Ultrastructure of Doxorubicin (Adriamycin)-induced Skin Ulcers in Rats

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ABSTRACT

Skin necrosis was produced in 24 male Fischer 344 rats by intradermal injection of 0.5 ml of doxorubicin (Adriamycin) at a concentration of 2 mg/ml. The resulting wounds healed slowly over 6 to 7 weeks with the reduced contraction rate paralleling the prolonged morbidity of doxorubicin ulcers in humans. Electron microscopy showed bizarre rough endoplasmic reticulum, double-walled vacuoles, and swollen mitochondria from 1 through 12 weeks after injury. Myofibroblasts with 60- to 80-A microfilaments with electron-dense bodies, intercellular connections, and prominent microtubules were seen from 4 through 12 weeks after injury. Although the appearance of myofibroblasts was delayed, their structure was normal. The delayed contraction of doxorubicin-induced skin ulcers thus appears due to persistent nonspecific cellular damage at the nuclear level rather than to specific derangement of myofibroblast function.

INTRODUCTION

Cutaneous ulcers caused by extravasation of doxorubicin (Adriamycin) in humans have a prolonged morbidity with slow healing rate (1, 2, 6, 8, 11, 13, 16). These ulcers heal at a much slower rate than do surgically created wounds of similar size (9). Grossly, they are similar to radiation-induced ulcers (8, 9) and yet on microscopic examination these wounds lack the blood vessel occlusion typical of radiation injury (14).

Recent electron microscopic studies have indicated that contractile fibroblasts (myofibroblasts) are the probable cause of wound contraction (3, 4). This study was designed to look at the ultrastructure of doxorubicin-induced skin ulcers with specific reference to their myofibroblast population to see whether the reduced rate of healing could be ascribed to specific alterations in myofibroblasts.

MATERIALS AND METHODS

Animal Injections. Twenty-four male Fischer 344 white rats, 200 to 280 g, were injected intradermally with clinically prepared doxorubicin in a concentration of 2 mg/ml (diluted with nonbacteriostatic 0.9% NaCl solution). Intradermal injections were done using a No. 25 needle, ballooning the dermis with solution as previously described (9). The animals were anesthetized with ether, and 2 injections were performed, one on the anterior and one on the posterior dorsal skin of each animal, after hair was shaved. The size of the skin wheal produced was measured at the time of injection.

The animals were weighed, and the diameter of the skin necrosis measured at 2, 4, 7, and 11 days and then weekly through 12 weeks. Additional measurements were done at 14 and 20 weeks after injection. At each time interval, multiple biopsies were taken from 2 ulcers. Biopsies were approximately 1-mm wide and were obtained from representative areas from the ulcers and adjacent normal skin. The complete depth of the skin and panniculus carnosus was included. No ulcer was biopsied more than once to avoid the biopsy process from interfering with later histology. Ulcers that were biopsied were not thereafter measured.

Electron Microscopy Preparation. Excised biopsy specimens were immediately placed into a solution of 4% paraformaldehyde and 5% glutaraldehyde buffered with 0.1 M sodium cacodylate at room temperature, 20°–22°. After 4 hr of fixation, the biopsy specimens were washed 3 times with cacodylate buffer and then were postfixed with 2% osmium tetroxide at 4°. Specimens were stained en bloc with 2% uranyl acetate, dehydrated with a graded series of ethanol washes, and placed in propylene oxide. Increasing concentrations of Epon 812-propylene oxide were used, proceeding to pure Epon 812 for embedding. During the embedding process, care was taken to orient the tissue so that cutting would be precisely cross-sectional. Thick sections were cut, stained with methylene blue, and studied to identify the proper areas for electron microscopic study. Attention was concentrated at the junction of necrotic tissue and live tissue based on the thick-section study. Thin sections were cut using an LKB ultratome, were counterstained with lead citrate, and were examined in a Zeiss 10 electron microscope at 4,000 to 16,000 magnification. All specimens were examined in the same uniform manner by the same electron microscopist. Approximately 15 to 20 cells were examined from each face block thin section.

RESULTS

Gross Appearance and Contraction. Doxorubicin uniformly produced skin necrosis with a diameter of 10 to 12 mm. The ulcers slowly contracted, requiring 6 to 7 weeks for full closure (Chart 1).

Light Microscopy. At 3 days after injection, necrosis of dermis was evident with pyknotic nuclei and dark-staining collagen. The epidermis was nonviable over the injection area. Acute inflammatory response was minimal, and vessels did not appear to be occluded. By 8 days, necrosis was accompanied by an inflammatory demarcation zone. Later, ulcers had chronic granulation tissue and once healed the lesions were composed of dense collagen with overlying epidermal hyperplasia.

Electron Microscopy. By the fifth day, intracellular struc-

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SEPTEMBER 1979 3689
tures underwent significant change. Rough endoplasmic reticulum was dilated or in small loops. Multiple vacuoles with double membranes were also seen. Through 8, 12, and 14 days, many of these vacuoles persisted (Figs. 1 and 2). Many of the cells were disrupted with lysis of the cell membrane. Considerable swelling of mitochondria was noted in some cells.

Rough endoplasmic reticulum persisted in unusual patterns with aggregation and rosettes through 3 weeks. Intracellular mitochondria were frequently disrupted, and many blood vessels were filled with cellular debris.

