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Variations in epidermal cytochrome oxidase activity after local irradiation

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Summary

Cytochrome oxidase activity was evaluated histochemically as an index of mitochondrial damage after local irradiation with X-rays. It was determined by microphotometry on the tail skin of newly born Wistar rats four days after irradiation with doses ranging from 2 to 16 krad.

The enzyme activity of the whole epidermis increased after irradiation, the increases being related to the increase in thickness of the epithelium which was observed as a response to irradiation injury. Within the dose range tested, the enzyme concentration (expressed per unit volume of tissue) decreased in relation to the dose applied.

At the electron microscopy level, the cytochemical demonstration of cytochrome oxidase revealed an irregular reaction over the cristae, intramitochondrial vacuolization and partial homogenization of the matrix. Positive membrane fragments were seen around lipid droplets. This reaction confirms the mitochondrial origin of these previously observed radiation-induced vacuoles.

Introduction

The pathological changes which occur in the skin after irradiation have been widely studied, because this tissue is frequently involved in accidental or therapeutic exposure to irradiation. The aims of these studies included a search for parameters for quantifying radiation damage. It has been shown that ionizing radiation produces metabolic changes of epithelial cells which become evident by variations in the activities of certain enzymes. A shift of activities from the Krebs cycle towards the pentose shunt has been shown to take place and the corresponding enzyme activity alterations have been quantified microphotometrically and related to the doses applied and elapsed post-irradiation times (Cabrini *et al.*, 1970; Klein-Szanto & Cabrini, 1970, 1972; Itoiz & Frasch, 1977). It has also been possible to correlate metabolic changes with morphological variations at the ultrastructural level (Rey & Cabrini, 1973a,b; Rey *et al.*, 1979).

To further our knowledge of the correlations between the biochemical and

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ultrastructural changes occurring in the skin epidermis, we have carried out a study on the irradiation damage of mitochondria using the histochemical activity of cytochrome oxidase as an evaluation index.

Materials and methods

The distal halves of the tails of five-day-old Wistar rats were irradiated with X-rays at 2,4,8,12 and 16 krad, with a Phillips 250 radiotherapy machine operated at 250 kV, 13 mA, employing 250 mm copper and 1 mm aluminium filters at a dose rate of 250 rad/min. The proximal portion of the tail, which was used as control, and the rest of the body were shielded with lead.

Each dose was delivered to 8–10 animals and all groups were killed three days after irradiation. This post-irradiation time was selected because the major enzyme and structural alterations were found to have taken place by this time in previous studies (Cabrini *et al.*, 1970; Rey & Cabrini, 1973a,b; Rey *et al.*, 1979). After three days, irreversible processes of cell death began to appear when the highest dose (16 krad) was delivered.

A specimen containing the irradiated and control areas of each tail was used for the histochemical demonstration of cytochrome oxidase at the optical microscope level. Other control and irradiated portions of each tail skin were processed for the demonstration of the enzyme at the electron microscopy level.

Light microscope histochemistry

Unfixed specimens were sectioned in a thermoelectrically cooled cryostat. Each tail specimen contained both control and irradiated areas. The sections were mounted on cover-slips and allowed to dry for 5 min. Cytochrome oxidase was then demonstrated according to the technique of Seligman *et al.* (1968). A diaminobenzidine concentration of 5 mM was used and an incubation time of 30 min was selected for all runs after having tested the linearity of the microphotometric readings in an incubation chamber described previously (Frasch *et al.*, 1978). After incubation, the sections were dehydrated and mounted in Canada balsam. They were not post-osmicated.

Microphotometric evaluation

Relative variations of reaction intensity were measured in a Cytoscan Zeiss microphotometer provided with an automatic scanning stage, following the conditions established previously (Frasch *et al.*, 1978). Six scans from the basal to the corneal layer were performed with a 2.5 μm diameter light spot on the irradiated and the non-irradiated area of each section. One section from each animal was measured. Blank measurements were taken through the slide, coverslip and mounting medium in an area adjacent to the section. The number of scans (six) for one section from each animal was determined by calculating progressive averages in a pilot set of measurements of 10 adjacent scans in five serial sections (Volco *et al.*, 1978). These sections were obtained from the irradiated tail of a rat exposed to 8 krad because it was found to exhibit the greatest variation of cytochrome oxidase activity on visual examination.

