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Tissue distribution of HRF20, a novel factor preventing the membrane attack of homologous complement, and its predominant expression on endothelial cells *in vivo*

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SUMMARY

A 20,000 molecular weight (MW) homologous restriction factor (HRF20), detected by 1F5 monoclonal antibody (mAb), is present on blood cell surfaces and inhibits the terminal stage of the formation of membrane attack complexes by homologous complement activation. The tissue distribution of HRF20 was studied by immunohistochemical analysis using 1F5. HRF20 was predominantly expressed on endothelial cells of systemic arteries, veins and capillaries, as well as on the surface of cultured human umbilical vein endothelial cells. HRF20 was also detected, to a lesser extent, on the Schwann sheath of peripheral nerve fibres, ependymal cells and certain epithelial cells such as acinar cells of the salivary gland, bronchial epithelium, renal tubules and squamous epithelium. The distribution pattern of HRF20 differed somewhat from that of decay-accelerating factor (DAF), which is another membrane inhibitor of homologous complement activation.

INTRODUCTION

Decay-accelerating factor (DAF) (Hoffman, 1969a, b; Nicholson-Weller *et al.*, 1982; Medof, Kinoshita & Nussenzweig, 1984) and membrane co-factor protein (MCP) (Colle *et al.*, 1985) prevent amplification of the complement reaction, thereby protecting autologous cell membranes from complement reaction. In addition, a 65,000 molecular weight (MW) homologous restriction factor (HRF) (Zalman, Wood & Müller-Eberhard, 1986) and C8 binding protein (C8bp) (Schonermark *et al.*, 1986) have also been reported to be membrane inhibitors that prevent the formation of membrane attack complexes (MAC) on autologous cell membranes. Recently, a monoclonal antibody (mAb), 1F5, has been obtained which allows haemolysis of neuraminidase-treated human erythrocytes by homologous complement to occur via the alternative pathway (Okada *et al.*, 1987). Since mAb 1F5 enhances reactive haemolysis of human erythrocytes sensitized with C5b67 by C8 and C9 (Okada *et al.*, 1989b, c), the antigen reacting with mAb 1F5 is thought to share a common function with HRF or C8bp. However, its MW of 20,000 differs from HRF. Therefore, the 1F5 antigen was designated a 20,000 MW homologous restriction factor (HRF20) (Okada *et al.*, 1989a, c). Subsequently, HRF20 was found to be identical to P18 (Sugita *et al.*, 1988) and CD59 (Stefanova *et al.*, 1989), because the base sequences of cDNA coding for HRF20 (Okada *et al.*, 1989a), P18 (Sugita *et al.*, 1989) and CD59 (Davies *et al.*, 1989) are essentially identical.

By flow cytometric analysis of HRF20 on blood cells using mAb 1F5, these molecules were shown to be expressed on most mononuclear leucocytes and erythrocytes (Hideshima, Okada & Okada, 1990), indicating that HRF20 may be playing a role in preventing autologous complement attack in the blood circulation. This is supported by the fact that erythrocytes and leucocytes from patients with paroxysmal nocturnal haemoglobinuria (PNH) are deficient in HRF20 and are sensitive to homologous complement attack (Okada *et al.*, 1987, 1989b; Holguin *et al.*, 1989). Furthermore, adsorption of HRF20 to erythrocytes from PNH patients rendered these cells resistant to haemolysis by homologous complement (Okada *et al.*, 1989d). Holguin *et al.* (1989) obtained similar results: an 18,000 MW membrane inhibitor from normal erythrocytes rendered PNH erythrocytes resistant to haemolysis by complement.

Both HRF20 molecules and DAF are expressed on cell surfaces of not only peripheral blood cells but also epithelial and parenchymal cells. Immunohistochemical analysis demonstrated that DAF is expressed predominantly on epithelial cells

Abbreviations: C8bp, C8 binding protein; DAF, decay-accelerating factor; HBSS, Hanks' balanced salt solution; HRF20, 20,000 MW homologous restriction factor; mAb, monoclonal antibody; MAC, membrane attack complexes; MCP, membrane co-factor protein; PBS, phosphate-buffered saline; PNH, paroxysmal nocturnal haemoglobinuria.

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of the cornea, conjunctiva, oral mucosa, salivary glands, esophageal epithelium, gastro-intestinal mucosa, renal tubules and urinary tract (Medof *et al.*, 1987). There are not, however, significant amounts of DAF on endothelial cells, as shown by immunohistochemical analysis *in vivo*, although it is present to a greater degree on cultured human umbilical vein endothelial cells (Asch *et al.*, 1986). In this study, it is demonstrated that HRF20 molecules are expressed on endothelial cells *in vivo* in a different distribution from DAF. Moreover, these molecules are present on Schwann sheath and ependymal cells originating from the neural tube and, to a lesser extent, on several epithelial cells.

