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Accelerated Death of Retinal Microvascular Cells in Human and Experimental Diabetic Retinopathy

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Abstract

To reconstruct the mechanisms for the vasoobliteration that transforms diabetic retinopathy into an ischemic retinopathy, we compared the occurrence of cell death in situ in retinal microvessels of diabetic and nondiabetic individuals. Trypsin digestion and sections prepared from the retinas of seven patients (age 67±7 yr) with 9±4 yr of diabetes and eight age- and sex-matched nondiabetic controls were studied with the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) reaction which detects preferentially apoptotic DNA fragmentation. The count of total TUNEL+ nuclei was significantly greater in the microvessels of diabetic (13±12 per one-sixth of retina) than control subjects (1.3±1.4, P = 0.0016), as were the counts of TUNEL+ pericytes and endothelial cells (P < 0.006). The neural retinas from both diabetic and nondiabetic subjects were uniformly TUNEL+. Retinal microvessels of rats with short duration of experimental diabetes or galactosemia and absent and minimal morphological changes of retinopathy, showed TUNEL+ pericytes and endothelial cells, which were absent in control rats. These findings indicate that (a) diabetes and galactosemia lead to accelerated death in situ of both retinal pericytes and endothelial cells; (b) the event is specific for vascular cells; (c) it precedes histological evidence of retinopathy; and (d) it can be induced by isolated hyperhexosemia. A cycle of accelerated death and renewal of endothelial cells may contribute to vascular architectural changes and, upon exhaustion of replicative life span, to capillary obliteration. (J. Clin. Invest. 1996. 97: 2883–2890.) Key words: endothelial cells † pericytes † apoptosis † vasoobliteration † experimental galactosemia

Introduction

The most consequential lesion of background diabetic retinopathy is the progressive obliteration of retinal microvessels, which leads to ischemia and sight-threatening unregulated angiogenesis. The process begins in isolated capillaries that become acellular and nonperfused, extends to groups of capillaries, and then advances centripetally to involve the arterioles and their side branches (1). The prodromes of retinal capillary closure are still speculative, but there is direct and indirect evidence that diabetes compromises the survival of vascular cells. Pericyte ghosts, the pockets in basement membranes marking the space from which pericytes have disappeared, are well known features in the histology of diabetic retinopathy (2, 3), and speak for the demise of pericytes. There is no corresponding morphological evidence of accelerated disappearance of endothelial cells until the time when acellular capillaries become evident. The observation, however, that acellular capillaries continue to develop in diabetic dogs years after institution of good metabolic control (4) is compatible with a paradigm in which exposure to the diabetic environment forces endothelial cells to expend a disproportionate amount of their replicative life span, a situation from which restored euglycemia cannot offer rescue.

The recent availability of methods to detect cell death in situ has prompted us to begin testing the hypothesis that accelerated vascular cell death may be an important event in the process of diabetes-induced retinal capillary obliteration. We studied retinal vessels from diabetic patients to investigate whether the phenomenon is a concomitant of human diabetic retinopathy. We then extended the studies to two animal models that are known to develop the early stages of retinopathy (the alloxan-diabetic rat and the galactose-fed rat, reference 5) to (a) control for confounding influences by pathologies that are present in the elderly donors of human specimens, and by events occurring in the postmortem period; (b) determine whether accelerated vascular cell death precedes morphological evidence of retinal microangiopathy; and (c) isolate the causative role of hyperhexosemia.

Methods

Specimens. The human eyes were provided by certified Eye Banks and obtained through the National Disease Research Interchange (Philadelphia, PA); the donors remained anonymous. Donors were selected along the following criteria: < 75-yr old, duration of diabetes < 15 yr to address mostly background retinopathy (6), the fewest possible chronic pathologies other than diabetes, the absence of retinal or hematological diseases and uremia, and absent administration of chemotherapy or life support measures. The characteristics of the diabetic patients and the age- and sex-matched nondiabetic controls are presented in Table I. The eyes of the diabetic and nondiabetic donors were enucleated 3±1 and 4±2 h after death, respectively. They were fixed in 10% buffered formalin by the Eye Banks 13±4 and 15±4 h after death, respectively, and shipped immediately to the laboratory.

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Table I. Clinical Characteristics of Eye Donors and TUNEL+ Cells in Their Retinal Trypsin Digests

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AA, aortic aneurysm; Ca, carcinoma; CAD, coronary artery disease; CHF, congestive heart failure; CPA, cardio-pulmonary arrest; CVA, cerebrovascular accident; DM, diabetes mellitus; MI, myocardial infarction; PE, pulmonary embolism; SU, sulfonylurea. *Counts were performed on trypsin digests, each representing one-sixth of the retina. The results for DM subjects 1 and 4 and control subjects 4 and 8 are the average of two experiments performed on two different trypsin digests.

