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Factor VIII-related antigen and lymphatic collecting vessels

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Summary. In order to investigate endothelial cells of lymphatic vessels for factor VIII-related antigen (FVIIIR:Ag), lymphatics from lo patients with malignant lymphoma or testicular carcinoma undergoing lymphangiography were examined. After the lymphangiography was terminated, a part of the cannulated lymphatic vessel was removed and prepared for immunohistochemical examination for FVIIIR:Ag. All the lymphatic vessels showed positive cytoplasmatic staining for FVIIIR:Ag in the endothelial cells, the reaction being weak and patchy when compared with blood vessels. Eletron microscopy was done on five lymphatic vessels and showed these to be lined with endothelial cells without a basal lamina.

Key words: Factor VIII-related antigen – Lymphatic vessels

It is important for the surgical pathologist to make a valid identification of the vascular system in tissue sections, especially when vessel invasion by tumour is in question. Identification of larger blood vessels poses no problem, but it is extremely difficult or impossible in a haematoxylin-eosin stained section to distinguish between blood capillaries and lymphatic capillaries.

The term "Factor VIII" refers to a single protein or complex of proteins which has three functionally distinct components: an antigen, a clot-promoting factor and von Willebrand factor (Jaffe 1977). It has been shown that vascular endothelium, megakaryocytes and platelets react with rabbit antiserum to "Factor VIII" (Bloom et al. 1973; Hoyer et al. 1973). Jaffe et al. (1973) confirmed by tissue culture studies the synthesis of Factor VIII-related antigen (FVIIIR: Ag) in endothelial cells from umbilical cord veins. There is agreement in localization of FVIIIR: Ag in endothelial cells of blood vessels, but agreement is not general concerning endothelial cells of lymphatics, partly because of difficulties in defining these vessels (Mukai et al. 1980; Burgdorf et al. 1981; Sehested and Hou-Jensen 1981).

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Table 1.

No. of patients	No. of tissue specimens removed after lymphangiography	No. of specimens containing lymphatic vessel in the histological section prepared as			
		cryostata-section	formalin-fixed	Bouin-fixed	glutaraldehyd fixed
10	18	7	11	11	5

The aim of this study was to clarify further the relationship of FVIIIR: Ag and the endothelial cells of lymphatic vessels defined by lymphangiography.

Materials and methods

In a six month period from June to December 1983 five patients with malignant lymphoma and five patients with malignant tumour of the testis underwent lymphangiography of the lower limbs as a staging procedure. The lymphangiography was done as described by Kinmonth et al. (1955) modified in the following way: under local anaesthesia (2 × 5 ml 0,5% Leostesin® with Noradrenalin) a five to eight cm long incision was made in the skin of the dorsum of the distal part of the foot over the first interstitium. A lymphatic vessel was dissected, punctured by a fine cannula after which an infusion of contrast was made (Lipiodol® ultrafluid 0,25 ml per kg body weight, approximately 4 ml per hour). To confirm that the infusion was in a lymphatic vessel, X-ray pictures of the limb were taken after infusion of 0,5 ml. When the infusion was terminated, X-ray pictures of the inquinal, pelvic and paraaortal lymph nodes were taken. Finally, five to ten mm of the lymphatic vessel was removed distal to the point of puncture, and immediately prepared for light microscopy and electron microscopy in the following manners:

Each vessel was divided into at least two parts. One part was fixed in ten per cent neutral formalin, the second was fixed in Bouin's fixative. If possible a third part was prepared for frozen section, and a fourth was fixed in three per cent glutaraldehyde for electron micrsocopy. The number of specimens available are illustrated in Table 1. The time of fixation was twelve to eighteen hours, following which the tissue was routinely processed for histological study and embedded in paraffin at 60° C. From the paraffin-embedded tissue blocks, sections were examined by the two-layer conjugated immunoperoxidase method consiting of the following steps: 1) Four micron sections were placed on glue-coated glass slides at 60° C. for thirty minutes and brought to water. 2) 0,1% pronase (protease type 14, sigma corp. P-5147), at 37° C. for two minutes. 3) 0,5% H_2O_2 in methanole for thirty minutes to block endogeneous peroxidase activity. 4) Wash in 0,1 M phosphate buffered saline (PBS) pH 7,4 for fifteen minutes. 5) 10% normal swine serum for twenty minutes to eliminate non-specific reaction. 6) Rabbit antiserum to FVIIIR: Ag diluted 1:200 for thirty minutes. 7) Wash in PBS for ten minutes, twice. 8) Peroxidase conjugated swine antirabbit innumoglobulin diluted 1:20 for twenty minutes. 9) PBS for ten minutes, twice. 10) 3-amino-9-ethylcarbazole and H_2O_2 for fifteen minutes to develop the colourreaction. 11) Counterstaining with Mayers hematoxylin.

