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SHORT COMMUNICATION

# A quantitative histochemical technique for the study of vascularization in tissue sections using horseradish peroxidase

The use of horseradish peroxidase (mol. wt. 40 000d and diameter 4 nm) injected intravenously as a tracer to study transport at an ultrastructural level was introduced by Graham & Karnovksy (1966) for analysing the early stages of absorption in the proximal tubules of mouse kidney. This method, which employs 3,3′-diaminobenzidine as an oxidizable substrate to reveal the injected peroxidase, is highly sensitive and affords a sharp localization of small amounts of the enzyme. The method has subsequently been used to study vascular transport at an ultrastructural level in other organs and tissues such as cardiac and skeletal muscle (Karnovsky, 1967; Williams & Wissig, 1975), the alveolar–capillary membrane (Keeley & Karnovksy, 1968), liver (Graham *et al.*, 1969) and lung (Clementi, 1970; Maisin, 1970; Schneeberger & Karnovsky, 1971).

The aim of the work reported in this Communication was to adapt this technique for quantitative studies under the light microscope of normal and altered vascular patterns and permeability for application as a model of experimental cutaneous irradiation. The small amount of intravenously injected peroxidase mixes immediately with the circulating plasma. Following the detection of its enzymatic activity in thick tissue sections, blood vessels are seen full of polymerized diaminobenzidine in quantities which increase with incubation time. This allows the visualization of all functional vessels, including capillaries. The method is thus superior to the standard methods of vascular filling such as Indian ink or Micropaque for the observation of fine vessels. Furthermore, since part of the injected peroxidase permeates the endothelium in quantities which increase as postinjection time elapses, passes into the perivascular connective tissue and is phagocyted by macrophages, information concerning changes in capillary permeability can be obtained from the same tissue sections.

Peroxidase type VI (Sigma) in 0.1 ml saline (0.02 mg/g body weight) was injected intravascularly in the jugular vein of newborn rats weighing approximately 20 g. Specimens of skin, 1 $cm^2$, were excised from the left cheek of locally irradiated and non-irradiated newborn rats and fixed immediately in fixative containing 2% glutaraldehyde, 1% paraformaldehyde, 1% sucrose, 0.02% calcium chloride and 0.067 M cacodylate buffer, pH 7.6, for 2 h and then washed in cacodylate buffer, pH 7.6, for 1 h. Sections, 70 $\mu$m thick, were cut in a thermoelectrically cooled cryostat and then

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incubated for 30 min at room temperature in a solution of 20 ml Tris–HCl buffer, pH 7.6, 0.035 ml $H_2O_2$ and 0.01 g diaminobenzidine. Sections were dehydrated and mounted in balsam.

Sections were placed directly on the slide of an electronic image processor (Kontron Messgeräte MOP/AM 03) and projected for quantitative evaluation at a total magnification of × 400. Image analysis renders possible the quantitative evaluation of morphological parameters once the structures to be analyzed have been appropriately stained by histochemical methods (Benno *et al.*, 1982; Fahimi *et al.*, 1982; Stoward & Ploem, 1982).

Vascular volume was measured as a percentage of the total volume of skin examined. As some experimental conditions cause shortening and widening of the blood vessels,