The right eye from each donor was processed to obtain retinal sections, the left to obtain isolated preparations of the vascular network through trypsin digestion of the neural retina. To prepare retinal sections, the eye was transferred from formalin to freshly prepared 4% paraformaldehyde for 1 h at 4°C, and cryopreserved by incubation in a 0.1 M sodium phosphate, 0.15 mM calcium chloride buffer containing 30% sucrose overnight at 4°C. The retina was dissected, cut in small pieces, and embedded in optimum cutter temperature compound (Miles Inc., Elkhart, IN); radial sections of 10 μm thickness were cut in a cryostat and set onto Silane-coated slides (Polysciences Inc., Warrington, PA), air dried, and stored at −20°C. Trypsin digestion of the retina was performed according to the method of Cogan et al. (2) with some modifications (7), after at least 48 h, but no more than 5 d of formalin fixation. The digestion buffer contained 0.2 M sodium fluoride to inhibit DNases contaminating the crude trypsin preparation.

Sprague-Dawley male rats (Harlan Sprague-Dawley Co., Indianapolis, IN) with alloxan-induced diabetes of 31 wk duration (n = 7) or experimental galactosemia of 24 wk duration (n = 7) were compared to age- and sex-matched normal animals (n = 5). Diabetes was induced with alloxan (45 mg/kg body weight intravenously) and insulin was given as needed (0–2 U of neutral protein Hagedorn insulin subcutaneously, two times per wk) to achieve slow weight gain without preventing hyperglycemia and glycosuria. Experimental galactosemia was produced by feeding a rat chow (5001; Ralston Purina Co., Richmond, IN) containing 30% d-galactose. Treatment of animals conformed to the Association for Research in Vision and Ophthalmology resolution on Treatment of Animals in Research. All rats had free access to food and water; food consumption and body weight were measured weekly. Shortly before death, the severity of blood hexose elevation was estimated after an overnight fast by measuring the level of glycohemoglobin (Glyc-Affin; Pierce Chemical Co., Rockford, IL). After 31 wk of insulin-deficient diabetes, or 24 wk of galactose feeding, the animals were killed and the eyes were removed and immediately placed in 10% buffered formalin. Trypsin digestion of the retina was performed as described for the studies in human eyes after at least 48 h, but no more than 5 d of formalin fixation.

**Figure 1.** Photomicrographs of retinal trypsin digests prepared from diabetic patients and stained with TUNEL (left) and periodic acid-Schiff hematoxylin (right). Each of the pictures showing TUNEL fluorescence is matched with the light microscopic view of the same area of vasculature to provide histological correlates for the positive nuclei. (A) Autofluorescent trains of RBC and a brightly fluorescent image corresponding to a pericycle nucleus that in the histologic view (arrow) shows pale chromatin suggestive of ongoing degeneration. The upper arrowhead points to a normal pericycle nucleus and the lower arrowhead to a normal endothelial cell nucleus. The brightly fluorescent image in B can be attributed to a

1. **Abbreviation used in this paper:** TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.
pericyte nucleus showing fragmentation (arrow). The small arrow points to a pericyte ghost which shows no TUNEL reaction, indicating that chromatin is no longer present. C and D show bright TUNEL positivity of groups of discrete and sharply delineated masses of chromatin, which have a counterpart in the histological view (arrows) and appear as apoptotic bodies of endothelial cells. The small arrow in C points to a polymorphonuclear cell which does not show TUNEL fluorescence, and the small arrow in D points to a pericyte ghost. Acellular capillaries are visible in C and D. Images are from diabetic patient 2 in Table I (A and B), patient 4 (C), and patient 1 (D). ×600 for the fluorescence views and ×700 for the color views.
Deoxynucleotidyl transferase catalyzes a template-independent addition of deoxynucleotides to free 3′-OH ends present in DNA breaks. This tailing reaction is especially sensitive to the type of DNA fragmentation occurring in apoptotic, rather than necrotic, cell death (9). The specimens were rehydrated in PBS, incubated in 3% H2O2 in water for 5 min to block endogenous peroxidase in anticipation of the possible need to perform peroxidase-catalyzed reactions, and permabilized with 0.5% Triton X-100 in PBS for 1 h at room temperature. The specimens were then incubated with TUNEL reagents for 1 h at 37°C under paraffin protection, and mounted in Slow-Fade (Molecular Probes, Inc., Eugene, OR). In each experiment, the negative control received only the label solution without the terminal transferase, and the positive control was exposed, before the TUNEL reaction, to DNase I (1 μg/ml in 40 mM Tris-HCl, 6 mM MgCl2, buffer, pH 7.5) for 10 min at room temperature.

