Histochemical Visualization of Lymphatic Capillaries in the Rat: A Comparison of Methods Demonstrated at the Posterior Pharyngeal Surface

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Summary. A sufficient differentiation of lymphatic capillaries from blood capillaries in conventional light microscopy still eludes researchers. The endothelium and media of lymphatic capillaries are characterized by a strong 5'-nucleotidase activity, whereas blood capillaries reveal no or significantly lower activity. Alkaline phosphatase activity, on the other hand, missing in the lymphatic capillaries is positive in most of the blood capillaries. For the histochemical visualization of the entire blood capillary bed, dipeptidyl peptidase IV-activity has to be used together with alkaline phosphatase. Various fixation and detection methods of 5'-nucleotidase are compared. In order to demonstrate 5'-nucleotidase activity, a method modified after HEUSERMANN (1979) is considered to be most suitable. The results obtained are discussed with regard to their significance concerning the visualization of lymphatic capillaries. They are compared with a series of investigations in which alkaline phosphatase and dipeptidyl peptidase IV-activity are visualized in blood capillaries additional to the 5'-nucleotidase reaction. Various color reactions reveal a differentiation between blood capillaries and small lymphatics. The isolated visualization of 5'-nucleotidase activity with a simultaneous inhibition of alkaline phosphatase with L-tetramisole is considered to be the best way to histochemically demonstrate lymphatic capillaries. It was shown for the first time that only in the presence of L-tetramisole can small lymphatics be adequately visualized. A satisfactory differentiation between blood and lymphatic capillaries succeeded by means of a different color intensity of the reaction product.

KÖLLIKER (1855) and FLEMMING (1876) were already able to describe a fundamental knowledge of the anatomy of lymphatic vessels and capillaries. After a period of stagnation in this field of research, great hopes arose over several injection methods for elucidation of the lymphatic system of organs. KAISERLING and SOOSTMEYER (1939) described a procedure of creating lymphatic congestion by cutting off a diverting lymphatic vessel. This technique proved to be of major consequence compared to later methods (RENyi-VAMOS, 1960). It became obvious that injection substances penetrate also into fissures of the connective tissue, and therefore any conclusions that striations of Indian ink were lymphatic capillaries ceased to be valid. As the use of KAISERLING's and SOOSTMEYER's method is limited to only specific examinations of tissue, other techniques of visualizing lymphatic capillaries had to be developed.

Early histochemical investigations of the wall of the lymphatic vessels were published in 1957 (PINTO et al.). This subject was repeatedly revised over the following
years. Since the absence of alkaline phosphatase and the existence of 5'-nucleotidase together with other enzymes can be detected in the endothelium as well as in the media, the histochemical reaction of these two enzymes was used to differentiate between blood capillaries and lymphatic vessels (Vetter, 1970; Heusermann, 1979). Histochemical methods to demonstrate 5'-nucleotidase activity are "Gomori-type" reactions using heavy metal precipitation (Gomori, 1939; Wachstein and Meisel, 1957). So far, a number of variations of these techniques have been described; however, instructions concerning the procedures are sometimes stated inaccurately or incompletely. In the present study frozen sections of the posterior pharyngeal surface of the rat are used in order to compare different procedures demonstrating 5'-nucleotidase activity in the lymphatic capillaries. In addition, a histochemical evaluation of all blood capillaries has been carried out using alkaline phosphatase and dipeptidyl peptidase IV (Lojda, 1979).

This study offers evidence to support a concept concerning histochemical differentiation between blood capillaries and small lymphatic vessels.

