Light and Electron Microscope Study of Osmotically Induced Tumor Necrosis

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ABSTRACT

Necrosis of 1.5-cm Walker 256 tumors was produced by injecting a strongly hypertonic solution of glucose in and around the growths and by delaying resorption of the solution with serotonin, given s.c. at a distance. The morphological changes occurring in 13 tumors were followed by light and electron microscopic analysis of samples taken 0.25, 0.5, 1, 1.5, 3, and 5 hr after treatment. The 0.25-hr samples showed mitochondrial swelling, loss of cristae, and flocculent material within the inner compartment. Swelling of the mitochondria persisted in the 0.5-hr specimens (as it did in all subsequent samples), and it was accompanied by clumping and margination of chromatin. These changes were more pronounced at 1 hr, at which time the nuclear and plasma membranes were frequently ruptured. The endoplasmic reticulum and Golgi complex could no longer be recognized. The 3-hr material revealed ruptures of the outer mitochondrial membrane with myelin figures and discontinuous cell membranes. In the 5-hr samples, the nuclei exhibited a dark nucleoplasm and large clumps of chromatin. The perinuclear membrane was not always recognizable.

INTRODUCTION

It was shown in rats that acute necrosis of a Walker 256 tumor, implanted in the quadriceps of the right thigh, could be induced by the injection of a strongly hypertonic solution of glucose in and around the growth and by the simultaneous administration of 5-hydroxytryptamine at a distance (15). In 84% of the animals, such treatment, administered once, produced a cure characterized by disappearance of the implanted tumor in 10 days on an average, with a normal increase of body weight, good general condition, and absence of macroscopic metastases on autopsy performed at the end of a 2-month observation period after treatment. The present light and electron microscopic studies were undertaken to gather information on the sequence of events leading to tumor necrosis.

MATERIALS AND METHODS

With a 17-gauge trocar, 3 fragments (1 mm) of a 7-day-old Walker 256 tumor were implanted in the quadriceps of the right thigh of female Sprague-Dawley rats weighing between 95 and 105 g. Fifteen animals, which had developed a growth of 1.5 cm mean diameter within 6 days of implantation, were selected for the experiments. Two were used as controls, and the remaining 13 received identical treatments under light ether anesthesia.

5-Hydroxytryptamine (serotonin creatinine sulfate; ICN Pharmaceuticals, Cleveland, Ohio) was administered under the skin of the left hemithorax at a dose level of 2 mg in 0.2 ml of distilled water.

Anhydrous glucose was injected at a concentration of 1 g in 1 ml of distilled water with a 25-gauge needle in (1 ml) and around (1.5 ml) the tumor. In the latter case, 1.2 ml were injected under the skin covering normal tissues, in the immediate vicinity of the tumor, and on the outer aspect of the thigh and 0.3 ml was injected in the connective tissue of the inner aspect of the thigh in contact with the medial aspect of the growth. The 25-gauge needle was used to minimize leakage of the glucose solution from the tumor. In spite of such a narrow gauge, the highly concentrated and sticky glucose solution could be injected fairly easily with a 1-ml tuberculin Luer-Lok syringe.

Tumors were excised from the 2 controls and 13 treated rats. The latter were divided into 7 groups from which the growths were removed 0.25, 0.5, 1, 1.5, 3, or 5 hr after treatment. Tissues were fixed for 1 hr in a 2.5% glutaraldehyde solution buffered (pH 7.3) according to the method of Millonig (14). They were postfixed for an additional hr in a similarly buffered 1% osmium tetroxide solution. The tissue blocks were dehydrated in ethanol and embedded in Epon. Thin sections were cut with an LKB ultratome and stained with lead nitrate and uranyl acetate. The grids were studied under a Siemens 1A electron microscope. Sections 1 μm thick were stained with toluidine blue for light microscopy.

RESULTS

Light Microscopy

Controls. Generally, the cells in these animals appeared to be closely packed. In some areas, they were separated by capillaries and fine collagen fibers. Large nuclei contained prominent, darkly stained nucleoli. Mitoses were observed occasionally. Large empty vacuoles occupying up to 25% of the cytoplasmic surface were present in about 10% of the cell population (Fig. 1). The cytoplasm was moderately to frankly dark in about 25 to 50% of the cells, whereas it was light in the others. Such variability in coloration may be related to the immersion technique used (10), although Earle (8) noticed that the cells of Walker 256 tumors did not stain uniformly in paraffin sections.