Identification of TUNEL+ cells was performed with knowledge of the identity of the human specimens, but in a masked fashion for the rat specimens. Each trypsin digest or retinal section was surveyed systematically under a Zeiss Axioshot (Carl Zeiss Inc., Thornwood, NY) fluorescence microscope by scanning the specimen with downward and upward motion beginning at the upper left margin. Specificity of any fluorescent signal was determined by switching to the rhodamine excitation wavelength; the fluorescein-specific signals compatible with nuclear material in phase contrast were photographed (Tri-X pan 400 films; Eastman Kodak Co., Rochester, NY), and their location in the specimen was identified by recording the coordinates on the microscope stage. The specimen was stained with periodic acid-Schiff and hematoxylin and the morphological counterparts of TUNEL images were identified by recording the coordinates on the microscope stage. The specimen was stained with periodic acid-Schiff and hematoxylin and the morphological counterparts of TUNEL images were identified and photographed (Lumiere 100X Ektachrome; Eastman Kodak Co.). These steps permitted the attribution of the majority of TUNEL+ images in trypsin digests to pericyte or endothelial cell nuclei; for rare TUNEL+ cells, generally of small size and/or with uninformative shape or topography, the cellular attribution remained undetermined. The results are thus reported as TUNEL+ pericytes, endothelial cells, or undetermined. Positive images found at intersections of vessels and not confidently attributable to a given cell were not counted to prevent inclusion of artifacts. Trypsin digests stained with periodic acid-Schiff and hematoxylin were also examined for the characteristic histological lesions of retinopathy (microaneurysms, pericyte ghosts, acellular capillaries). In the human specimens, the lesions were not seen as being present or absent; in the rat specimens, the frequency of pericyte ghosts and acellular capillaries was quantitated in a masked fashion as reported previously (5). Saccular microaneurysms were not observed in the rat specimens.

**Immunohistochemistry.** To examine whether TUNEL+ images attributable by shape and topography to endothelial cell nuclei colocalized with an endothelial cell-specific marker, selected specimens were reacted, after photography of the TUNEL signals, with vWF antibodies as previously described (7). Negative controls received preimune mouse IgG1. Bound vWF antibodies were detected with the Vectastain Elite ABC peroxidase system (Vector Laboratories Inc., Burlingame, CA), and the reaction product was developed with diaminobenzidine for 10 min. The specimens were counterstained lightly with hematoxylin.

**Statistical analysis.** The data are summarized with the mean±SD. Statistical analysis of the characteristics of patients and specimens was performed with the unpaired t-test; analysis of the prevalence of TUNEL+ cells was performed with the Mann-Whitney rank-sum test because the distribution of values was skewed. Analysis of the characteristics and microvascular lesions of diabetic, galactosemic, and control rats was performed with ANOVA, followed by Fisher’s multiple comparisons test to isolate differences between groups.

**Results**

Two types of nuclei are present in retinal microvessels: the round or slightly oval nuclei of pericytes, which (albeit fully engased in the basement membrane) often appear to protrude from the vessel wall and stain heavily with hematoxylin, and the larger ellipsoid nuclei of endothelial cells lying in the axis of the capillary and taking a lighter stain (Fig. 1 A). TUNEL+ nuclei were attributed to pericytes or endothelial cells when they satisfied both the criteria of shape and topography. No TUNEL+ nuclei were detected in retinal trypsin digests not exposed to exogenous terminal transferase (negative controls), whereas all nuclei were positive in digests pretreated with DNase I (positive controls) indicating specificity of TUNEL staining.