Not until 4 weeks after doxorubicin injection were cells seen suggestive of early myofibroblasts. At this time, small bundles of 60- to 80-Å microfilaments occurred with electron-dense bodies plus occasional desmosomes (Fig. 3). Of the fibroblasts present at 4 weeks, 10 to 15% had small bundles of microfilaments. At 6 weeks, cells were again seen containing microfilament bundles suggestive of myofibroblasts and also containing many microtubules. Weekly biopsies through 11 weeks continued to demonstrate cells with characteristics of myofibroblasts but with quite small bundles of microfilaments and vaguely defined electron-dense bodies. Microtubules were prominent in the cells during this period (Fig. 4). Mitochondrial degeneration and dilated rough endoplasmic reticulum persisted in some cells. By 12, 14, and 20 weeks, cells characteristic of myofibroblasts were no longer seen, and by 14 and 20 weeks, the rough endoplasmic reticulum had returned to a normal appearance. Dense collagen was noted in all specimens after the second week.

Thus, degenerative changes including bizarre rough endoplasmic reticulum, double-membrane vacuoles, and swollen mitochondria were seen from the first through 12 weeks, with the tissues appearing more normal at 14 and 20 weeks. Myofibroblasts containing microfilament bundles with electron-dense bodies, desmosomes, and microtubules were seen only from 4 through 11 weeks.

**DISCUSSION**

The prolonged morbidity of clinical doxorubicin ulcers suggests persistent damage to the tissue. In previous experimental studies, we have shown that surgically induced lesions of the same size heal at a much faster rate than do doxorubicin-induced ulcers (7, 9). Two possible mechanisms have been suggested as the cause of this prolonged morbidity (9). Doxorubicin binds directly to the bases of DNA (12) and hence interferes with nuclear function. Thus, it would interfere with cell replication necessary for healing of tissues.

An additional possible mechanism might be direct interference with wound contraction mechanisms. Contractile fibroblasts (myofibroblasts) have been demonstrated by Gabbiani et al. (3) to be the probable cause of wound contraction (4, 15). These cells share electron microscopic characteristics of both fibroblasts and smooth muscle cells. In addition, pharmacological stimulation and relaxation with agents known to cause effects in smooth muscle produce similar effects in granulation tissue. Finally, immunofluorescent studies have demonstrated similarity of myofibroblasts to smooth muscle cells (3, 4).

Myofibroblasts contain microfilament bundles with electron-dense bodies similar to those of smooth muscle cells. In addition, they have intercellular desmosomes and gap junctions which connect the individual cells and allow them to exert pull on each other. We have also demonstrated prominent microtubules in actively contracting myofibroblasts and have theorized that these intracellular structures are necessary for effective cellular contraction (10).

Jaenke (5) demonstrated via electron microscopy that in heart muscle damaged by doxorubicin, disruption of the intracellular contractile microfilaments occurred in addition to nuclear damage. Our study was conducted to evaluate whether any of the myofibroblast structures related to effective contraction were specifically affected by doxorubicin injury.

In fact, doxorubicin injury leads to delayed development of myofibroblasts. In surgical wounds in rats, myofibroblasts began to appear within 2 days (7), whereas in this study, they were not clearly seen until 28 days. The myofibroblasts themselves did not appear to be unusual, although the microfilament...
bundles were somewhat smaller than in normally contracting wounds. Extracellular basal lamina and convoluted nuclei, originally described as features of myofibroblasts (3), were rarely seen, as is typical of myofibroblasts in rats versus those in humans or pigs (7). No specific alteration in intracellular myofibroblast appearance could be demonstrated in the doxorubicin-damaged tissues.

Electron microscopy can never be truly quantitative because of sampling error problems, and thus, it is possible that intracellular effects might not have been seen on the samples studied. However, multiple samples were taken and studied in exactly the same fashion by the same electron microscopist, experienced from previous studies (7, 10) of myofibroblasts in identifying them and their characteristics. Thus, this study represents a reasonable approximation of the myofibroblast population. In contrast to the lack of changes in the myofibroblasts, multiple intracellular degenerative changes occurred which persisted through 14 weeks after doxorubicin injury. The persistent swollen mitochondria, rough endoplasmic reticulum appearing dilated or in small circles, and vacuoles with double membranes all suggest chronic intracellular damage.

Probably, these changes represent disruption of cellular processes due to persistent damage of the nucleus. In cardiac muscle, the muscle bundles are preexisting, whereas in doxorubicin-damaged skin, contractile cells must be developed by the local tissues to produce contraction. A reduced ability of the damaged tissues to differentiate and produce contractile cells probably explains the reduced contraction of doxorubicin-induced skin ulcers.

REFERENCES

Fig. 1. Fibroblast in 8-day-old doxorubicin ulcer. Rough endoplasmic reticulum is dilated and in smaller loops than in normal active fibroblast. Multiple vacuoles with double membranes (arrows) are present. × 20,000.

Fig. 2. Persistent abnormality (small loops) of rough endoplasmic reticulum in fibroblast in 2 week doxorubicin skin ulcer. Mitochondria are swollen. × 25,000.
Fig. 3. Myofibroblast features in cells at 4 weeks after doxorubicin skin injury. Rough endoplasmic reticulum more normal (cf. Figs. 1 and 2). A, small bundle of microfilaments with electron-dense bodies (arrows); B, intercellular connection (desmosome). × 40,000.

Fig. 4. Myofibroblast at 9 weeks. Microfilament bundles with electron-dense bodies (*) plus prominent long microtubules (arrow). × 40,550.
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