Treated Animals. Small clumps of chromatin surrounded
by clear areas were evident in most nuclei 15 min after treatment with glucose and serotonin. Nucleoli, being indistinguishable from the chromatin clumps, were no longer prominent. Numerous small vacuoles made it difficult to differentiate the clear and dark cells observed in control sections. Large vacuoles were still present. There was intercellular edema, and small areas of hemorrhage, probably due to the trauma of the injection, were noted. All these cellular changes occupied about 50% of the field area of the sections examined.

The clumping of chromatin was even more striking 30 min after treatment. The previously noted small vacuoles were more numerous. The cellular changes now involved 75 to 100% of the field area of the various sections.

Considerable edema and isolated erythrocytes were noted between cells 1 hr after treatment. There was marked clumping and margination of chromatin. About 10% of the nuclei were pyknotic. The cytoplasm was clear and contained numerous fine vacuoles. Large vacuoles were still present in some cells. All these cellular changes were observed in 75 to 100% of the field area of the sections examined (Fig. 2).

Several nuclei were pyknotic, and most cells showed a large number of well-delineated vacuoles 1.5 hr after treatment. There was considerable intercellular edema. Erythrocytes were again observed. The cellular alterations now involved the entire field area of all sections, just as they did in later time sequences.

Intercellular edema, cytoplasmic vacuolation, and clumping as well as margination of chromatin were again pronounced at 3 and 5 hr posttreatment. In certain 5-hr sections, up to 25% of the nuclei were pyknotic.

**Electron Microscopy**

**Controls.** Our findings correlate well with those reported by some investigators (1, 9, 13). The tumor cells did not show any form of intercellular junctions. Numerous pseudopods projected from their surfaces. More than 1 nucleolus could be seen within some nuclei. The nuclear membrane and pores displayed a normal pattern. The chromatin was regularly dispersed. Ribosomes were evenly distributed throughout the cytoplasm. The mitochondria appeared to be small, round or ovoid, and sparsely distributed without any particular site of concentration. Their membranes and cristae looked normal. The endoplasmic reticulum was poorly developed, its granular form being more common than the smooth variety. The Golgi complex was scantily represented by thin elongated saccules, usually in the vicinity of the nucleus. Small dense bodies, probably corresponding to lysosomes, were discernible. The large intracellular vacuoles, already noted by light microscopy, were limited by a membrane. They were usually optically empty, although some contained highly dense clumps of glycogen or unidentified debris (Fig. 3).

**Treated Animals.** The mitochondria were swollen to roundness 15 min after treatment. The cristae had disappeared in many cases, and marked matrical pallor was evident. Flocculent material was found frequently in the inner compartment. Chromatin was beginning to condense within the nucleus. An early degree of margination was noticeable. The perinuclear space was sometimes enlarged. The nucleoli appeared to be a little smaller. Their granular/fibrillar ratio was unchanged. The ribosomes, endoplasmic reticulum, and Golgi complex remained unaltered (Fig. 4).

High-amplitude swelling of mitochondria was seen 30 min after treatment. Nearly all the cristae had disappeared. Flocculent material was present in the expanded pale inner compartment. Chromatin clumping and margination had progressively increased. The nuclear sap was pale. The nucleoli were more dense and homogeneous, although a few lighter areas were still seen within the agglomerated mass of fibrillar and granular components. No change was observed in the endoplasmic reticulum and Golgi complex. The cytoplasm was more pale than in control specimens. The pseudopods had become more discrete. The glycogen granules seemed unchanged (Fig. 5).

Cellular damage was strikingly obvious 1 hr after treatment. High-amplitude mitochondrial swelling persisted. Frank pyknosis of nuclei could be observed. In most cases the nucleoplasm was pale and the chromatin was marginated. The perinuclear membrane was frequently ruptured. The nucleoli showed changes similar to those observed after 30 min. The ribosomes were clumped, and in the completely disorganized and frequently vacuolated cytoplasm the endoplasmic reticulum and Golgi complex could no longer be recognized. The pseudopods of the cell surface had disappeared. Frequent ruptures of the cell membrane were noted. On the other hand, some cells revealed no more damage than that commonly observed 15 min after treatment (Fig. 6).