MATERIALS AND METHODS

Monoclonal antibodies

1F5 (mouse IgG1) was obtained from the hybridoma clone 1F5 derived from spleen cells of a human erythrocyte-immunized BALB/c mouse and P3U-1 myeloma cells (Okada *et al.*, 1987, 1989b, c). Purified 1F5 from the culture supernatant of 1F5 clone cells in serum-free medium (Nissui Pharm. Co., Tokyo) was biotinylated af follows. 1F5 (500 μg) was suspended in 2400 μl of 0·05 M phosphate-buffered saline (PBS) containing 0·2 M $NaHCO_3$ and 400 μg of D-biotinyl-ε-aminocapronic acid *N*-hydroxysuccinimide ester (Boehringer-Mannheim, Mannheim, FRG) and incubated at 4° overnight. The reaction mixture was applied to a PD-10 column (Pharmacia, Uppsala, Sweden) equilibrated in PBS (pH 7·2). The void fraction was collected and used as the source of biotinylated 1F5. Biotinylated anti-DAF mouse IgG1 mAb (1C6) was kindly donated by Teizo Fujita, University of Tsukuba.

Immunohistochemical study

Autopsy tissues were frozen in OCT compound (Miles Lab. Inc., Kankakee, IL). Cryostat tissue sections were cut to a thickness of 3 μm and fixed in cold acetone for 5 min. Histochemical procedures were based on those of Hsu, Raine & Fanger (1981). After drying and washing with PBS, the fixed sections were immersed in 0·3% hydrogen peroxide-methanol solution for 20 min to inactivate endogenous peroxidase and then were rinsed well in PBS. The sections were overlaid with PBS containing 2% normal human serum, left for 15 min at room temperature and washed. The sections were then treated with biotinylated antibodies at the optimal concentration in a moist chamber at 4° overnight. After thorough washing with PBS, the treated sections were incubated with horseradish peroxidase-labelled avidin-biotin complex (ABC kit PK400, Vector Lab. Inc., Burlingame, CA) at room temperature for 30 min, washed with PBS and then immersed for 5–15 min in 0·05 M Tris-HCl buffer (pH 7·6) containing 0·05 mM 3,3′-diaminobenzidine, 2 mM hydrogen peroxide and 3 mM sodium azide. The stained sections were washed with PBS and counterstained for 2 min with haematoxylin or methylgreen. Control studies performed using the same procedure, except without biotinylated antibodies, were negative in all experiments.

Endothelial cell culture and immunostaining

Cultured human endothelial cells were obtained from an umbilical cord according to the method described by Jaffe (1984). Endothelial cells (2×10^4) in tertiary passages were put on a coverglass, 15 mm in diameter, previously coated with type I collagen (Sigma, St Louis, MO) and cultured in Daigo T medium (Nippon Pharmaceutical Co., Tokyo) supplemented with 20% fetal calf serum. After a 2-day period, the live cells were washed with Hanks' balanced salt solution (HBSS), overlaid with biotinylated antibodies dissolved in HBSS containing 0·02% sodium azide and then incubated at 4° for 1 hr. After washing in the above HBSS, FITC-labelled avidin (Vector Lab. Inc.) was added at room temperature. The specimens were left standing for 30 min, washed with HBSS and then mounted in 90% glycerin-Dulbecco's PBS. Cells were visualized under a fluorescent microscope (Zeiss Axioplan, Oberkochen, FRG).

RESULTS

Endothelial cells *in vivo*

To examine HRF20 (1F5 antigen) expression on endothelial cells *in vivo*, specimens obtained from various tissues were treated with mAb 1F5. Strong anti-HRF20 staining of endothelial cells was observed in arteries, veins and capillaries of various organs (Fig. 1 a–d). Renal glomerular endothelial cells were also significantly stained (Fig. 1d), while anti-DAF staining of the same tissue was unsuccessful (Fig. 1e). 1F5 antigens were also localized on splenic sinus and hepatic sinusoid (not shown).

Cultured endothelial cells

To determine whether HRF20 was present on the surface of live endothelial cells, cultured human endothelial cells obtained from an umbilical cord were stained with biotinylated 1F5 followed by FITC-avidin. As shown in Fig. 1f, surface fluorescence was visible on almost all endothelial cells. Staining was negative when anti-trinitrophenyl (TNP) antibodies (mouse IgG1) were used instead of 1F5 (not shown).

