

IMMUNOHISTOCHEMICAL LOCALIZATION OF FACTOR VIII-RELATED ANTIGEN IN HUMAN ENDOTHELIAL CELLS

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ABSTRACT

Immunohistochemical localization of factor VIII-related antigen was studied in human tissues obtained at autopsy or renal biopsy, umbilical cord vessels, smears of washed blood cells and cultured human endothelial cells. Factor VIII-related antigen was demonstrated in the vascular intima throughout the body and cells lining the sinusoids of the lymph node, spleen and liver by an indirect immunofluorescent antibody technique. The existence of factor VIII-related antigen was also demonstrated in the cultured endothelial cells derived from umbilical cord veins. Specific granular immunofluorescent stainings were demonstrated in the cytoplasm of cultured endothelial cells. The findings shown by a direct method were similar to those shown by an indirect method. The localization of factor VIII-related antigen was also demonstrated in the intima of umbilical cord veins by the peroxidase-labeled antibody technique. Immunofluorescent staining for the HMW component of factor VIII was detected in the vascular intima and cultured endothelial cells. However, the direct fluorescent method using homologous anti-factor VIII antibody did not show immunofluorescence in the intima of umbilical cord veins or cultured endothelial cells. This finding suggests that VIII:CAg is not localized in the endothelial cells. The postculture media of human endothelial cells contained factor VIII-related antigen (1.2%), Willebrand factor (0.6%) and no factor VIII coagulant activity. These results indicate that factor VIII-related antigen is localized in endothelial cells, but VIII: CAg is probably not present in endothelial cells.

Key words: factor VIII-related antigen, cultured endothelial cell, immunofluorescent

microscopy.

INTRODUCTION

Factor VIII coagulant activity is reduced in patients with hemophilia A and von Willebrand's disease.^{1),2)} Recent studies indicate that the protein of factor VIII has four measurable biological properties and can be separated into two subunits: a low molecular weight (LMW) component and a high molecular weight (HMW) component.^{1),2),3),45)} The former is the antihemophilic factor (VIII: C or factor VIII-related antigen (VIIR: Ag) or von Willebrand factor (VIIR: WF),^{1),2),3),45)}

Although many other coagulation factors are synthesized in the liver, the site of production of factor VIII remains unclear, because VIII: C is not reduced even in patients with severe liver failure.⁴⁾ Previous studies have suggested that factor VIII is produced in the liver, spleen, kidneys, lymphocytes, macrophages or reticuloendothelial system.^{1),2),4)} Recently, Hoyer and his associates⁵⁾ have reported that factor VIII-related antigen was identified in the endothelial cells in human tissues throughout the body using the fluorescent antibody technique. Jaffe and associates^{6),7)} have also shown that cultured human endothelial cells synthesize and release VIIR: Ag and VIIR:WF without coagulant activity. In this paper, the site of production of factor VIII is studied using immunohistochemical techniques with three kinds of anti-factor VIII antibodies. The possible synthesis and release of factor VIII is discussed in terms of its significance in hemostasis and thrombosis.

MATERIALS AND METHODS

Preparation of Antisera: Rabbit anti-VIIR:Ag antibody was obtained from fresh normal human plasma (400 ml) according to the method of Owen and Wagner.⁸⁾ A single line was identified when this absorbed antiserum was tested by Ouchterlony gel diffusion against factor VIII-rich concentrates of normal human plasma. The antiserum was diluted (1: 40) in 0.01 M phosphate, 0.14 M NaCl, pH 7.2 (PBS). The heterologous anti-HMW subunit antiserum was obtained according to the method of Owen and Wagner.³⁾ The homologous antibody against factor VIII was obtained from a patient with classical hemophilia A having an inhibitor due to frequent infusions of cryoprecipitates. This antibody neutralized VIII: C but did not form a precipitation line against purified normal human factor VIII concentrates. Different FITC-labeled antibodies against α_2 -macroglobulin, IgG, IgA and IgM, in addition to unlabeled anti-fibrinogen antibody (Hoechst) were also used.