The lesions examined 1.5 hr after treatment were closely similar to those in the preceding time sequence (Fig. 7).

The necrotic process was accentuated 3 hr after treatment. The outer membrane of the dilated mitochondria was frequently ruptured or had disappeared. All the cells under study had ruptured membranes. Myelin figures were observed in the cytoplasm (Fig. 8).

The demarcation between nucleus and cytoplasm was generally uncertain 5 hr after treatment. In some cases, the perinuclear membrane had disappeared. Large clumps of chromatin were dispersed throughout the nucleus, instead of being concentrated at its periphery, as in earlier sections. The nucleoplasm was very dark, as opposed to the remarkable pallor usually observed 3 hr after treatment. The nucleoli were indistinguishable from the clumps of chromatin (Fig. 9).

**DISCUSSION**

Necrosis being the common denominator of many forms of cellular assault, we observed lesions that have been well documented previously (12, 16, 17, 22–25). However, the treatment we used and the tissue that it was applied to seemed to give a few particular features to the alterations we have just reported. Acute dilation of mitochondria (16, 17, 22–25) affecting the inner compartment took place very rapidly, i.e., within 15 min. It was accompanied by the virtually complete disappearance of cristae. Flocculent densities (16, 17, 22–25) remained small and were inconstant. Dilation of the endoplasmic reticulum (16, 17, 22–25)
was remarkably absent 15 and 30 min after treatment. Because of extensive damage, this structure could not be recognized in later specimens.

Although much has still to be done to analyze the mechanism of the alterations produced, we may already suggest a few likely factors. Local anoxia of an already poorly vascularized structure (26) comes first to mind. Serotonin contributes to it (3-6, 19-21), as does the increased pressure generated within the growth by the 1-ml glucose injection, the volume of which represents about 50% of the initial tumor volume. Such heightened intratumoral pressure might be compounded by the further addition of water from the extravascular compartment in an effort to dilute the excess glucose. We would, however, expect this extravasation of water to be limited by the circulation slowdown caused by serotonin (3-6, 19-21) and the increased intratumoral pressure as well as by the competition of the larger amount of glucose injected around the tumor (1.5 ml as compared with 1 ml into the growth) in the loose connective tissue where considerable edema rapidly develops. The large quantity of hypertonic glucose injected in comparison to the blood volume of the animal should be kept in mind even if we consider that the rat has free access to drinking water. Such hematocrit-elevating conditions would facilitate the development of thrombosis (21) in the small vessels going to and coming from the tumor as well as in the growth itself. Local thrombosis would further impede the extravasation of water and the resorption of the excess glucose. These various factors would contribute to the maintenance of a high osmotic pressure within the tumor. It is likely that this raised osmotic pressure, affecting at first the extracellular fluid, causes the cells to contract in an effort to dilute the excess glucose in their environment. Such shifts of water may occur very rapidly. However, we would not expect the cellular contraction to last very long. The increased lucency of the cytoplasm and the nuclear sap, which we observed in our electron micrographs from 30 min onward, indicates the entry of water, a common event in multiple forms of cellular injury (2, 11, 12, 17, 18, 22-25).

Control of cell volume under normal conditions requires a tremendous amount of energy. For instance, more than one-third of the energy metabolism of resting muscle cells is used up for the extrusion of sodium (11). Failure of the sodium pump and an increase of membrane permeability cause leakage of potassium and magnesium from cells (11, 18, 22-25) and penetration by sodium, chloride, and calcium (11, 18, 22-25). Such shifts are known to interfere with mitochondrial respiration, oxidative phosphorylation, and protein synthesis (23). Under conditions of severe metabolic disturbance and failure of the sodium pump, exchanges of water are governed by the intracellular colloid osmotic pressure (18), and swelling of cells takes place.

