Proc. Natl. Acad. Sci. USA
Vol. 77, No. 9, pp. 5445–5447, September 1980
Medical Sciences

Protection of cultured renal tubular epithelial cells from anoxic cell swelling and cell death

(ischemia/*in vitro*/polyethylene glycol)

J. I. KREISBERG*†, J. W. MILLS‡§, J. A. JARRELL¶, C. A. RABITO¶, AND A. LEAF¶

Departments of ‡Anatomy, *Pathology, and ¶Medicine, Harvard Medical School; and The Renal Unit, Medical Services, Massachusetts General Hospital, Boston, Massachusetts 02114

Contributed by Alexander Leaf, May 27, 1980

ABSTRACT **In order to study the relationship between cell swelling and cell death due to ischemia, we have developed an *in vitro* model by using primary cultures of renal tubular epithelial cells. With this model, we have studied two components of ischemia—namely, anoxia along with substrate deprivation. After 2 hr of anoxia in the absence of substrate, the cultured cells swelled and blebbed. Cells similarly treated in the presence of 8% polyethylene glycol, an oncotic agent, did not swell and bleb, and when cells were counted 18 hr later, similar numbers of cells were seen as in the untreated cultures. However, tubule cells exposed to anoxia without 8% polyethylene glycol had 50% fewer cells 18 hr later. Therefore, if cell swelling is prevented during 2 hr of anoxia, cell viability is improved.**

Little is known about the cellular mechanisms that render cells unable to survive a period of ischemia or substrate deprivation. In fact, the precise event that dooms the cell irreversibly to cell death due to ischemia is not known. Understanding the mechanisms of irreversible cell damage is essential to designing rational prophylactic or therapeutic interventions.

Much has been written concerning ischemic injury in the kidney (1–9); however, although we can induce renal failure in a variety of ways, the pathogenesis of renal failure after an ischemic insult has yet to emerge. Current evidence, largely from micropuncture studies, indicates that the functional failure of the kidney subjected to an ischemic insult results from tubular obstruction and increased back reabsorption of glomerular filtrate through damaged, leaky tubular epithelia (6–9). Frega *et al.* (5) have found that when kidneys were flushed with 8% (wt/vol) polyethylene glycol (PEG), an oncotic agent, and the renal arteries were clamped for 45 min, proximal tubule cells were prevented from swelling and blebbing and renal function was preserved. Contrary to this finding, in kidneys flushed with saline followed by a similar period of ischemia there was swelling and blebbing of proximal tubule cells and acute renal failure (5). The addition of 8% PEG to the extracellular fluids during ischemia balances the osmotic activity of the intracellular nondiffusable macromolecules and prevents cells from swelling (10, 11). A similar preservation of cell viability has been demonstrated in ischemic cardiac tissue when mannitol was used as the osmotic agent (12, 13).

Although the results with 8% PEG in the kidney confirmed the importance of cell swelling to ischemic renal injury, it did not clarify the nature of the relationship. As pointed out by Frega *et al.* (5), cell swelling may obstruct renal tubules or increase vascular resistance. For these reasons, we developed a system for obtaining, culturing, and observing primary cultures of renal tubular epithelial cells. With this preparation, the cell environment can be strictly controlled in order to study the pathogenesis of anoxic injury *in vitro*. Because of the complex effects of ischemia on tissue, we have simultaneously examined two components of ischemia—namely, anoxia and substrate deprivation—by means of our cultured renal cells. Cultured renal tubule cells swell, bleb, and undergo cell death after anoxia, similar to ischemic renal tubule cells *in vivo*. In addition, we can protect the cultured cells from swelling, blebbing, and subsequent cell death with 8% PEG added to the anoxic perfusate.

METHODS

Male Sprague–Dawley rats weighing 150–250 g were used. Animals were anesthetized with 35 mg of sodium pentobarbital per kg of body weight. The kidneys were perfused *in situ* via the aorta with Hanks' balanced salt solution at a pressure of 120 mm Hg (1 mm Hg = 1.33×10^2 Pa) to remove erythrocytes. The kidneys were excised, capsules were removed, and cortices were dissected from each kidney. Cortical slices were minced with a razor blade, pressed with a spatula through a stainless steel screen of 60 mesh (250-μm pore), and rinsed with balanced salt solution onto a screen of 150 mesh (150-μm pore) (14). Tissue on a 150-mesh screen, consisting of tubules, was transferred to a plastic tube containing balanced salt solution with antibiotics (penicillin, streptomycin, and fungizone) and washed twice. After the final wash, tubules were suspended in tissue culture medium for outgrowth of cells. An exact volume of medium containing the tubule fragments (0.2 ml, which contains 100 tubule fragments) was plated onto 25-mm glass coverslips in 35-mm plastic tissue-culture dishes. After 4 days, which allows the tubule fragments to attach to the glass, 2 ml of medium was added. The tissue-culture medium was Roswell Park Memorial Institute medium (RPMI) with 20% (vol/vol) fetal calf serum supplemented with 10% (vol/vol) conditioned medium (from Swiss 3T3 cells in the logarithmic phase of growth) and 0.33 international unit of insulin per ml.