Specimen for Immunohistochemical Studies: Human tissues obtained at autopsy were rapidly frozen and stored at -70°C until use. A specimen obtained by renal biopsy from a patient with glomerulonephritis was also used. Sections were cut in a cryostat and stored at -20°C until use. Smears prepared from washed red cells, lymphocytes and platelet suspension were air dried, fixed in acetone for 5 min and stored at -20°C.

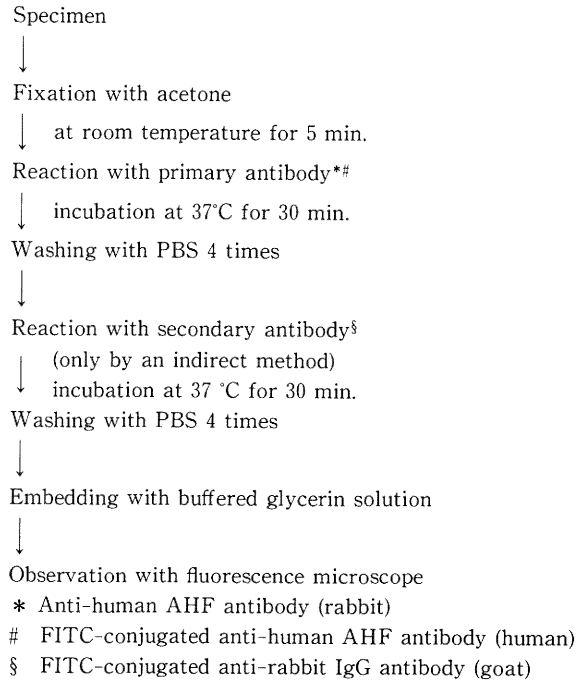


Fig. 1. Procedure of fluorescent antibody techniques.

Cultured monolayer endothelial cells on the cover slips were washed with PBS and fixed with acetone.

Fluorescent Antibody Techniques: The procedure for immunofluorescent study was based on the method of Kawamura^{9),10)} modified from the method of Marshall et al¹¹⁾ and direct methods were employed in the present study. FITC-conjugated goat anti-rabbit gamma globulin (Seikagaku Ind. Co.) was used. Conjugation of the anti-factor VIII homologous antibody with FITC was done by a modification of the method of Hamajima and Kyogoku.¹⁰⁾ The stained specimens were observed by fluorescent microscope (Olympus and Nikon Optical Co. Ltd.).

Peroxidase-labeled Antibody Technique: The anti-factor VIII antibody was labeled with peroxidase according to the method of Hamajima and Kyogoku.¹⁰⁾

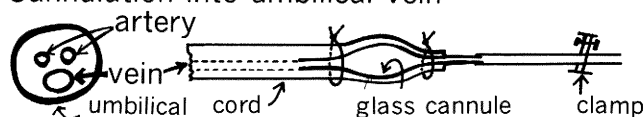
Specificity of Antisera: The specificity of the antisera used was evaluated by the method of Hoyer et al.⁵⁾

Cell Culture Techniques: The culture of human endothelial cells derived from umbilical cord vein was carried out according to the method of Jaffe et al,¹²⁾ based on the method of Maruyama¹³⁾ with a slight modification by using 0.2% trypsin (DIFCO 1: 250) instead of collagenase. The details of the procedure are summarized in Fig. 2. The monolayer endothelial cells were cultured in 35 mm Petri dishes or culture chambers (Lab-Tek, Olympus).

1. Preparation of fresh umbilical cord

stored in cord buffer (PBS, pH 7.4) at 4°C

2. Cannulation into umbilical vein

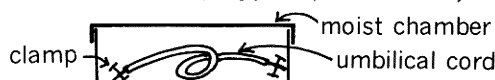


3. Perfusion with cord buffer

to wash out blood

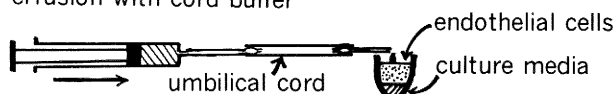
4. Trypsinization (37°C, 15 min.)