MATERIALS AND METHODS

The pharynx was visualized and carefully removed from ether-anesthetized male Wistar rats weighing from 180 to 210 g. After preparing the posterior pharyngeal surface, it was immediately frozen in liquid nitrogen. Sections (15-18 μm thick) were cut in a cryostat and mounted on gelatine coated slides. Air-dried sections were used with and without fixation. For fixation the following methods were compared with each other: 1) 2 min in 4% formaldehyde at room temperature; 2) 30 min in ice-cold formaldehyde-CaCl₂ (6% paraformaldehyde, 1% CaCl₂) (Klaushofer et al., 1974); 3) 10 sec in a solution of 3% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 at 4°C. One part of these sections was later rinsed with distilled water, while another part was washed in 0.1 M cacodylate buffer pH 7.2 for 15 min. The remaining sections were also washed in 0.1 M cacodylate buffer pH 7.2 for 15 min and then air-dried for 90 min. Sections treated this way were incubated for 15, 30 or 60 min at a temperature of 37°C as follows:

a) Standard medium (I), according to Wachstein and Meisel (1957), (Klaushofer et al., 1974). This medium contains 1 mM AMP, 3.6 mM Pb(NO₃)₂ and 10 mM MgSO₄ dissolved in 0.2 M trismaleate buffer at pH 7.2.

b) Incubation medium II containing 5 mM MgSO₄, 2 mM Pb(NO₃)₂, 135 mM saccharose, 2 mM AMP in 0.2 M trismaleate buffer pH 7.2 (modified after Heusermann, 1979).

c) Medium III containing 1.35 mM AMP, 1.95 mM Mg SO₄ and 2 mM lead citrate in 0.28 M trismaleate buffer, pH 7.2 (according to Mayahara et al., 1967).

When demonstration of 5'-nucleotidase activity was not followed by alkaline phosphatase reaction, each of the incubation media I-III contained 1 mM L-tetramisole. Histochemical visualization of alkaline phosphatase activity was achieved by incubation in medium A and B for 60, 90 or 120 min at a temperature of 37°C.

d) Medium A: 10 mg naphthol AS-MX phosphate (Sigma, Munich) are dissolved in 0.5 ml N,N-dimethylformamide and added to 10 ml 0.1M tris-HCl buffer, pH 9.2 containing 40 mg varianmine blue salt RT (Sigma, Munich) (Lojda, 1979).

e) Medium B: 25 mg naphthol AS-BI (Sigma, Munich) is dissolved in 0.5 ml N, N-dimethylformamide and added to 50 ml 0.1 M tris-HCl buffer pH 9.2 containing 50
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mg Fast Red TR (HEUSERMANN, 1979).

In some sections, dipeptidyl peptidase activity was visualized following a combined reaction of 5'-nucleotidase (a, b or c) and alkaline phosphatase (d). These sections were fixed in a chloroform-acetone mixture instead of 4% formaldehyde for 5 min and dried.

f) For demonstrating dipeptidyl IV-peptidase activity, certain sections were incubated at 37°C for 45 min in a medium which contained 4 mg glycyl-L-proline-4-methoxy-2-naphthylamide (Bachem, Budendorf, Switzerland), dissolved in 0.5 ml N, N-dimethylformamide and 10 mg Fast Blue BB salt (Sigma, Munich), dissolved in 10 ml 0.1 M phosphate buffer pH 7.4.

g) Control experiments: 1) Incubation without AMP. 2) Inhibition of 5'-nucleotidase activity with NiCl₂ (50 mM) in distilled water for 10 min before being incubated in medium I.

After incubation (a, b, or c), the sections were washed in distilled water and the reaction product converted into lead sulphide by immersing for 2 min in a 1% ammoniumsulphide solution. Before mounting in glycerin, the sections were thoroughly rinsed in distilled water. Slides were examined using a Zeiss-microscope, and micrographs were taken with Ektacrome 160-film.

RESULTS

Satisfying tissue preservation could be achieved with sections fixed either in 4% formaldehyde at 37°C for 2 min or in ice-cold formaldehyde-CaCl₂ (6% paraformaldehyde, 1% CaCl₂) for 30 min. In contrast, sections without fixation prior to the incubation, always displayed an irregular distribution of metal precipitation together with a great number of artefacts in the tissue. In the case of fixation with glutaraldehyde, tissue preservation particularly improved. Glutaraldehyde, however, shows an inhibitory influence on 5'-nucleotidase. In contrast, there is no inhibition from formaldehyde on the 5'-nucleotidase. Tissue artefacts will be avoided considerably if the sections are washed in 0.1 M cacodylate buffer, pH 7.4, for 15 min subsequent to fixation and then air-dried for 90 min. As shown in the control experiments, it is more the drying process than the washing in cacodylate buffer which is responsible for this improvement.