Fig. 1 (left) shows images of TUNEL+ retinal pericytes and endothelial cells in three of the diabetic patients studied, and (right) the corresponding morphological correlates. In the fluorescence views, dimly fluorescent (autofluorescent) trains of RBC do not interfere with the identification of brightly fluorescent (TUNEL+) round, eccentric images corresponding to pericyte nuclei (Fig. 1, A and B), and clusters of discrete smooth bodies, evocative of apoptotic bodies, corresponding to nuclei that by shape and topography can be attributed to endothelial cells, (Fig. 1, C and D). Figure 1 also shows pericyte ghosts (Fig. 1 B), noticeable loss of pericytes (Fig. 1, C and D), and acellular capillaries (Fig. 1, C and D). This type of histological abnormality, as well as microaneurysms, was observed in the trypsin digests of all diabetic patients, with the exception of patient 3 in Table I. The count of total TUNEL+ nuclei in trypsin digests, each representing one-sixth of the retina, was significantly (P = 0.0016) greater in the diabetic than in age- and sex-matched control subjects (Table I), as were the counts of TUNEL+ pericytes (P = 0.005) and endothelial cells (P = 0.006). Statistical significance of the difference between the diabetic and control groups was maintained even when the large counts registered in diabetic subject 6 were excluded from analysis. Only one nondiabetic subject (control subject 4 in Table I) showed a substantial number (six) of TUNEL+ nuclei, mostly attributable to endothelial cells. However, in a second trypsin digest from the same subject, only one positive nucleus (endothelial) was identified. In three other subjects (two diabetic and one control), the study of a second trypsin digest showed satisfactory reproducibility of observations (5, 10, and 0 TUNEL+ nuclei vs 8, 11, and 2 in the first digest, respectively). The average of the counts obtained in the two studies was used in the final computation.

To obtain additional confidence that TUNEL+ signals were attributable to endothelial cells, colocalization was sought with vWF, especially in capillaries devoid of pericytes. Despite the fact that vWF staining is variable among and within retinal vessels (7), and that degenerating endothelial cells may no longer produce or store the glycoprotein, several instances of colocalization of apoptotic bodies and vWF were observed (Fig. 2).

The TUNEL reaction was also performed in radial sections of human retinas. The positive controls showed beautifully the scattered, variable-size nuclei of the ganglion cell layer, and the smaller, more abundant, and densely packed nuclei of the inner and outer nuclear layers. The retinal sections obtained from both diabetic and nondiabetic subjects were uniformly negative for TUNEL fluorescence.

Because most of the diabetic patients had histological evidence of retinopathy, it could not be determined whether evidence of microvascular cell death precedes morphological changes in vessels. Rats were thus studied after a duration of diabetes (31 wk) or galactosemia (24 wk) expected to result in only minimal, if any, retinopathy. Diabetic and galactose-fed rats showed (Table II) increased glycohemoglobin levels (P <
0.001 vs controls), documenting the effects of hyperhexosemia. Whereas only one of the control animals showed TUNEL images in its retinal microvessels (two images classified as undetermined), all the diabetic and galactose-fed rats manifested TUNEL nuclei. Fig. 3 presents fluorescence images and morphological counterparts in the retinal vessels of galactose-fed rats, and Table II presents the summary of the data relative to the TUNEL reaction and microvascular histology in rat retinal microvessels. In the diabetic rats, TUNEL cells occurred in capillaries showing counts of pericyte ghosts and acellular vessels not different from those in control rats. In the galactosemic rats, the count of pericyte ghosts was not different from controls, but a slightly larger number of acellular capillaries was observed ($P < 0.003$). These counts reflect incipient stages of retinopathy as documented by comparison with the counts obtained after 18–22 mo of diabetes or galactose feeding (5).

**Discussion**

We have documented an increased prevalence of death of both pericytes and endothelial cells in the retinal vessels of diabetic patients and experimentally diabetic or galactosemic animals when compared to control subjects. Excess cellular death in diabetes and galactosemia was observed at early stages of retinopathy or even in its absence, and it was confined to the microvessels; the neural retina did not show evidence of TUNEL$^+$ cells. Insofar as experimentally galactosemic animals lack many of the hormonal and metabolic abnormalities of diabetes (10), hyperhexosemia appears to be a sufficient determinant of the accelerated demise of vascular cells.