Perfusion of 0.2% trypsin (Difco 1:250)



5. Collection of endothelial cells

Perfusion with cord buffer



6. Washing with culture media (2 times)

Centrifugation (250 G, 10 min.) and resuspension

Yield: $0.5 - 1.5 \times 10^6$ cells

7. Culture at 37°C under 5% CO₂

Petri dish (35 mm)

Culture media (TC-199—20% FCS, SM & PC)

※ Subculture: 0.01% EDTA-0.2% trypsin

Fig.2. Culture method of human endothelial cells derived from umbilical cord veins.

Staining of Cultured Endothelial Cells: Cultured endothelial cells were rinsed with prewarmed PBS, fixed with methanol for 5 min and then stained with May-Grünwald and Giemsa solution.

Observation of Cultured Endothelial Cells: Phase contrast and light microscopy were used for observation of unfixed and fixed cells, respectively.

Assay of Factor VIII: VIII: C was measured by a one-stage method using AHF-deficient substrate plasma (DADE).¹⁴⁾ VIII: Ag was measured by the method of Zimmerman et al.¹⁵⁾ using Laurell's immunoelectroassay.¹⁶⁾ VIII: WF was assayed according to the method of Weiss et al.¹⁷⁾

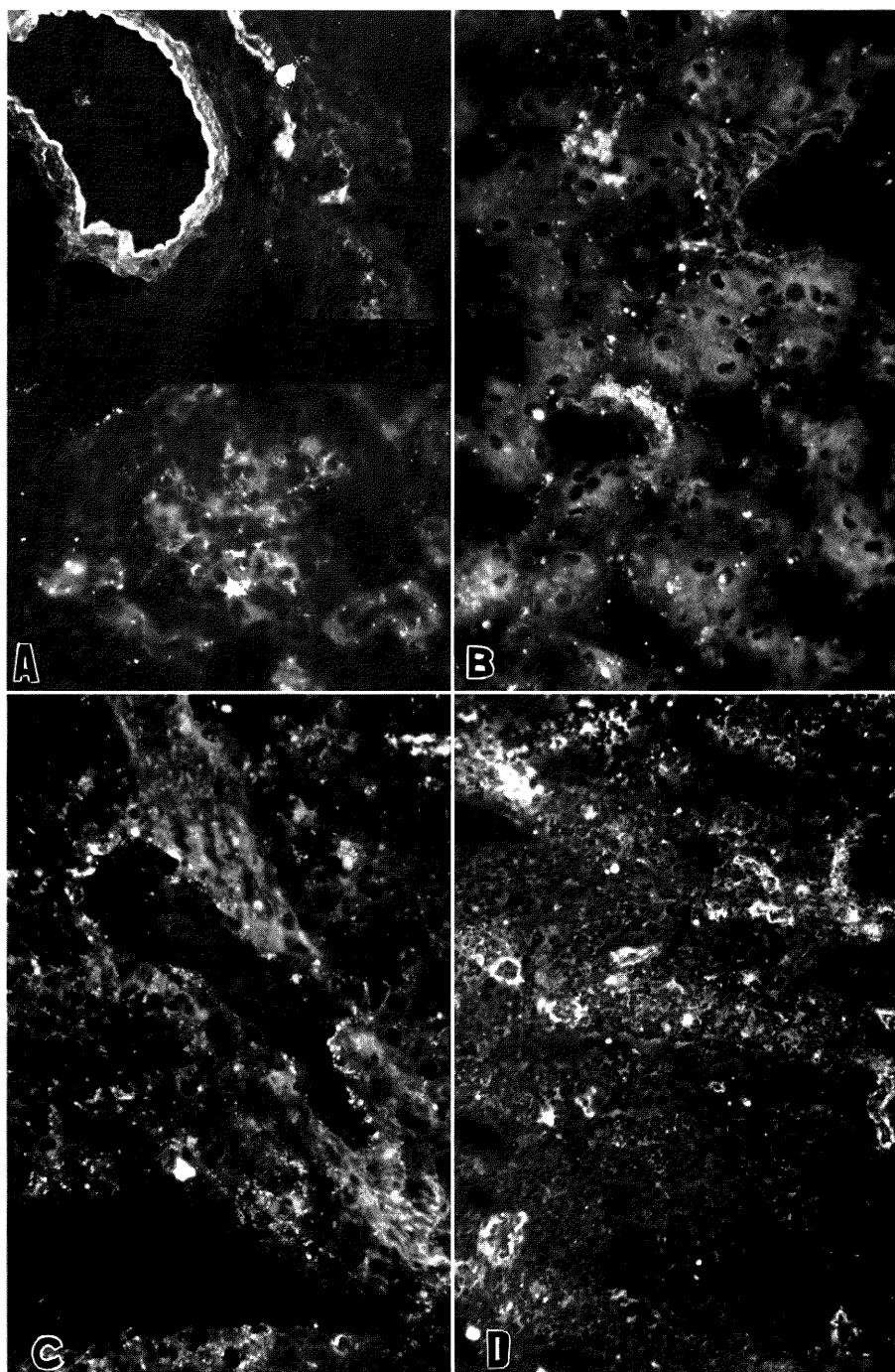


Fig.3. Localization of factor VIII-related antigen in human tissues by indirect fluorescent antibody technique. A: $\times 200$ B: $\times 300$ C: $\times 200$ D: $\times 400$

RESULTS

Localization of Factor VIII in Human Tissues: In all tissues examined, such as liver, spleen, lymph node, kidney, lung, heart, abdominal aorta, common iliac artery and vein, and pancreas, the indirect immunofluorescent method