Frozen sections were fixed in acetone at 4° C. for fifteen minutes, and then prepared for immunoperoxidase staining as described above, beginning at stage four. All sera was purchased from Dakopatts A/S, Copenhagen.

The following controls were performed on separate sections from every case: 1) Non-immune rabbit serum was substituted for the anti-FVIIIR: Ag in stage six, and 2) the primary antibody in stage six was omitted. Finally a section from normal lung tissue fixed in formalin was processed as a positive control for staining of FVIIIR: Ag in the blood vessels.

Tissue for electron microscopy was 1) fixed in 3% buffered glutaraldehyde and 2) postfixed with 1% osmium tetroxide for one hour at 4° C. 3) Rinsed with PBS. 4) Dehydrated with graded

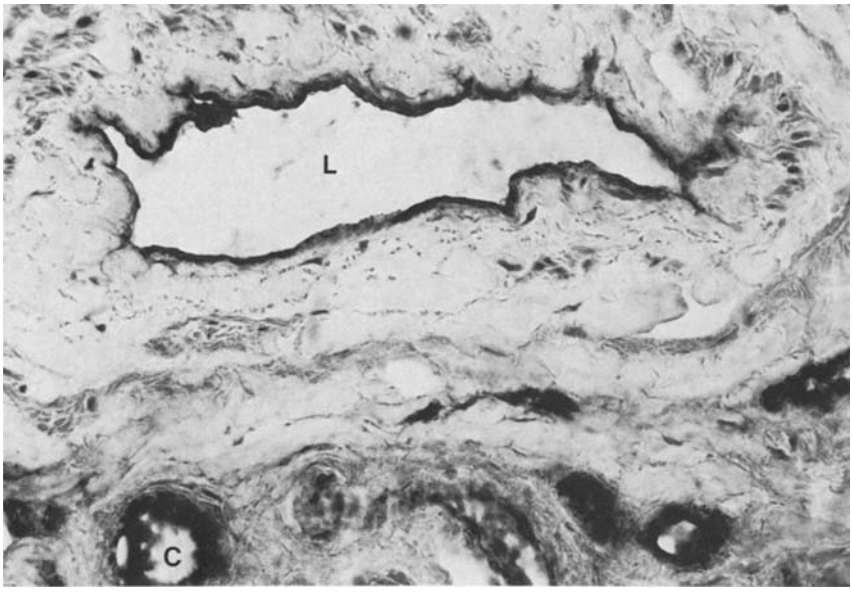

Fig. 1. Lymphatic collecting vessel with FVIIIR:Ag localised diffuse in the endothelial cells lining the lumen (*L*). Endothelial cells of blood capillaries outside the wall of the lymphatic vessel show a more intense reaction (*C*). Cryostat section. Original magnification ×320, paper magnification ×3,5

bath of alcohol to absolute alcolhol. 5) Embedded in Epon. 6) One micron sections for orientation were stained with touluidine blue. 7) Ultrathin sections were stained with uranyl acetate and lead citrate.

Results

The lymphograms of six patients were normal while four patients had pathological or possibly pathological changes.

As seen in Table 1, not all the tissue specimens prepared in the different ways contained a lymphatic vessel. This is primarly because some of the tissue samples were damaged by the operative procedure making the identification of the vessel impossible by microscopy. Secondly, some of the vessels were sectioned tangentially and the lumen was not visualized. In all specimens containing a lymphatic vessel, a positive reaction for FVIIIR: Ag was seen as cytoplasmatic staining of the endothelial cells. In three of the frozen sections and in one of the formalin fixed sections, the positive reaction was seen as a diffuse staining of the cytoplasm in all endothelial cells lining the whole circumference of the lumen of the lymphatic vessels (Fig. 1). In the rest of the sections containing a lymphatic vessel, the intensity of the staining varied (Table 2), and the staining reaction was patchy, not involving all the endothelial cells. The cytoplasm of the endothelial cells of the small blood vessels outside the wall of the lymphatic vessels were positive for FVIIIR : Ag, the reaction being diffuse and more intense compared to the endothelial

Table 2.