Coverslips for the histochemical demonstration of alkaline phosphatase were fixed for 1 min in 10% (vol/vol) formalin/1% (wt/vol) calcium chloride. Slides were incubated in 0.25%

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviation: PEG, polyethylene glycol.

† Present address: Department of Pathology, University of Texas Health Science Center, San Antonio, TX 78284.
§ To whom requests for reprints should be sent at: Laboratory of Renal Biophysics, Massachusetts General Hospital, Boston, MA 02114.

naphthol AS-MX buffered with phosphate at pH 8.6 with 0.05% fast red violet LB as the capturing agent (Sigma) for 30 min at room temperature and washed in deionized water (15).

Anoxic experiments were performed on 5-day explants of rat tubular epithelial cells. Glass coverslips containing tubule explants were placed into Dvorak–Stotler chambers (Nicholson, Bethesda, MD). The chamber was housed on the specimen stage of a Zeiss photomicroscope and the cells were observed through a ×40 water-immersion phase-contrast lens. Perfusion was by gravity feed, and the effluent was connected to a Radiometer oxygen electrode type D616 (Rainin, Brighton, MA) run in the continuous mode. The advantages of this system are: (*i*) the shape of the cells can be observed continuously and recorded by photography; (*ii*) the perfusate can be readily modified for studying the pathogenesis of anoxia; and (*iii*) the perfusate can be passed over the cells at controlled flow rates (0.1 ml/min). The perfusate was phosphate-buffered saline (140 mM NaCl/1 mM $CaCl_2$/2.7 mM KCl/1.4 mM KH_2PO_4/0.5 mM $MgCl_2$/8 mM Na_2HPO_4). Substrate was not present.

For each experiment, the cells were exposed to oxygenated perfusate for 30 min. Then the perfusate was changed to phosphate-buffered saline that had been equilibrated with 100% nitrogen, and this medium was allowed to flow through the chamber for 2 hr. In order to establish whether a correlation between cell swelling and cell death exists, 8% PEG (M_r 6000) with a reflection coefficient of 1.0 (16) was added to the anoxic perfusate and the experiments were conducted as described above. After the anoxic insult, coverslips were returned to normal culture conditions for 18 hr, after which the cells were trypsinized to separate them. Cell viability was determined by counting the cells.

RESULTS AND DISCUSSION

After 5 days in culture, cells could be seen growing out from rat kidney tubule fragments. These cells were polygonal and had a cobblestone-like appearance. Ninety percent of these cells had histochemically demonstrable alkaline phosphatase activity. Such cells formed tight junctions in culture and grew in tissue culture medium in which D-valine was substituted for L-valine, a condition under which fibroblasts cannot survive (17). For these reasons, we believe that these cells are proximal tubule cells.

When these renal tubule epithelial cells were maintained under anoxic conditions for 2 hr, they swelled and some demonstrated blebbing of their plasma membranes. When exposed to the anoxic and substrate-deprived perfusate in the presence of 8% PEG, there was no detectable swelling or formation of membrane blebs. Counting of cells after the 18-hr recovery period showed that the coverslips exposed to anoxic conditions in the presence of 8% PEG had approximately the same number of cells as coverslips maintained continuously in culture (Table 1). Those coverslips exposed to anoxic conditions without PEG had approximately 50% fewer cells than either the 6-day culture or the PEG-protected one (Table 1 and Fig. 1).

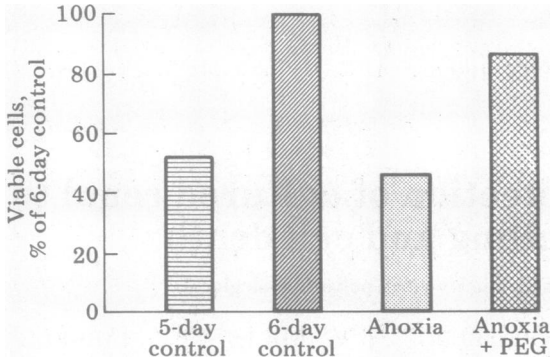

FIG. 1. Viability of renal tubular epithelial cells exposed to 2 hr of anoxia (on day 5) followed by overnight culture. Data are expressed as the percent of cells in culture compared to 6-day normal control culture. Cells exposed to anoxic perfusate with 8% PEG grow similarly to controls, whereas anoxic perfusate alone causes a 44% decrease in cell number.