demonstrated that VIIIIR: Ag was restricted to the intima of the vessels (Fig. 3). Positive staining was detected in the intima of the vessels (aorta, artery, arteriole, vein and capillaries). The thin and linear positive staining along the lumen of the arteriole in the lung was clearly detected, as shown in Fig. 3A. However, staining was not identified in the layers of the vessel wall other than the intima. Similar findings were observed in sections of the heart, aorta, common iliac artery and vein. The autofluorescence of the internal elastic lamina could be distinguished from the endothelial cell lining. The immunofluorescent stainings of the renal glomeruli of the specimens obtained from autopsy and renal biopsy appeared as small rings (Fig. 3B). Sections of the liver showed granular and linear fluorescent stainings of sinusoidal lining cells, but hepatic parenchymal cells were not stained (Fig. 3C). The intima of the vessels in the Glisson's sheath showed a positive staining. Granular and linear fluorescent stainings were observed in the red pulp of the spleen and the medulla of the lymph node (Fig. 3D and E). Linear stainings were considered to show sinusoidal lining cells in the spleen and the lymph node. The fluorescent stainings which appeared as small rings might have derived from the endothelial cells of the capillaries in the spleen and the lymph node. On the other hand, smears of washed red cells and lymphocytes did not show positive stainings for VIIIIR: Ag, while normal washed platelets