The optical density values (absorbances) obtained during the scanning were integrated, thus obtaining information about the total enzyme activity (TEA) registered through the whole epithelial thickness. The mean enzyme activity (MEA), which is the activity per unit of tissue, was obtained by dividing TEA by the length of the epithelial scans. The ratio (I/C) of the irradiated and control values was determined for each section in order to average the values from different animals. Using this ratio, the errors that may arise from variations in the thickness of the sections and in technical factors are eliminated. (In our experience, the variations in thickness between sections of different specimens, using the same cryostat settings, are within the order of 10%. This variation increases for specimens sectioned with different knives or on different days.)

A statistical programme was used to assess the significance of the differences between control and experimental values within each irradiation dose group and to obtain correlations between variations of the irradiated/control ratios and the applied doses.

Electron microscope cytochemistry

Fresh 1 mm × 3 mm skin specimens were fixed in 3% glutaraldehyde phosphate buffer, pH 7.4, at 4° C for 30 min and rinsed for 1 h in phosphate buffer. Non-frozen sections, about 50–80 μm thick, were cut under a stereoscopic microscope in order to keep the orientation of epithelial layers. The sections were then incubated for cytochrome oxidase in the medium of Seligman *et al.* for 2 h, and afterwards treated with 2% osmium tetroxide in phosphate buffer for 1 h and embedded in Epon 812. Ultrathin sections were examined without contrast staining in a Phillips 300 electron microscope.

Inhibition control

In all experiments, control sections were incubated with 1 mM KCN in the substrate medium for the inhibition of cytochrome oxidase activity.

Results

The distribution of enzyme activity within the epithelium, as observed in light microscope preparations, showed a decrease in activity from the basal to the subcorneal layers. All irradiated epithelia were thicker than the respective controls. This was due to the well-known acanthotic response to irradiation (Rey & Klein-Szanto, 1972; Klein-Szanto *et al.*, 1977; Lanfranchi *et al.*, 1979). The enzyme reaction product in the irradiated zones was less homogeneously distributed than in control zones. In the former, small areas of intense reactivity and more extensive areas of very low reactivity were observed. This resulted generally in lower activity in the irradiated areas overall (Fig. 1).

The microphotometric measurements indicated an increase in total enzyme activity (TEA) in the irradiated epidermis. The increments were proportional to the increase in epithelial thickness. Both the enzyme content and the acanthosis were related to the irradiation dose. The mean enzyme activities (MEA) (the activities per unit volume of tissue regardless of the increment in epithelial thickness) indicated a lower enzyme activity in the irradiated epidermis (Fig. 2). The actual values were closely related to the dose delivered ($r = 0.72$, $P = 0.001$).

At the electron microscope level, the cytochemical reaction clearly marked the mitochondrial structures. The epidermis of animals irradiated with the highest dose (16 krad) showed an increase in mitochondrial size frequently associated with swelling or vacuolization, with an irregular enzyme reaction over the cristae (Fig. 3). In some mitochondria, areas of matrix were partially replaced by a homogeneous and electron-dense material (Fig. 4). These areas seemed to be transitional stages towards other advanced lesions in which dense vacuoles surrounded by membrane remnants with a positive cytochrome oxidase reactivity were observed (Fig. 5). These reactive membrane fragments, like other mitochondrial membranes, were negative in the enzyme inhibition controls.