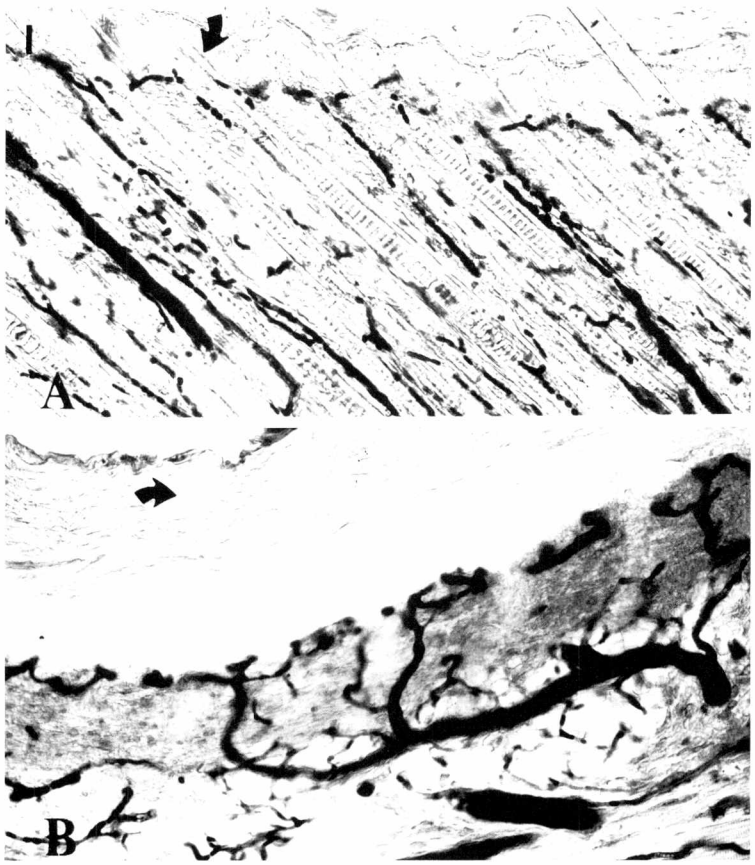


**Fig. 1.** Blood vessels in (A) normal and (B) irradiated skin. Note the vascular pattern altered by the presence of telangiectatic vessels and the increase in connective tissue staining caused by enhanced permeability induced by irradiation. Radiation also causes the loss of hair follicles, epithelial acanthosis and narrowing of the dermis. A remaining hair has been marked (arrow) as evidence that both photographs were taken with the same magnification. × 20.

vascular length and blood vessel diameter were measured. Vascular length was recorded with the same computer program as above. The diameter of vessels was estimated by superposing projections of sections and a grid of parallel lines and recording the calibres of those vessels which intersected the lines.

The amount of peroxidase-positive macrophagic cells can be taken as a morphological index of vascular permeability. Cells 15–30 $\mu$m in diameter were clearly visible and were counted directly in areas of 0.25 mm$^2$ of each section under the light microscope with the aid of a grid fitted in the eyepiece. It is always necessary to run noninjected controls simultaneously to identify any peroxidatic activity that may appear due to haemoglobin released as a result of haemolysis or to any phagocytic cells having endogenous peroxidatic activity. Connective tissue staining, which is another marker of vascular permeability, was evaluated by performing microphotometric readings in a Zeiss Cytoscan. The validity of microphotometry for measuring the amount of final reaction product of oxidized 3,3′-diaminobenzidine has been demonstrated previously (Frasch *et al.*, 1978). Ten measurements at 480 $\mu$m were taken, using a spot 23.4 $\mu$m in diameter in the subepithelial connective tissue of each section. Care was taken to avoid vascular structures, hair follicles and peroxidase-positive cells.

As an example of the application of the method described, we present results from animals in which irradiation produced telangiectatic changes and an increase in vascular permeability. The full results and radiobiological significance of this work will be reported elsewhere.