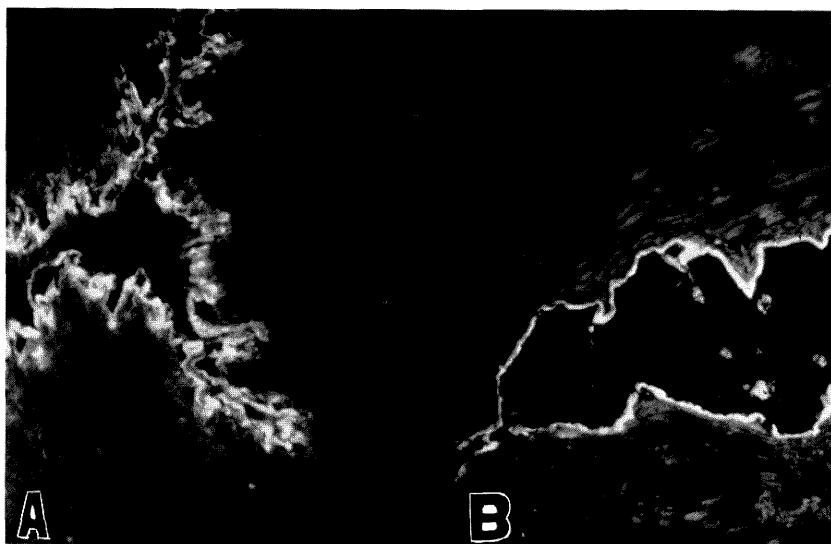


Fig. 4. Localization of factor VIII-related antigen on the intima of umbilical cord vessels by indirect fluorescent antibody technique (A:artery, B: vein). $\times 200$

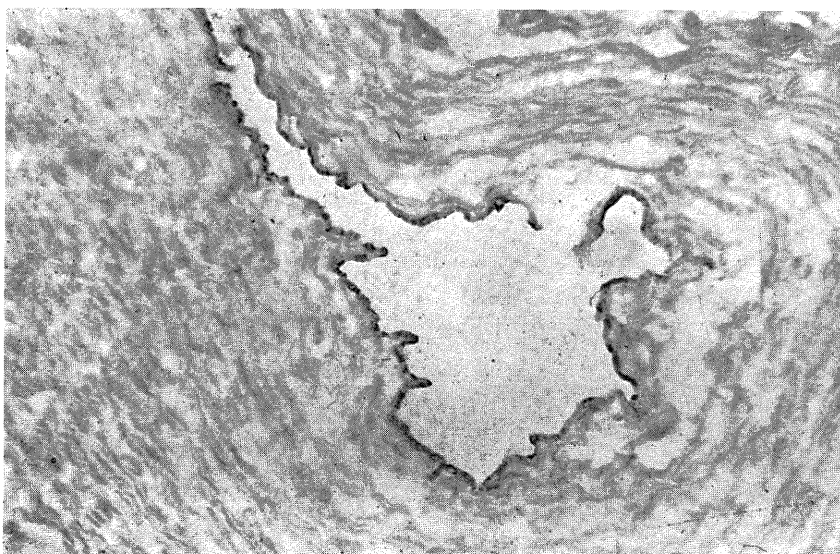


Fig. 5. Localization of factor VIII-related antigen on the intima of umbilical cord vein by peroxidase-labeled antibody technique. $\times 200$

showed strikingly positive stainings. Smears of washed platelets from a patient with mild von Willebrand's disease who had 17% VIII: C and 11% VIIIIR:Ag showed a weak positive fluorescence for VIIIIR: Ag.

Localization of Factor VIII-related Antigen in the Intima of Umbilical Cord Vessels: The immunofluorescent staining by the indirect method using heterologous anti-VIIIIR: Ag antibody was observed only in the intima of the umbilical cord artery (Fig. 4A) and vein (Fig. 4B). Similar findings were obtained by the peroxidase-labeled antibody technique (Fig. 5.).

Localization of Factor VIII-related Antigen in Cultured Human Endothelial Cells Derived from Umbilical Cord Veins: The intima, including the endothelial cells, disappeared after trypsinization (Fig. 6A and B). Phase contrast microscopy (Fig. 7A) revealed closely opposed large polygonal cells (20–50 μm) in monolayer. Light microscopy of May-Giemsa stained cells showed large, round or polygonal cells with a few cytoplasmic vesicles (Fig. 7B). The shape and the mode of the growth of these cells were characteristic of endothelial cells. Positive fluorescent stainings for VIIIIR: Ag were detected within cultured endothelial cells. The distribution of positive immunofluorescence within endothelial cells was cytoplasmic and appeared mostly granular (Fig. 8A and B).