The histochemical demonstration of 5'-nucleotidase activity was particularly successful after incubation in media I or II. The brown coloring ranged from a light shade at 15 min to dark tones at 60 min of incubation. 5'-Nucleotidase activity, however, showed slightly higher in sections incubated in medium II compared to those incubated in medium I. The often disturbing appearance of a white sediment during preparation of the incubation medium does not occur in the arrangement of media II and III. With the use of medium III, however, the 5'-nucleotidase reaction is much more weakly stained compared with media I and II.

Concerning the vessels, 5'-nucleotidase activity is found in lymphatic capillaries (Fig. 1-3). In blood capillaries, a very weak 5'-nucleotidase activity was found only in exceptional cases. If the incubation mixture does not contain L-tetramisole as an inhibitor of alkaline phosphatase, there is a brown coloring in blood capillaries, whereas no change is revealed in the lymphatic capillaries.

Both methods applied for alkaline phosphatase staining show identical results as far as localization and intensity. The activity of the enzyme is visualized in blue by
Fig. 1-8. Legends on the opposite page.
method A (Fig. 4, 5, 7) and in red by method B (Fig. 6, 8). Increasing the incubation time from 60 to 90 min had most of the blood capillaries reveal positive alkaline phosphatase activity, in contrast to results from a shorter incubation time. No essential increase in color intensity was achieved after incubation for longer than 90 min.

This reaction pattern of alkaline phosphatase does not change in connection with the 5'-nucleotidase assay. Sufficient demarcation between alkaline phosphatase and 5'-nucleotidase depends on the intensity of the brown staining caused by lead sulphide during visualization of 5'-nucleotidase. If 5'-nucleotidase activity is stained dark brown (Fig. 4, 6) alkaline phosphatase activity, demonstrated as red or blue reaction products, will contrast noticeably. With a lighter brown (Fig. 5), a clear demarcation of alkaline phosphatase is achieved with method A (blue reaction product).

Dipeptidyl peptidase IV activity is visualized brown-red with the corresponding reaction. A significant color differentiation from the alkaline phosphatase activity succeeds in connection with the alkaline phosphatase reaction (medium A). A clear demarcation of the positive dipeptidyl peptidase IV-reaction from the brown color of the 5'-nucleotidase reaction is not always found, however, especially when dealing with different shades of light brown.

**DISCUSSION**

The identification of lymphatic vessels in conventional light microscopy, especially of lymphatic capillaries, still today causes considerable difficulties. Characteristics of the lymphatic capillary in light microscopy are worked out as follows: the lymphatic capillary consists of a closed layer of endothelial cells with a thickness of 0.1 to 0.2 μm. A basement membrane is either missing or developed incompletely (WENZEL, 1972). Even with these characteristics, the light microscopical differentiation of blood and lymphatic capillaries remains elusive, as lymphatic capillaries are often found strongly collaborated between bundles of connective tissue.

KAISERLING and SOOSTMEYER (1939) provoked interstitial edema by the ligature of large draining lymph vessels, thereby better revealing lymphatic capillaries. MILLER (1963, 1972) confirmed the advantage of this method in his investigations. With this technique, KAISERLING and SOOSTMEYER were able to demonstrate the insufficiency of injection methods, which had previously been practiced (GEROTA, 1896; MAGNUS, 1922; FISCHER, 1933; v. BRZEZINSKI, 1963; WELSH et al., 1983). Another possibility of differentiating small lymph vessels from the blood capillaries was carried out in the heart by retrograde perfusion of the coronaries with different fixation solutions (BORGERT and HART, 1977; LEAK et al., 1978; POGGI et al., 1986). However, all these methods are limited to specific investigations of tissue.