The addition of a large amount of glucose to the extracellular fluid is liable to create special problems. Some of it might be actively transported across the cell membrane as long as phosphorylation of the hexose is maintained, and simple diffusion might occur later on. If the experiments of Davey and Skegg (7) on kidney slices are regarded as a guide in the interpretation of our own results, we would expect glucose to reach a roughly similar concentration in the intracellular and extracellular water. Although at this stage we do not know the metabolic fate of the hexose under our experimental conditions, it is likely that some splitting of the molecule takes place with further increase of the local osmotic pressure. Enhanced acid production under a lack of oxygen would create a situation highly reminiscent of that reported by von Ardenne and Reitnauer (27-29), who raised intratumoral glycolysis by a 4-fold increase of blood sugar through prolonged systemic perfusion of glucose. They thus obtained a lower pH, which facilitated the activation and release of lysosomal enzymes (28, 29) and the damage of capillaries (27).

Although several mechanisms are involved in the production of the described cell damage, we feel that it would be appropriate to regard the damage as osmotically induced. The large amount of glucose administered and the high tonicity of the solution are decisive factors. A slowdown of the circulation protects the injected hexose from rapid dilution and resorption. Thrombosis of the small vessels further isolates the tumor from corrective influences. A loss of selectivity of membranes under anoxic conditions helps to spread the hypertonicity into the intracellular compartment. The catabolism of glucose and cell constituents increases the number of molecules.

We feel that the principal merit of electron microscopy in the study of osmotically induced tumor necrosis was to show that considerable damage could be produced within a very short period. This remains in keeping with the observation that cyanosis of the skin overlying the tumor is obvious 30 min after the treatment. The morphological information obtained thus far already invites analysis of the functional damage caused to the cells and their organelles in relation to time. Knowing how soon after treatment malignant cells may be rendered nonviable would not be of mere academic interest.

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REFERENCES
Osmotically Induced Tumor Necrosis

Fig. 1. Photomicrograph of a semithin section from a control Walker 256 tumor. The coloration of the cells is not uniform. Some cells contain large vacuoles. Toluidine blue, × 875.

Fig. 2. Photomicrograph of a semithin section from a Walker 256 tumor 1 hr after treatment with serotonin plus hypertonic glucose. There is marked edema. The cytoplasm is clear and contains numerous fine vacuoles apart from the large ones seen in control sections. Note the marked clumping and margination of chromatin. Toluidine blue, × 875.

Fig. 3. Electron micrograph of control Walker 256 tumor. Intercellular junctions are not visible. The cell surfaces are irregular. A large nucleus contains a prominent nucleolus. Small, ovoid mitochondria are sparsely distributed. × 7,000.

Fig. 4. Electron micrograph of a Walker 256 tumor 15 min after treatment with serotonin plus hypertonic glucose. The mitochondria are markedly swollen, and most cristae have disappeared. The pale inner compartment contains flocculent material. × 28,000.

Fig. 5. Electron micrograph of a Walker 256 tumor 30 min after treatment. High-amplitude swelling of mitochondria is evident in the upper part of the picture. Most cristae have disappeared. Note clumping and margination of chromatin. The nuclear sap is pale, and a nucleolus looks more dense. Erythrocytes are present between cells. × 5,000.

Fig. 6. Electron micrograph of a Walker 256 tumor 1 hr after treatment. Ribosomes appear to be absent in some areas of the cytoplasm and clumped in others. The high-amplitude swelling of mitochondria persists. Clumping and margination of chromatin are very pronounced. The nuclear sap is pale. × 7,000.

Fig. 7. Electron micrograph of a Walker 256 tumor 1.5 hr after treatment. The nucleolus appears more dense and homogeneous than normal. There is a clear contrast between the perinucleolar chromatin (C) and the nucleolus (N). × 17,500.

Fig. 8. Electron micrograph of a Walker 256 tumor 3 hr after treatment. The cell membranes are ruptured. The mitochondria are extremely dilated. Most of the cristae have disappeared. Erythrocytes can be seen between cells. × 5,500.

Fig. 9. Electron micrograph of a Walker 256 tumor 5 hr after treatment. Large clumps of chromatin are dispersed throughout the nucleus. The nuclear sap is very dark. Massive dilation of mitochondria is evident, and most of their cristae have disappeared. × 5,500.
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