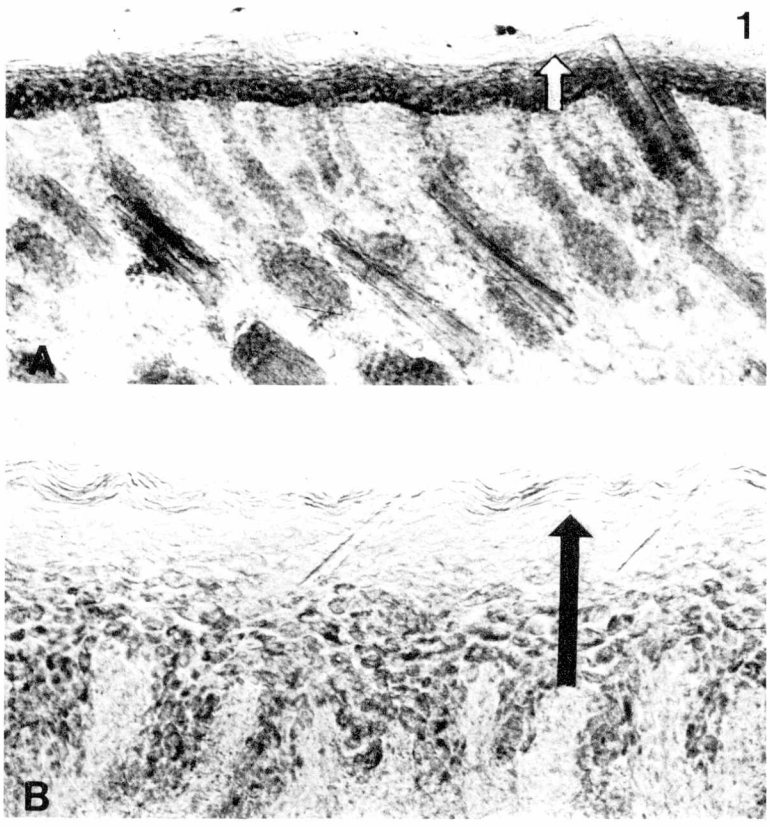

Fig. 1. Cytochrome oxidase reaction in tail epidermis. (A) An unirradiated control area. (B) Irradiated (16 krad) area of the same section showing a marked increase in epithelial thickness. The staining is less homogeneous than in the control area. Small zones of intense reactivity and unreactive larger zones are evident. Arrows indicate the direction of the microphotometric scannings. × 160.

Discussion

The topographical distribution of reaction product in the different epithelial strata was consistent with a mitochondrial distribution and mitochondrial enzymes patterns (Itoiz *et al.*, 1977; Rey *et al.*, 1979). This normal distribution pattern was not altered in irradiated epithelia although there was, however, some irregular arrangement in the latter. At the ultrastructural level, this patchy arrangement of the reaction product was found to be due to the presence of grouped enlarged mitochondria. The cytochemical technique for cytochrome oxidase was thus found to be especially useful as a mitochon-

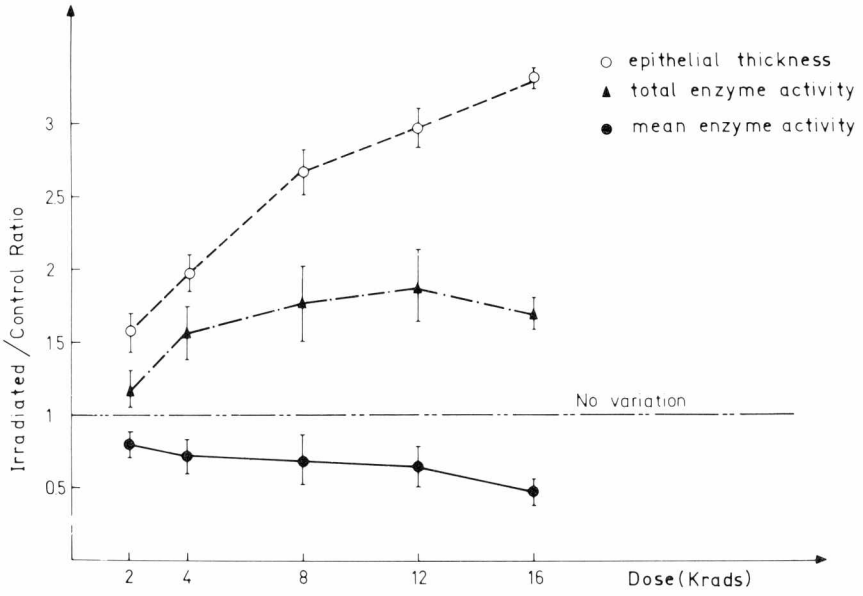

Fig. 2. Variation of total and mean enzyme activity and epithelial thickness after irradiation. Ordinate shows the ratio between experimental and control values plotted against irradiation dose. Each point represents the mean ± S.D. of 8–10 samples (animals).