About 100 years after KOLLIKER's work, fundamental investigations concerning the histochemistry of the walls in the lymphatic vessels were conducted (PINTO et al., 1957; POBERAI et al., 1963; WEGMANN, 1967; VETTER, 1970). In addition to numerous

**Fig. 1-8.** Cryostat sections of the posterior pharyngeal wall of the rat. Fig. 1-3. Isolated staining of 5'-nucleotidase. Lymphatic capillaries are marked with black arrows in (1) and (2). In (3) lymphatic capillaries are lying between bundles of muscles. Fig. 4-8. Combined staining of 5'-nucleotidase and alkaline phosphatase. Blood capillaries are stained red or blue. Fig. 1: x165, Fig. 2 and 3: x420, Fig. 4-6: x260, Fig. 7 and 8: x165
other enzymes (Wenzel, 1972), 5′-nucleotidase is localized in the endothelium as well as in the media. 5′-nucleotidase, an important enzyme in the metabolism of nucleotides, is widely used as a marker enzyme of plasma membrane (Song and Bodansky, 1967; De Pierre and Karnovsky, 1973; Böck and Klauschofer, 1975). Heusermann (1979) has identified this enzyme in the lymphatic vessel endothelia of the human spleen, skin, thyroid gland, lung, appendix, kidney, liver and skeletal muscles. Therefore, the occurrence of 5′-nucleotidase in lymphatic vessels endothelium is regardless of the organ.

All histochemical methods of visualizing 5′-nucleotidase activity in light and electron microscopy are "Gomori-type" reactions using heavy metal precipitation (Gomori, 1939; Wachstein and Meisel, 1957).

Barka and Anderson (1962) described diffuse artefacts and the nonspecific staining of cell nuclei with lead ions, provided the frozen sections are not fixed in formaldehyde. In addition, it is advantageous to wash the sections in cacodylate buffer pH 7.4 after fixation for 15 min and then air-dry them for 90 min. A special problem with all "Gomori-type" reactions concerns the occurrence of artefacts (Mühlbach, 1977). According to Deane et al. (1960) and Pearse (1968), the extent of such artefacts depends on the composition of the incubation solution. According to Böck and Klauschofer (1975) it is only the poor quality of frozen sections which is responsible for an unsatisfactory resolution of structural details. Cytological details of the localization of enzymes can be visualized with excellent morphological appearance and high sensitivity and specificity of the histochemical 5′-nucleotidase reaction in glycol methacrylate and in paraffin embedded tissue (Mitrenga et al., 1974; Klauschofer and Mayersbach, V. 1979).

The composition of the incubation medium and the performance of the detection method are of great importance. The visualization of 5′-nucleotidase activity depends on the presence of the substrate AMP in the incubation medium. Besides 5′-nucleotidase, alkaline phosphatase also metabolizes AMP (Reis, 1951). Taking this into account, attention must be paid to the fact that by using this substrate, the activity of alkaline phosphatase will also be found in blood capillaries and lymphatic capillaries, which might question the possibility of differentiation between blood capillaries and small lymphatics. Since Borgers (1973) has described L-tetramisole as an inhibitor of alkaline phosphatase, the 5′-nucleotidase reaction can be carried out specifically, owing to that L-tetramisole has no influence on 5′-nucleotidase activity (De Pierre and Karnovsky, 1974). Previous studies on the histochemical differentiation between small lymphatics and blood capillaries have not utilized L-tetramisole. However, according to the present results, there is a positive staining of blood capillaries during the demonstration of 5′-nucleotidase activity in the absence of L-tetramisole due to an additional metabolizing of AMP by the alkaline phosphatase (Reis, 1951). Therefore a complete inhibition of alkaline phosphatase with at least 0.5 mM L-tetramisole (Borgers, 1973; Mühlbach, 1977) is required.