These results demonstrate that if cell swelling is prevented during a 2-hr period of anoxia, cell viability is dramatically improved. Experiments performed with PEG *in situ* in the rat kidney demonstrated that if proximal tubule cells were prevented from swelling during an ischemic insult, renal function was preserved and subsequent tissue necrosis was largely prevented (5). Deprivation of cell energy supplies, a consequence of anoxia, prevents the extrusion of sodium ions, which therefore accumulate with chloride and water in the cell resulting in osmotic swelling of the cell (19). Extracellular sodium normally balances the oncotic swelling pressure of obligatory intracellular colloids, but cell membranes are permeable to sodium ions. Thus, the extracellular position of sodium is maintained by the continuous, energy-requiring active transport of sodium out of the cell by membrane-bound Na^+,K^+-ATPase. This establishes a steady state and stabilizes cell volume. When energy metabolism is inhibited by anoxia, this regulatory mechanism fails and cells swell. The addition of 8% PEG to the extracellular fluids during ischemia balances the activity of the intracellular, nondiffusible macromolecules and therefore prevents cells from swelling (10, 11). The results with PEG *in situ* confirmed the importance of cell swelling to renal injury but did not clarify the nature of the relationship—for example, cell swelling could affect renal hemodynamics, which then leads to necrosis. Because of the complexity of tissue ischemia, we have examined two factors of ischemia *in vitro*: anoxia along with substrate deprivation. We found that if we protected our cultured cells from swelling with 8% PEG, we increased their viability after an anoxic insult. The mechanisms of this protective effect with 8% PEG can now be examined.

Table 1. Protective effect of PEG on cultured renal tubular epithelial cells from anoxic cell death*

Condition	Days of culture	*n*	No. of cells (mean ± SEM) × 10^{-3}
Pre-anoxia	5	12	25.67 ± 3.28
Anoxia	6	9	21.78 ± 1.33†
Anoxia + PEG	6	9	41.22 ± 5.03†
Control	6	10	48.0 ± 3.91†

* Cultured cells were exposed to 2 hr of anoxia with or without PEG followed by 18 hr of culture, after which they were trypsinized and counted in a hemocytometer. Note that the cells exposed to anoxia and PEG divided similarly to the 6-day controls.

† Means were significantly different in the anoxia compared to the PEG group ($P < 0.05$) and in the anoxia compared to the control group ($P < 0.005$) by one-way analysis of variance and a sums of squares simultaneous test procedure (18).

We thank Mr. Mark Rothman and Ms. Mary Whelan for technical assistance and Ms. Sylvia Wells for typing the manuscript. This work was supported by U.S. Public Health Service Grant HL 06664.

1. Trump, B. F., Croker, B. P. & Mergner, W. J. (1971) in *Cell Membranes: Biological and Pathological Aspects*, eds. Richten, G. W. & Scarpelli, D. G. (Williams & Wilkins, Baltimore, MD), p. 84.
2. Trump, B. F., Berezesky, I. K., Collan, Y., Kahng, M. W. & Mergner, W. J. (1976) *Beitr. Pathol.* **158,** 363–388.
3. Kreisberg, J. I., Bulger, R. E., Trump, B. F. & Nagle, R. B. (1976) *Virchows Arch. Cell Pathol.* **22,** 121–133.
4. Venkatachalam, M. A., Bernard, D. B., Donohoe, J. R. & Levinsky, N. G. (1978) *Kidney Int.* **14,** 31–49.
5. Frega, N. S., DiBona, D. R. & Leaf, A. (1979) *Pfluegers Arch.* **381,** 159–164.
6. Arendhorst, W. J., Finn, W. F. & Gottschalk, C. W. (1975) *Circ. Res.* **37,** 558–568.
7. Arendhorst, W. J., Finn, W. F. & Gottschalk, C. W. (1976) *Kidney Int.* **10,** 100–105.
8. Tanner, G. A. & Sophasan, S. (1976) *Am. J. Physiol.* **230,** 1173–1181.
9. Tanner, G. A. & Steinhausen, M. (1976) *Kidney Int.* **10,** 65–73.
10. Wiggins, P. M. (1964) *Biochim. Biophys. Acta* **88,** 593–605.
11. Robinson, J. R. (1971) *J. Physiol. (London)* **213,** 227–234.
12. Powell, J. W., DiBona, D. R., Flores, J. & Leaf, A. (1976) *Circulation* **54,** 603–615.
13. DiBona, D. R. & Powell, J. W., Jr. (1977) *Circulation Suppl. 3,* **70,** abstr. 264.
14. Burlington, H. & Cronkite, E. P. (1973) *Proc. Soc. Exp. Biol. Med.* **142,** 143–149.
15. Burstone, M. S. (1958) *J. Natl. Cancer Inst.* **20,** 601–615.
16. Little, J. R. (1965) Dissertation (Univ. of Otago, Otago, New Zealand).
17. Gilbert, S. F. & Migeon, B. R. (1975) *Cell* **5,** 11–17.
18. Gabriel, K. R. (1964) *Biometrics* **20,** 459–477.
19. Leaf, A. (1956) *Biochem. J.* **62,** 241–248.