Nervous system

HRF20 was unexpectedly detected in the Schwann sheath of nerve fibres such as the periarterial autonomic nerve (Fig. 2a) and the craniospinal nerve (Fig. 2b). Moreover, ependymal cells in plexus chorioideus were positive (Fig. 2c). Neither glial cells nor myelin sheath in the central nervous system were stained (not shown).

Epithelial cells

In several organs, epithelial cells were not stained to any great degree, except in a few areas. Bronchial epithelium was strongly stained with 1F5 only in the cilia but not in the cytoplasm (Fig. 2d). Alveolar and bronchiolar epithelium were negative (Fig. 1c). In the salivary gland, 1F5 staining was significant in acinus, especially in the serous gland (Fig. 2e), although staining in the excretory duct was not remarkable. The pancreas, however, another excretory organ, was negative for HRF20 expression (Fig. 2f). The distribution of HRF20 in the kidney was almost the same as that for DAF (Fig. 1b, d, e).

DISCUSSION

C5 convertase is formed at the site of complement activation, usually on foreign particles such as micro-organisms. C5

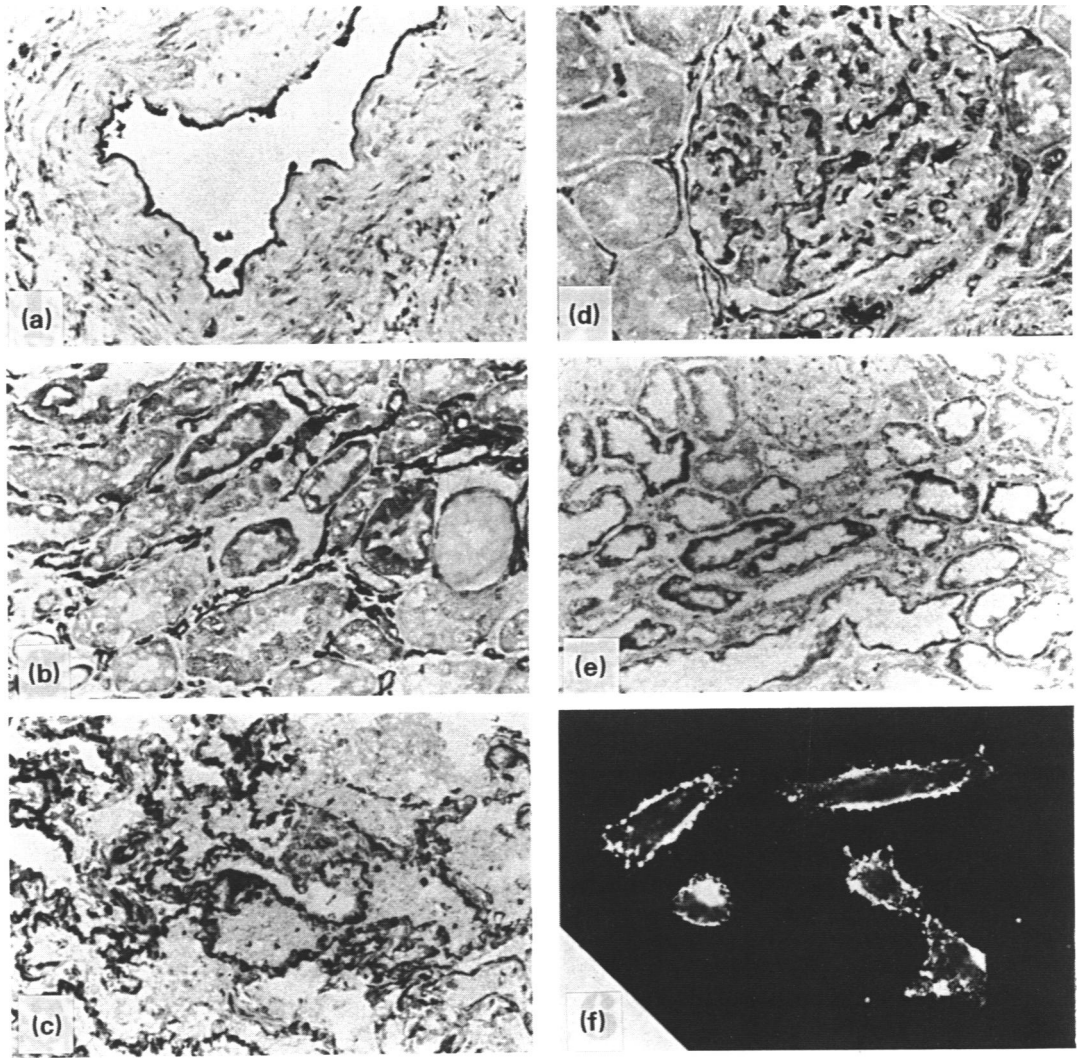

Figure 1. (a) 1F5 staining of endothelial cells of central artery in spleen. Strong staining is observed in the cytoplasm. Basement membrane of the vascular wall is negative. Immunoperoxidase staining, × 496. (b) 1F5 staining of the kidney. 1F5 antigens are present in capillary endothelial cells of the interstitium and, to a lesser extent, in several proximal and distal convoluted tubules. Immunoperoxidase staining, × 496. (c) 1F5 staining of lung. Capillary endothelial cells in alveolar septum are positive. Neither alveolar epithelium nor alveolar macrophages are stained. Immunoperoxidase staining, × 248. (d) 1F5 staining of renal glomerulus. Glomerular endothelial cells are positive as well as podocytes and mesangial cells. Immunoperoxidase