Immunofluorescent Studies by Use of Other Kinds of Anti-Factor VIII Antibodies and Different Antibodies: The indirect method using the heterologous anti-HMW subunit revealed the specific fluorescence of the HMW subunit in the intima of umbilical cord vessels and cultured endothelial cells. In contrast, the direct method using the

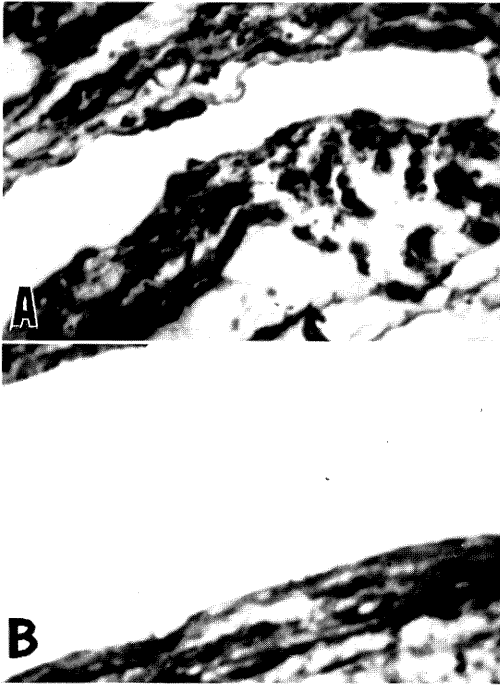


Fig. 6. Histology of umbilical cord vein (Hematoxylin-eosin stain) A: Before trypsinization B: After trypsinization. $\times 100$

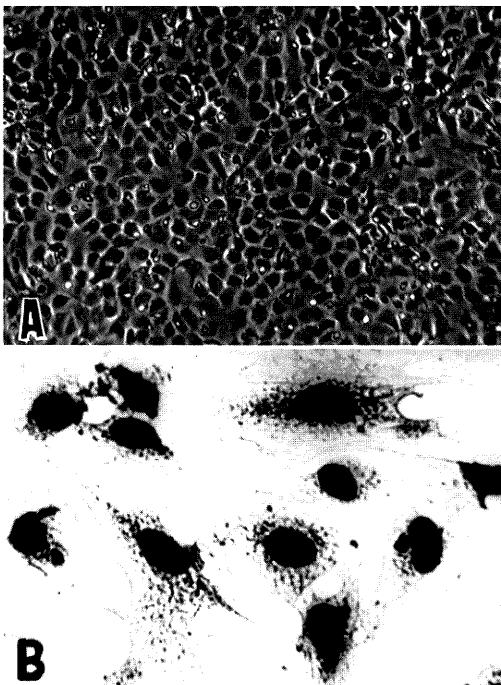


Fig. 7. Cultured human endothelial cells derived from umbilical cord veins (A: Phase contrast microscopy $\times 100$ B: May-Giemsa stain $\times 200$).

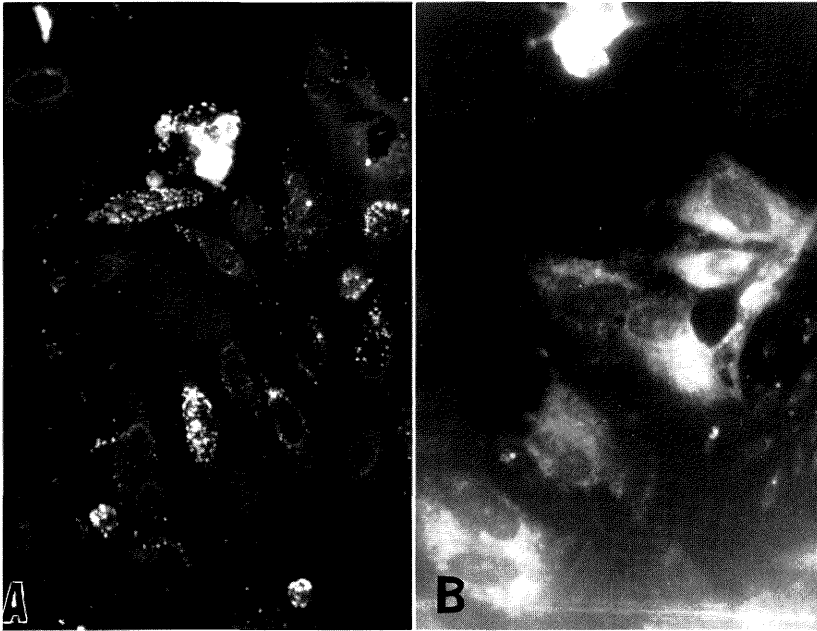


Fig. 8. Localization of factor VIII-related antigen in cultured human endothelial cells by indirect fluorescent antibody technique (A: $\times 200$ B: $\times 400$).