We detected cell death with a technique that uncovers DNA fragmentation, an early event in the form of suicidal and regulated cell death known as apoptosis (11, 12). Because studies to document the internucleosomal DNA cleavage and ultrastructural features characteristic of apoptosis (12) promise to be labor intensive in our specimens in view of the miniscule proportion of TUNEL$^+$ cells, and have not yet been performed, we refrained from defining microvascular cell death as apoptotic. A large body of circumstantial evidence points, however, in this direction. The extremely bright signals obtained within 1 h of incubation with the tailing enzyme are consistent with the presence of a large number of DNA double-
Figure 3. Photomicrographs of retinal trypsin digests prepared from rats after 24 wk of galactose feeding. Methods are as described in Fig. 1. A and B present TUNEL$^+$ pericytes, with evidence of chromatin fragmentation (A). C shows an example of TUNEL$^+$ chromatin that could not be attributed to a specific cell type and was thus classified as undetermined. D shows apoptotic bodies oriented with the long axis of the vessel and attributed to an endothelial cell. The vessels appear histologically normal. Images are from four different rats. Magnifications are as in Fig. 1.
strand breaks, which are hypothesized to occur earlier and with much greater frequency in apoptosis than in the nonspecific DNA fragmentation of the late stages of necrosis (9). The clustered clumps of chromatin giving positive signals are identical to apoptotic bodies as observed in light microscopy (12), and the appearance of TUNEL chromatin in pericytes and endothelial cells is compatible with the different degrees of cellular budding and nuclear fragmentation occurring in different cell types (12). Although we cannot exclude underestimation of the number of dying cells because we do not know the duration of a positive TUNEL signal in a microvascular cell undergoing apoptosis, the pattern was one of death occurring in random, scattered cells, another distinguishing feature of apoptosis. The histological appearance of necrosis typically involves groups of adjoining cells and the presence of inflammation (12), which also is remarkably absent from the histology of diabetic retinopathy. Finally, experiments performed in vitro have shown that elevated ambient glucose accelerates apoptosis of vascular endothelial cells (13).

The fact that pericytes and endothelial cells are dying in the very early stages of diabetic and diabeticlike retinopathy, and that these dying cells were mostly observed in vessels that looked histologically normal, indicate that cell death is not a sequela of grossly compromised conditions of the vascular wall. In fact, accelerated death of microvascular cells may have a causative role in the lesions of diabetic retinopathy. Insofar as pericytes probably do not replicate in the adult organism (14), the death of pericytes is likely to account directly for the pericyte ghosts and the overall loss of pericytes from vessels that eventually become acellular. Retinal capillary endothelial cells have a slow but measurable turnover (14) and undergo mitosis in response to injury (15). A cycle of accelerated death and consequent accelerated renewal of endothelial cells would not be morphologically apparent, yet would subject the vessel wall to continued remodeling and might thus contribute to the architectural changes (microaneurysms, dilatation) of diabetic retinal vessels. A similar paradigm has been invoked to explain alterations of large vessels’ caliber during the progression of atherosclerotic lesions (16). Eventually, accelerated death of endothelial cells may lead to capillary closure as the increased turnover would prematurely exhaust the cell’s replicative potential and compromise the maintenance of the antithrombotic lining. This sequela could be hastened if the process of endothelial cell replication is hampered in diabetes (17). Increased frequency of endothelial cell death has also been noted in the aorta of streptozotocin-diabetic animals (18), and the evidence provided by our studies for accelerated death of both pericytes and endothelial cells in diabetic retinal vessels disputes the long-held view (2,3) that pericytes are preferentially lost in diabetic retinopathy and that the demise of pericytes is the initial lesion in the pathogenesis of the disease.

The fact that we did not detect evidence of neural cell death in the retinas of diabetic patients militates against accelerated loss of neurosensory elements as a mechanism for the retinal dysfunction reported to often precede demonstrable lesions of diabetic retinopathy (19,20).

The occurrence of accelerated microvascular cell death in galactosemic rats, combined with evidence from in vitro studies that elevated glucose levels induce increased death (21), DNA damage (22), and apoptosis (13) of vascular endothelial cells, points to at least an initiating role of high levels of hexose in the demise of endothelial cells and, possibly, pericytes. It is of note that galactose-fed rats showed the same extent of accelerated microvascular cell death as the diabetic rats, despite substantially lower elevation in glycohemoglobin levels. Insight into the reasons underlying this observation, as well as into the biology of diabetic retinopathy, should derive from the detailed study of the cellular events that precede and perhaps permit cell death. The likelihood that the accelerated microvascular cell death is by apoptosis, which is a highly regulated phenomenon (11,16), prompts questions on how microvascular cells are normally equipped to resist the many extrinsic triggers of apoptosis (23) and whether high hexose levels act as positive inducers of apoptosis or rather alter survival programs or signals. As we learn more about the cellular aspects of diabetic retinopathy, it becomes apparent that the long latency period described between the onset of diabetes and the appearance of retinopathy applies mostly, if not solely, to the histology of the disease. Vascular cell responses to hyperglycemia are likely to begin early, and this has important clinical corollaries and implications for the conceptual as well as practical aspects of the pathogenetic quest. In this context, accelerated microvascular cell death may become a useful surrogate end-point in pharmacological studies of experimental diabetic retinopathy since it reflects events intrinsically relevant to the traditional histological end-points, but detectable months or years before an informative number of morphological abnormalities.

Acknowledgments

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