staining, × 496. (e) DAF staining of kidney. Renal tubules are stained in the same manner as that of 1F5 antigens, but it is worthy of note that capillaries in neither the interstitium nor glomerulus are positive. Immunoperoxidase staining, × 248. (f) 1F5 staining of cultured human umbilical vein endothelial cells. Surface fluorescence is prominent. Immunofluorescence, × 1080.

convertase acts on C5 to yield C5a and C5b, the latter of which combines with C6 to form the heteromolecular complex C5b6 (Lachmann & Thompson, 1970). This complex is relatively stable until it interacts with C7 to form C5b67, which assumes a hydrophobic character facilitating deposition on to a hydrophobic surface such as the lipid bilayer of cell membranes (Lachmann *et al.*, 1970). C5b6 can migrate from the site of complement activation, and a delay in reaction with C7 may result in its deposition on an autologous cell membrane rather than on the particle on which complement activation was initiated. In other words, autologous cell membranes can be exposed to C5b67 deposition as a result of complement activation on micro-organisms in their vicinity.

MAC formation by C5b67 following interaction with C8 and C9 on autologous cell membranes is restricted by HRF20 as well as the 65,000 MW HRF (Zalman *et al.*, 1986) or C8bp (Schonermark *et al.*, 1986). Flow cytometric analysis of peripheral blood cells demonstrated that most of these cells expressed HRF20 detectable by 1F5 (Hideshima *et al.*, 1990). Since Western blot analysis showed that the antigen reacting with 1F5 on nucleated cells has the same molecular size as that on erythrocytes (Harada *et al.*, 1990), it is believed that the antigen detected with 1F5 must be HRF20 and not another cross-reacting molecule.

Tissue distribution of HRF20 was examined immunohistochemically using biotinylated 1F5 and compared with that of