	Postculture media	Preculture media
VIII: C	<0.1%	<0.1%
VIII: Ag	1.2%	<0.1%
VIII: WF	0.6%	<0.1%

cf. 1) Mean, $n=3$

2) F. VIII of reference pooled normal citrated plasma = 100%

3) Cultivation time: 72 hours

Fig. 9. Factor VIII in the postculture media of human endothelial cells.

FITC-conjugated homologous anti-VIII: C antibody stained neither the intima of umbilical cord vessels nor the cultured endothelial cells. Similar findings were detected in the tissue sections, smears of washed platelets, umbilical cord vessels and cultured endothelial cells using FITC-unlabeled anti-fibrinogen antibody or FITC-labeled antibodies against α_2 -macroglobulin and IgM, While no fluorescence for IgG and IgA was detected. Specific fluorescence for fibrinogen was observed in the peripheral area of the liver parenchymal cells as well as in the endothelial cells and the platelets.

Factor VIII in Postculture Media of Human Endothelial Cells: Factor VIII in the postculture media (72 hours) contained less than 0.1% VIII: C, 1.2% VIII: Ag and 0.6% VIII: WF, while all three activities in the preculture media were less than 0.1%, as shown in Fig. 9. Control studies using normal rabbit serum instead of the antibodies were negative, Antiserum did not stain specimens in the absorption studies and the

blocking test. Absorption by an ethanol concentrate of plasma from a patient with severe von Willebrand's disease (less than 1% VIII: C) did not decrease positive staining of endothelial cells. Control studies were negative for all antibodies against fibrinogen, α_2 -macroglobulin, IgG, IgA and IgM, but absorption studies and blocking tests were not done.

DISCUSSION

The present paper indicates that factor VIII-related antigen (VIII: Ag) is localized in the vascular intima, cells lining the sinusoids of the spleen, liver, and lymph node, platelets and cultured endothelial cells derived from human umbilical cord veins. Our results also suggest that factor VIII procoagulant antigen (VIII: CAg) is not localized in the vascular intima and the cultured endothelial cells.

These results coincide with the reports by Hoyer *et al.*^{2),46)} and Holmberg.^{43),44)} Moreover, our results also show that factor VIII with VIII: Ag and VIII: WF but not VIII: C was detected in the postculture media of human cultured endothelial cells. These data suggest that endothelial cells synthesize and release factor VIII molecules, without procoagulant activity,^{5),6)} or that they synthesize a molecule with all biological properties characteristic of factor VIII, although VIII: CAg or VIII: C determinant sites of the molecule may be destroyed by traces of serine proteases or by other mechanisms in the culture media or in the circulating blood soon after it is released.¹⁸⁾ Recently, Tuddenham *et al.* has reported that the cultured human endothelial cells do not synthesize and release factor VIII procoagulant antigen (VIII: CAg). As the factor VIII molecule can be separated into two subunits—LMW subunit with VIII: CAg and VIII: C and HMW subunit with VIII: Ag and VIII: WF,^{2),3),19)} it is reasonable that HMW subunit as well as VIII: Ag was detected in endothelial cells and platelets.