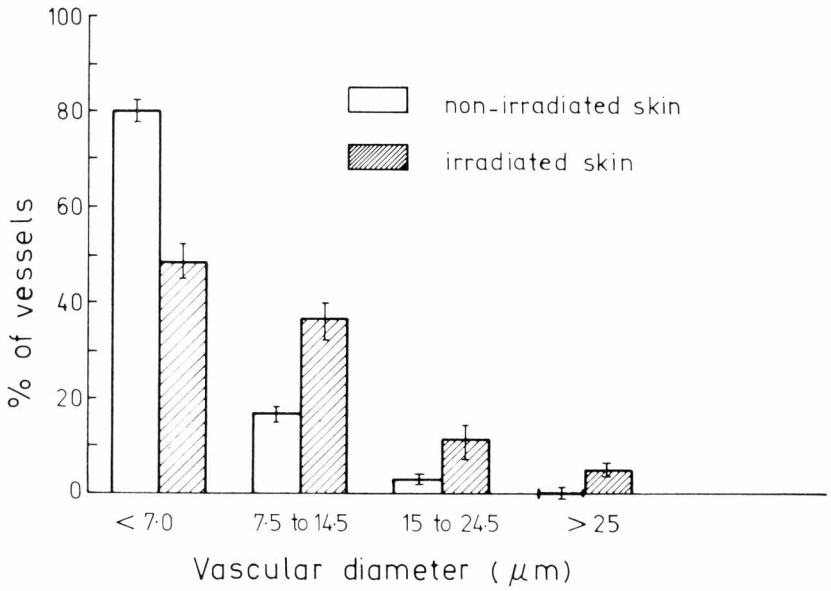


**Fig. 2.** Frequency distribution of vascular diameters measured on projections of sections of five cases of normal skin and five cases of skin six days after irradiation with 6 krad of X-rays.

Fig. 1 illustrates alterations produced six days after irradiation with 6 krad of X-rays. Measurements obtained from a group of five animals under these conditions revealed the marked increase in relative vascular area: 16.20 ± 1.06% for irradiated tissue and 9.20 ± 0.77% for controls. Vascular length exhibited a tendency to decrease: 8800 ± 323 $\mu$m/mm of epidermal surface and 9638 ± 871 for controls. A change in frequency distribution of vessel diameters was likewise detected (see Fig. 2). Changes in vascular volume, length and diameter characterize the alteration of the vascular pattern produced by irradiation and allow a quantitative comparison with other dose and time experiments.

The quantification of phagocytic cells reveals the enhanced permeability produced by irradiation. Fig. 3 illustrates changes produced in samples obtained from cheeks three days after a dose of 12 krad of X-rays. Peroxidase-positive phagocytic cell counts gave the following values: 705.2 ± 67.6 cells/mm$^2$ for irradiated tissue and 206 ± 50.4 for controls.

Microphotometric readings revealed an increase in connective tissue staining: 0.59 ± 0.004 units of optical density for irradiated tissue and 0.13 ± 0.012 for controls.

The method used in this work has proved particularly useful for studying vascular alterations in a comparatively large area of tissue, rendering it possible to obtain parameters which can be evaluated statistically.

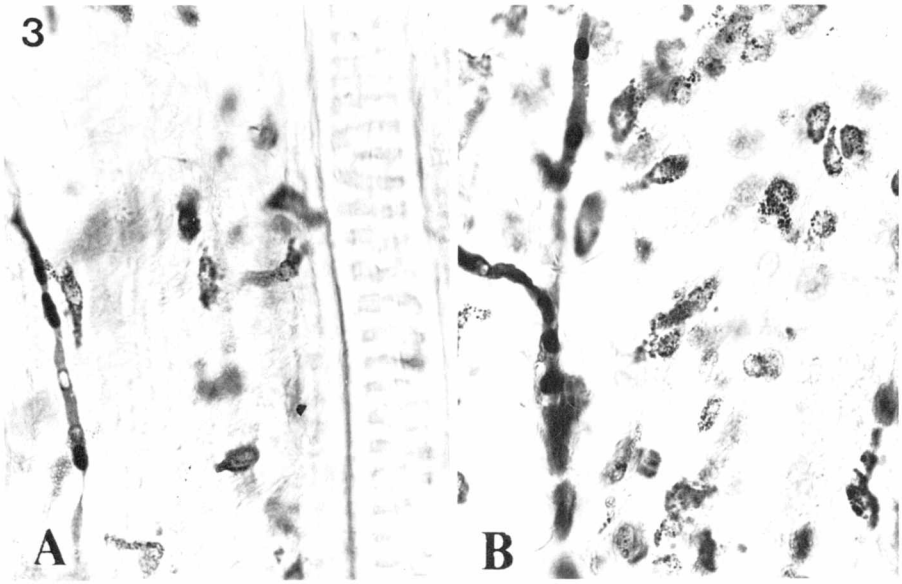


**Fig. 3.** Positive phagocytic cells five minutes after intravascular injection of peroxidase in (A) normal skin and (B) skin three days after irradiation with 12 krad of X-rays. The increase in the amount of positive cells reveals the enhanced permeability caused by irradiation. × 180.

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