Lymphatic vessel prepared as	No. of histological sections with lympahtic vessel	Reaction for FVIIIR:Ag			
		Negative	Weak	Moderate	Strong
Cryostat section	7	0	0	3	4
Formalin-fixed	11	0	9	2	0
Bouin-fixed	11	0	10	1	0

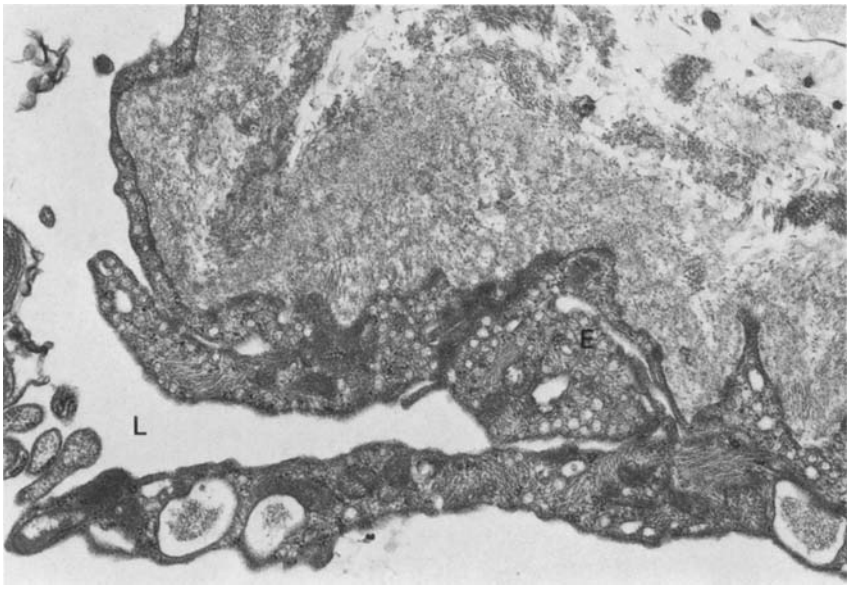

Fig. 2. Small lymphatic collecting vessel with endothelial cells (*E*) lining the lumen (*L*). No basal lamina is seen. ×25000

cells of the lymphatics. None of the negative controls showed positive staining for FVIIIR: Ag.

By electron microscopy five of seven specimens were found to include the lumen of the lymphatic vessels, and all of these were found to be lined by a single layer of endothelial cells without a basal lamina (Fig. 2).

Discussion

It is recommended by WHO (1977) that the pathologist report the finding of vessel invasion when describing malignant gastric neoplasms. Unfortunately it is often impossible to distinguish small blood vessels, lymphatics and arteficial clefts in a stroma infiltrated by tumour. There is agreement that endothelial cells of blood vessels are positive for FVIIIR: Ag (Jaffe et al.

1973; Hoyer et al. 1973; Mukai et al. 1980; Burgdorf et al. 1981; Sehested and Hou-Jensen 1981; Crocker and Smith 1984). Mukai et al. (1980) found a negative reaction for FVIIIR: Ag in normal lymphatic vessels and sinuses of lymph nodes, while Sehested and Hou-Jensen (1981) found endothelial cells of lymphatics to be positive. Burgdorf et al. (1981) found positive FVIIIR: Ag in endothelial cells of haemangioma and angiokeratoma, and less commonly in the endothelial cells of lymphangioma. Crocker and Smith (1984) found no positive reaction for FVIIIR: Ag in sinuses of lymph nodes. However, the authors who have mentioned the reactivity of FVIIIR: Ag in lymphatic vessels have not defined how to separate lymphatics from blood vessels. Our material included small lymphatic collecting vessels identified by lymphangiography and the lymphatic nature of five of the vessels was further supported by electron microscopy, demonstrating the lack of a basal lamina in the endothelial cells. In all the lymphatic vessels we found the endothelial cells to be positive for FVIIIR: Ag independent of the fixative used but most intense in cryostat sections. The intensity was most often weak and patchy when compared with the endothelial cells of blood vessels

The lymphatic collecting vessels examined in this study all originated from patients with malignant disease. The expression of FVIIIR: Ag by lymphatic endothelial cells could, therefore, be abnomal secondary to chronic lymphoedema following malignant infiltration of regional lymph nodes, or secondary to lymphonodal fibrosclerosis as described by Rada et al. (1984). The lymphograms in six of our patients were normal while four patients had pathological or possibly pathological lymphograms. We found no difference in the staining reaction for FVIIIR: Ag in endothelial cells of lymphatic collecting vessels from patients with normal and pathological lymphograms which makes us believe that expression of FVIIIR: Ag is a normal phenomenon.

From this study we conclude that endothelial cells of lymphatic collecting vessels contain FVIIIR: Ag. To document the positivity for FVIIIR: Ag in lymphatic capillaries further studies are necessary, using techniques to identify lymphatic capillaries as described by Barsky et al. (1983), combined with methods for FVIIIR: Ag.

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