Our immunofluorescent studies were performed using three kinds of anti-factor VIII antibodies. It is known that heterologous anti-VIII: Ag and HMW subunit antibodies react strongly with factor VIII antigen and Willebrand factor,¹⁵⁾ while homologous or antologous factor VIII inhibitors (antibodies) react with factor VIII coagulant activity to a greater degree than heterologous antibodies. Unlike heterologous antibodies, homologous antibodies (inhibitors) usually neutralize factor VIII procoagulant activity, but do not usually form a precipitation line with factor VIII-related antigen and do not affect Willebrand factor.^{20),21)} However, Thomson reported a very rare case with a high titer of inhibitor which interacts with VIII: WF.²²⁾

Homologous antibody can bind with VIII: CAg because homologous antibody (inhibitor) is used for the immunoradiometric assay of VIII: CAg.⁴²⁾

Bloom *et al.*^{23),24)} reported that antihemophilic factor was detected by antibody neutralization in the washed homogenates of human tissues. Hoyer *et al.*⁵⁾ and Gruson *et al.*²⁵⁾ reported that factor VIII-related antigen is localized in the endothelial cells of blood vessels, the cells lining splenic and hepatic sinusoids, platelets and megakaryocytes.

Jaffe et al^(6,7) also reported that cultured endothelial cells derived from umbilical cord veins synthesize and release factor VIII-related antigen and Willebrand factor without procoagulant activity. Jaffe and Nachman⁽²⁶⁾ further demonstrated that VIIIIR: Ag synthesized by cultured endothelial cells was found to contain the same single polypeptide subunit (mol wt 225,000) present in plasma VIIIIR: Ag. These findings suggest that endothelial cells are a major site of synthesis of circulating factor VIII-related antigen.

Many investigators have reported that VIIIIR: Ag is demonstrated in platelets^(24,27,29,30,31) and megakaryocytes.^(31,43) Piovella⁽³²⁾ has suggested that megakaryocyte is able to synthesize VIIIIR: Ag and secrete it. Although the present paper and others⁽⁵⁾ show that lymphocytes do not show the specific fluorescence to VIIIIR: Ag, some investigators have reported that the lymphoid tissues synthesize factor VIII activity.^(33,34)

Holmberg et al⁽³⁵⁾ could not detect the protein of VIIIIR: Ag in the vessel wall intima of three patients with severe von Willebrand's disease using a direct method, but did find it in the intima of patient with a moderately severe form of the disease. We observed the presence of VIIIIR: Ag in the endothelial cells derived from umbilical cord veins of the baby delivered by a patient with mild von Willebrand's disease. VIIIIR: Ag of this baby was not decreased, so endothelial cells might have had the ability to synthesize VIIIIR: Ag.

Giddings et al⁽³⁶⁾ has demonstrated that factor V is localized in the endothelium of normal vessels.

Although our studies show that immunofluorescent stainings are observed in endothelial cells for α_2 -macroglobulin, IgM and fibrinogen and in hepatic parenchymal cells only for fibrinogen, the conclusion that these proteins are localized in the endothelial cells has to be held because absorption studies and blocking tests were not done. Hoyer et al⁽³⁶⁾ have reported that fibrinogen is localized in endothelial cells and hepatic parenchymal cells, using the monospecific antibody prepared in their laboratory.

Benson and Dodds⁽³⁸⁾ demonstrated that hepatic perfusates contain both HMW-factor VIII-stimulating material and large amounts of factor VIII-neutralizing antigen lacking procoagulant activity, and suggested that the liver produces a biologically inactive precursor of factor VIII, which is then rapidly converted to active VIII by the spleen, i. e.: the spleen has factor VIII-stimulating material for hepatic perfusates.

Mannuci et al^(39,40) reported that VIIIIR: Ag was not immunohistochemically detected in the buccal mucosa obtained from a patient with von Willebrand's disease in whom all three biological properties of factor VIII, except for bleeding time, were corrected after the infusion of cryoprecipitate. Similar findings⁽⁴¹⁾ were observed in the vascular endothelium in the same disease. Factor VIII in the vascular intima seems to be more important for hemostasis than plasma factor VIII.

It is very interesting that factor VIII molecule is synthesized by human endothelial cells, because endothelial cells play an important role in hemostasis and thrombosis. The site of production of VIII: C and VIII: CAg still remains unclear, though.

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