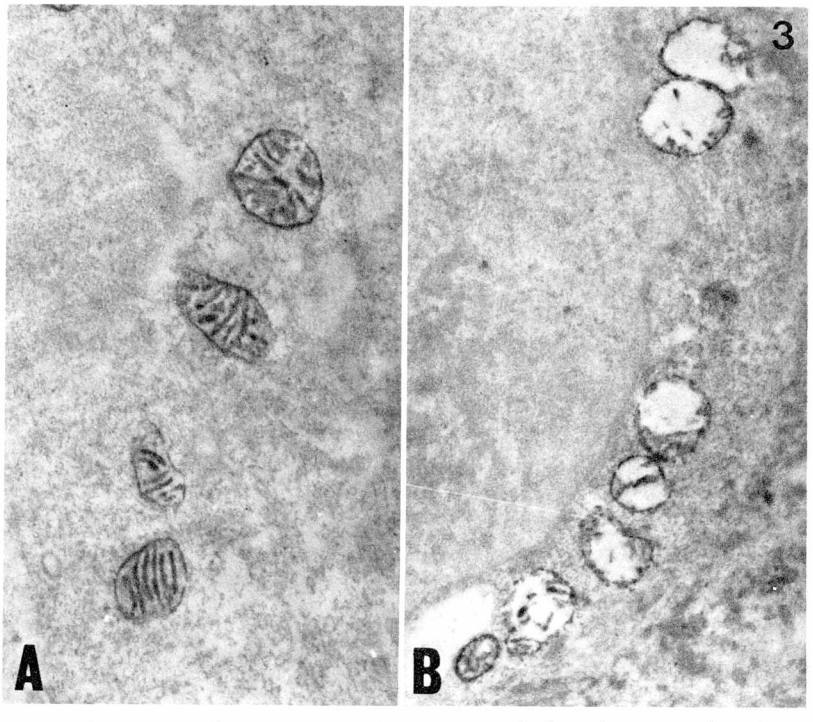

Fig. 3. (A) Cytochrome oxidase reaction in a normal keratinocyte showing perinuclear mitochondria with a distinctive reaction in the membranes. (B) Basal keratinocyte after 16 krad of irradiation. Intense swelling and mitochondrial vacuolization are evident. × 14 000.

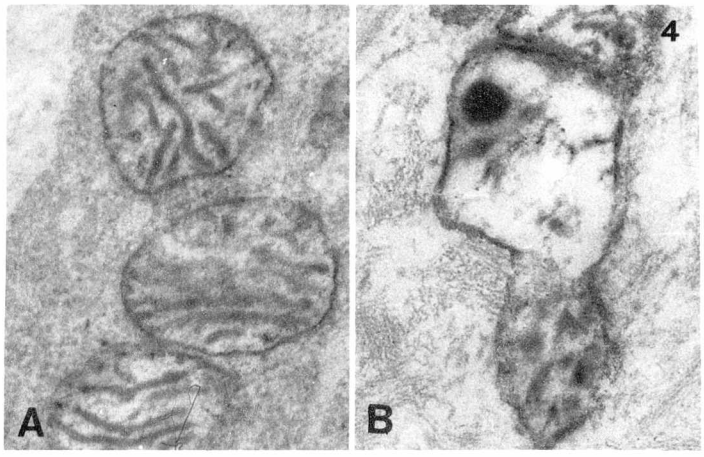

Fig. 4. (A) Mitochondria in a control cell. Cytochrome oxidase reaction product is localized in the space between the inner and outer mitochondrial membranes. (B) Mitochondria of an irradiated cell showing an irregular enzyme reaction over the cristae and the deposition of homogeneous dense material in the matrix. × 20 000.

drial marker, and allowed mitochondrial damage in our material to be detected easily. Swelling, vacuolization and disarrayed cristae have been described previously in epidermis and other animal tissues injured by radiation (Gartner, 1973; Rey & Cabrini, 1973a, b). The presence of dense membrane-coated vacuoles has also been reported and it has been suggested that they had a lipid content and were of mitochondrial origin. Our observation of such vacuoles containing structural elements with a positive cytochrome oxidase reactivity confirms this hypothesis.

Our microphotometric data for the activity of the enzyme can be analysed in relation to previous data on the amount and volume of mitochondria determined by applying stereological methods to the same model of rat tail skin irradiated with 16 krad (Rey *et al.*, 1979). The relative mitochondrial volume in the basal and spinous layers of normal control skin was 32 mm^3 per cm^3 of epithelium. This value decreased to 23 mm^3/cm^3 three days after irradiation ($I/C = 0.72$). The mean concentration of cytochrome oxidase showed an I/C ratio of 0.51 under the same experimental conditions, and a similar ratio was also found when another mitochondrial enzyme, succinate dehydrogenase, was assayed microphotometrically (Klein-Szanto & Cabrini, 1970; Itoiz *et al.*, 1977). The difference in the ratios would indicate that a significant mitochondrial volume with diminished or no activity remains after irradiation. This non-functional volume became evident in the electron micrographs of irradiated tissue subjected to the cytochrome