Phosphate liberated during the 5′-nucleotidase reaction can be precipitated as calcium salt (Naidoo and Pratt, 1954) and lead salt (Pearse and Reis, 1952). However the precipitation of phosphate with calcium has a disadvantage in that the reaction product can be visualized only after treatment with silver nitrate or after transformation into cobalt phosphate (transformation into sulphide). In contrast, lead phosphate can be directly detected as black lead sulphide (Böck and Klauschofer, 1975). Therefore, the sections should be immersed in a dilute ammonium sulphide solution (1%) for 2 min. The solubility product of the desired precipitate should be exceeded at the place
of enzyme activity in order to prevent the diffusion of products of the enzymic hydrolysis and their unspecific adsorption at different places (GOMORI, 1950; PEARSE, 1968). Therefore, the concentration of lead ions in the incubation medium and the enzymic release of phosphate has to be sufficiently strong (MUHLBACH, 1977).

The purpose of the present study is to establish the most suitable concentration for the detection of 5'-nucleotidase. For this reason, the methods in comparison are mainly based on the technique described by WACHSTEIN and MEISEL (1957). 5'-Nucleotidase activity in lymphatic capillaries is visualized most impressively either by the method according to KLAUSHOFER et al. (1974) or by a method modified after HEUSERMANN (1979). This reduction of the concentration also prevents the appearance of a white sediment caused by the precipitation of lead salt during the preparation of the incubation medium. Therefore no CO₂-free distilled water (KHAMAS and GHOSHAL, 1986) is needed anymore. The disturbing white sediment does not occur with the method according to MAYAHARA et al. (1967) either, but the 5'-nucleotidase reaction turns out much weaker. The comparatively low concentration of magnesium is responsible for this, the purpose of magnesium being to activate 5'-nucleotidase (AHMED and REIS, 1957).

HEUSERMANN (1979) could not find any 5'-nucleotidase activity in blood capillaries. BÖCK and KLAUSHOFER (1975) noticed a varying 5'-nucleotidase activity in endothelial cells in different sections of blood vessels of the rat. GAZDZIK and KAMINSKY (1968) described a positive, diffuse reaction within the walls of blood vessels of the rat testis. The present investigation, however, also shows only in exceptional cases a very slightly positive 5'-nucleotidase reaction in blood capillaries after inhibition of alkaline phosphatase with L-tetramisole. Compared to the lymphatic vessel endothelium, though, it is of a much lower intensity. Therefore, L-tetramisole has to be added to the incubation medium as an indispensable substance to histochemically differentiate lymphatic capillaries from blood capillaries. The addition of L-tetramisole is not required during a combined histochemical visualisation of 5'-nucleotidase and alkaline phosphatase. By means of different color reactions, the two enzymes are distinguished. Using this color differentiation necessitates the consideration that alkaline phosphatase activity is not present in all blood capillaries. For example, VETTER (1970) was able to demonstrate a higher alkaline phosphatase activity in the arterial capillary loop compared to the venous capillary loop. LOJDA (1979) showed that alkaline phosphatase does not reveal all blood capillaries and that the combined detection of dipeptidyl peptidase IV and alkaline phosphatase efficiently demonstrates the entire capillary bed in most organs. The number of blood capillaries staining for alkaline phosphatase activity is consistently greater than of dipeptidyl peptidase IV-positive blood capillaries (GRIM et al., 1986).

GRIM et al. were able to demonstrate in their investigations that alkaline phosphatase activity spreads over an increasing length of the capillary bed when the incubation time is prolonged to 60 min—an observation which is confirmed by the present investigation. Therefore, the combined detection of 5'-nucleotidase and alkaline phosphatase can be considered as a possible differentiation between lymphatic capillaries and blood capillaries, always keeping in mind the possible absence of alkaline phosphatase in blood capillaries. When alkaline phosphatase activity is lacking, blood capillaries show a much weaker brown coloring compared to lymphatic capillaries, because of low 5'-nucleotidase activity. However, this possibility of differentiating is not applicable, as an additionally applied dipeptidyl peptidase IV reaction contributes a further red-brown coloring of the blood capillaries.
Considering all these results, it can be stated that lymphatic capillaries are visualized the easiest and also the best with an isolated 5'-nucleotidase reaction. An additional visualization of alkaline phosphatase is of no further advantage, because the isolated demonstration of 5'-nucleotidase activity in the presence of L-tetramisole shows a very intensive color reaction in lymphatic capillaries.

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