expected to increase with increasing liposome dose and the opposite is observed. Furthermore, it has previously been established that the cytotoxic activity of clodronate is liposome-dependent (Van Rooijen, 1989). The observation that apoptotic endothelial cells decrease in number with increasing liposome dose and that injected bone-marrow macrophages can mediate regression provides the strongest evidence that macrophages induce endothelial cell apoptosis.

These observations indicate that macrophages drive most, if not all aspects of capillary regression. Such an active role for macrophages in pupillary membrane remodeling explains the persistence of these vessels in mice expressing an ocular not all aspects of capillary regression. Such an active role for macrophages in pupillary membrane remodeling explains the persistence of these vessels in mice expressing an ocular.

REFERENCES


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