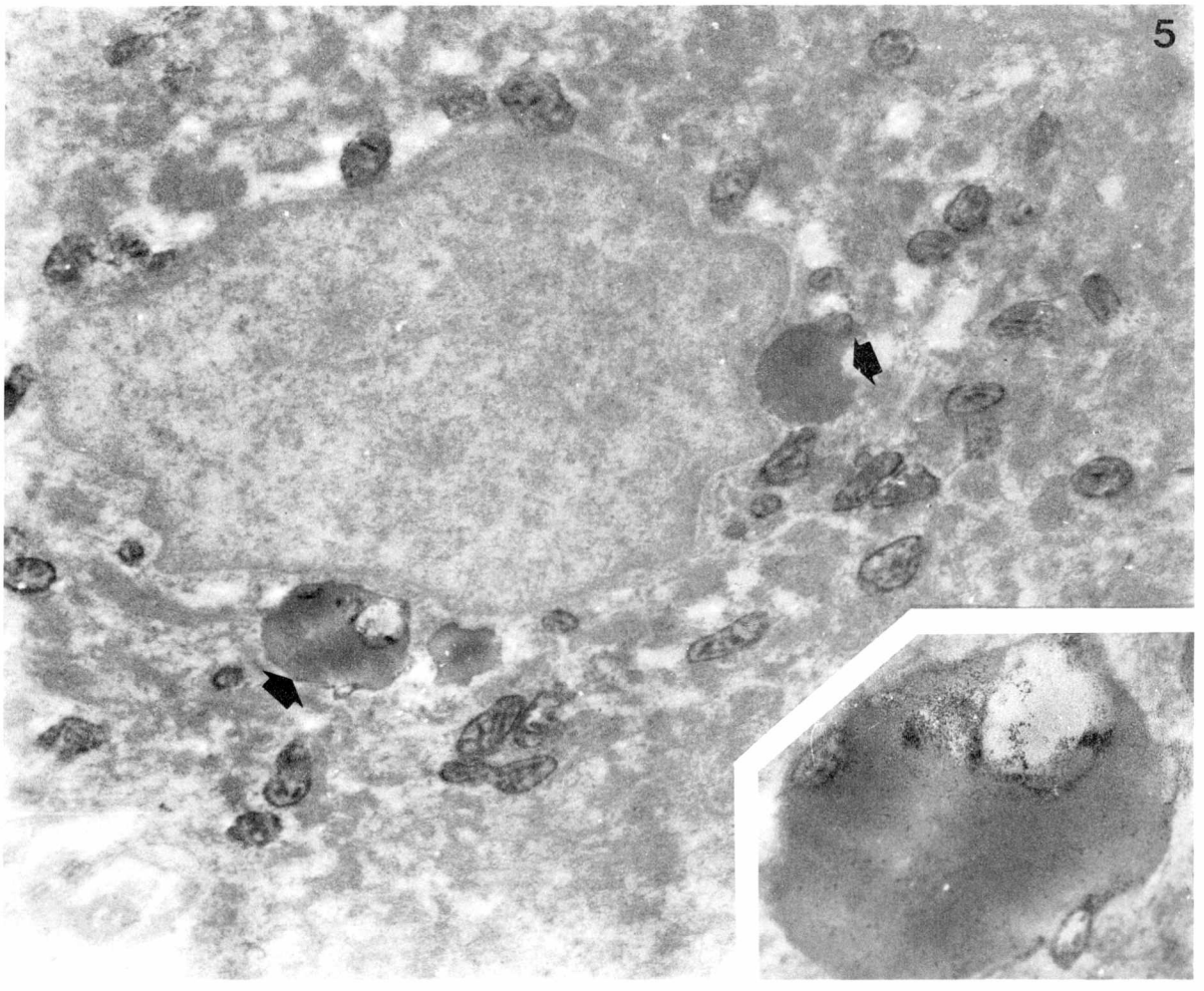

Fig. 5. Irradiated keratinocyte showing irregular distribution of mitochondria. Arrows indicate dense vacuoles in which membrane remnants with a positive cytochrome oxidase reaction is evident. × 7500; inset, × 16 000.

oxidase technique in which large mitochondrial spaces without reaction product were seen.

Other alterations in mitochondrial metabolism produced by irradiation have been reported by Benjamin & Yost (1960), Yost *et al.* (1967) and Trump *et al.* (1974) who found, using biochemical models, that important changes occurred in respiratory chain phosphorylation.

The study of variations in mitochondrial metabolism after irradiation is at present fundamental rather than applied. However, the correlation between ratio values of

mean cytochrome oxidase activity and doses between 2 and 16 krad point to a practical use of the microphotometric method as an easy and rapid tool for evaluating the degree of skin damage in experimental models of irradiation.

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