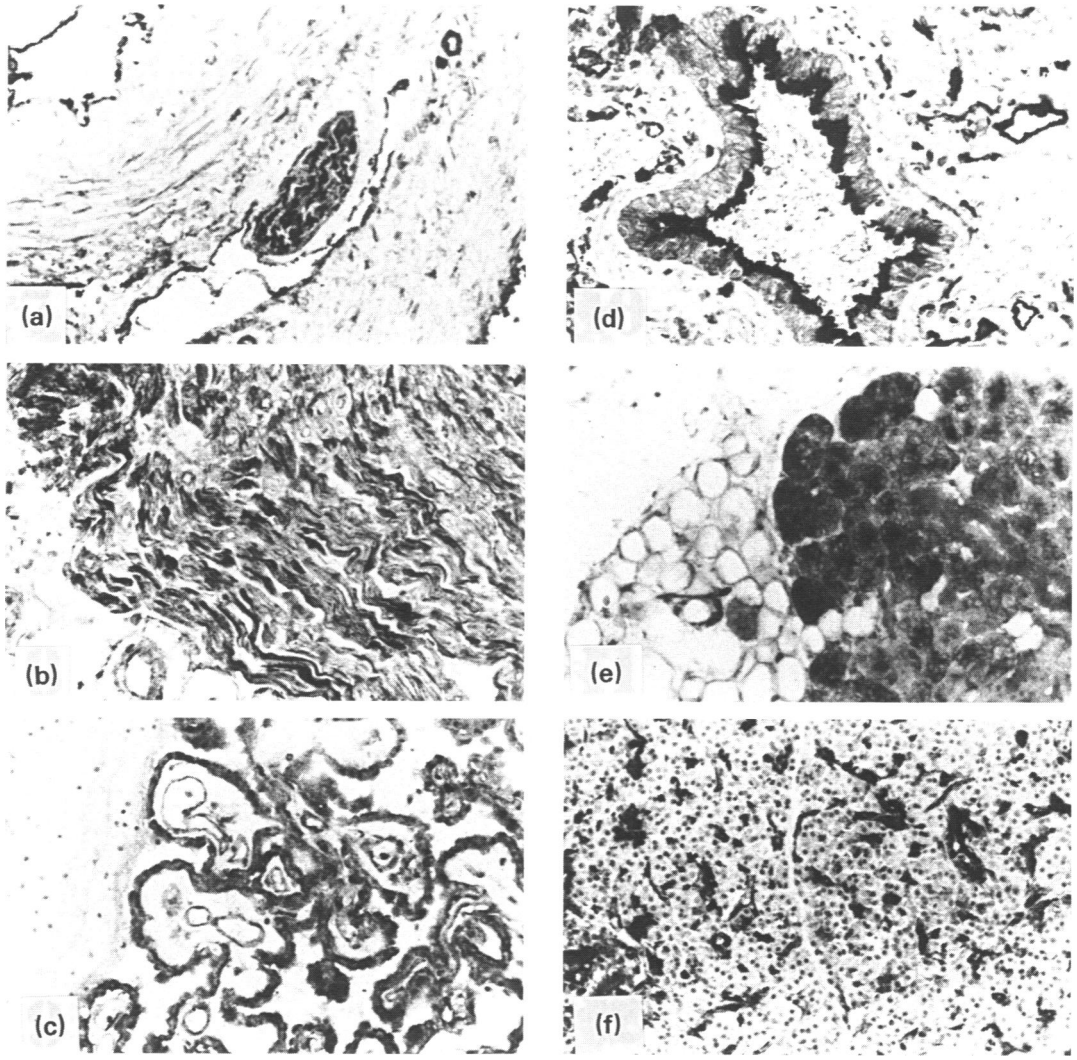

Figure 2. (a) 1F5 staining of peripheral nerve fibres in splenic artery. The Schwann sheath is strongly positive. Endoneurium and perineurium are not prominently stained. Immunoperoxidase staining, × 496. (b) 1F5 staining of trigeminal nerve. The Schwann sheath alone is positive. Axons are not stained at all. Immunoperoxidase staining, × 496. (c) 1F5 staining of ependymal cells in plexus chorioideus. The cytoplasm and microvilli are strongly positive, although the cilia are not. Chorioideal capillaries are well stained. Immunoperoxidase staining, × 248. (d) 1F5 staining of bronchial epithelium. On these cells only cilia are strongly stained. Cytoplasm and secretory vacuoles are negative. Immunoperoxidase staining, × 496. (e) 1F5 staining of submaxillary gland. Serous acinar cells located in a peripheral region are predominantly stained. There is no significant staining of excretory glands. Immunoperoxidase staining, × 496. (f) 1F5 staining of pancreas. There is no staining of either acinar cells or excretory ducts. Capillary endothelial cells are strongly positive. Immunoperoxidase staining, × 248.

DAF. The greatest expression of HRF20 was on endothelial cells of systemic vessels, including splenic sinus and hepatic sinusoid. HRF20 was also detected on the surface of cultured human umbilical vein endothelial cells. With regard to DAF, in agreement with the findings presented here, immunohistopathological studies by Medof *et al.* (1987) revealed no significant levels in endothelial cells *in vivo*. However, using flow cytometric analysis, cultured human umbilical vein endothelial cells express DAF in quantities four times higher than those of peripheral blood cells (Asch *et al.*, 1986). It should be noted that glomerular endothelial cells were positive for HRF20 and negative for DAF (1C6), even though the same individual specimen was used (Fig. 1d, e).

Epithelial cells of several organs expressed HRF20 with almost the same distribution as DAF. Renal convoluted tubules (Fig. 1b, d, e), salivary gland (Fig. 2e) and squamous epithelium (data not shown) were significantly stained by 1F5.

Interestingly, HRF20 was strongly expressed in the Schwann sheath of peripheral nerve fibres and ependymal cells (Fig. 2a–c), both of which are known to be derived from matrix cells in the neural tube (Fujita, 1963). However, glial cells and myelin sheath from the same origin in the central nervous system were not positive. Although the physiological significance of these observations remains unclear, it is likely that the spinal fluid contains a high amount of HRF20.

Factors such as HRF20 and DAF, which prevent the

activation of homologous complement, may be present in various tissues and display different distribution patterns. They function in a manner which differs from that of the intrinsic factors by protecting the self from autologous complement-mediated tissue injury *in vivo*.

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