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BRIEF COMMUNICATION

Immunolocalization of Endothelin and Nitric Oxide-Synthase in Lymphatic Vessels and Cultured Lymphatic Endothelial Cells

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INTRODUCTION

It is generally accepted that lymph flow is determined by both passive and active driving mechanisms. The passive mechanism is determined by compression of lymphatic vessels due to extrinsic activities of the surrounding tissue, whereas the active one consists of intrinsic contractility of lymphatic vessels due to the activity of lymphatic wall smooth muscle cells (Ohhashi *et al.*, 1980; Drake *et al.*, 1985; Ohhashi, 1993). Functional studies have demonstrated that several vasoactive substances, including ET and NO, might be involved in lymphatic vessel contractility and lymph flow (Dabney *et al.*, 1988, 1991; Fortes *et al.*, 1989; Dobbins *et al.*, 1991; Dobbins and Dabney, 1991; Ferguson and DeFilippi, 1994). Moreover, the release of vasodilator agents from lymphatic endothelium has been demonstrated, thus suggesting that lymphatic vessels may be able to regulate their own tone (Ohhashi and Takahashi, 1991; Bohlen and Lash, 1992; Ohhashi and Yokoyama, 1994).

The aim of our study was to investigate the occurrence and distribution of ET and constitutive NOs in lymphatic vessels using immunocytochemistry. Moreover, we have studied the expression of ET and NOs in primary cultures of endothelial cells isolated from lymphatic vessels.

MATERIALS AND METHODS

Collection of Lymphatic Vessels and Culture of Lymphatic Endothelial Cells

Lymphatic vessels were obtained from bovine mesenteries at the local slaughterhouse. Some vessels were fixed in 4% paraformaldehyde in 0.1 *M* phosphate buffer, pH 7.3, and washed overnight in Tris-buffered saline (TBS), pH 7.4, containing 15% sucrose. The specimens were then embedded in Tissue Tek II OCT (Miles,

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Naperville, IL), frozen in isopentane cooled by liquid nitrogen, and cut in a cryostat.

Endothelial cells for cultures were obtained by collagenase digestion from isolated lymphatic vessels cannulated at both ends, washed with normal saline solution, and incubated at 37° for 10 min. The vessels were then drained with culture medium TC 199 containing Earle's salts and L-glutamine, and supplemented with 20% fetal calf serum, 2.2 mg/ml $NaHCO_3$, 200 U/ml penicillin, 200 mg/ml streptomycin. After washing, the vessels were incubated with 0.2% collagenase in saline solution at 37° for 5 min. The fluid was collected and placed in Costar well plates containing coverslips for cell cultures. Cells were grown at 37° in a humidified incubator with air and 5% CO_2. Finally, the cells were fixed for 5 min in 4% paraformaldehyde in 0.1 *M* phosphate buffer and washed in TBS before immunostaining.

Light Microscopy Immunocytochemistry

Sections of lymphatic vessels and primary cultures of lymphatic vessel cells were processed for the immunocytochemical detection of ET and endothelial constitutive NOs using indirect immunofluorescence and immunoperoxidase (Polak and Van Noorden, 1986). The immunolocalization of the von Willebrand factor (vWf) was also performed to demonstrate the endothelial nature of cultured cells.

Before immunostaining, the vessels and the cells were treated with 0.3% Triton X-100 in TBS for 1 min and incubated with 0.3% hydrogen peroxide in TBS for 30 min to remove endogenous peroxidase activity. The samples were then incubated overnight at 4° with rabbit polyclonal antisera to ET, NOs, and von Willebrand protein, at dilutions between 1:50 and 1:1000. After washing in TBS, the samples were incubated with biotinylated goat anti-rabbit IgG, diluted 1:100 in TBS for 30 min, washed in TBS, and finally incubated with streptavidin-biotinylated peroxidase complexes diluted 1:200 in TBS for 1 hr at room temperature. After washing in TBS, peroxidase was visualized by incubation with 0.03% 3,3′-diaminobenzidine tetrahydrochloride solution in 0.05 *M* Tris-HCl, pH 7.4, to which 0.02% hydrogen peroxide was added before use.

Antisera and Specificity Controls of the Immunocytochemical Reactions

The antisera against synthetic endothelin-1 and endothelial constitutive nitric oxide synthase were raised in rabbits and have been characterized previously (Yoshizawa *et al.*, 1990; Casasco *et al.*, 1991; Springall *et al.*, 1991, 1992; Buttery *et al.*, 1994). Pertinent specificity tests were performed, including adsorption of the specific antisera with related and unrelated antigens, omission of the first layer, and substitution of an inappropriate antiserum or a nonimmune serum for the specific primary antisera (Polak and Van Noorden, 1986).

As for ET antiserum, immunostaining was prevented by adsorption of antiserum with synthetic endothelin-1, -2, or -3 (10 nmol in 1 ml antiserum diluted 1:700 in TBS), indicating that the antiserum cross-reacts with different ET isoforms.

NOs antiserum was raised against a synthetic 15-residue peptide, based upon deduced amino acid sequences of cDNA encoding the bovine and human endothelial constitutive NOs (Lamas *et al.*, 1992; Marsden *et al.*, 1992). The peptide sequence was VTSRIRTQSFSLQER-C, corresponding to amino acids 1172–1186 of the deduced amino acid sequence, with a cysteine residue at the C-terminus to assist coupling to carrier. It represents a sequence identical between the bovine and human endothelial NOs (Lamas *et al.*, 1992; Marsden *et al.*, 1992), and having low homology with other isoforms of NOs (less than 50% with rat brain NOs and virtually no homology with macrophage NOs). The peptide was coupled to maleimide-activated keyhole limpet hemocyanin and used to immunize rabbits.

The antiserum to human von Willebrand protein and the secondary antibodies were purchased from Dakopatts (Glostrup, Denmark). Biotinylated probes were purchased from Amersham International (Amersham, England).

RESULTS

Immunoreactions to detect the presence of ET and NOs were positive both in lymphatic vessels and in cultured endothelial cells. Lymphatic vessel sections ex-

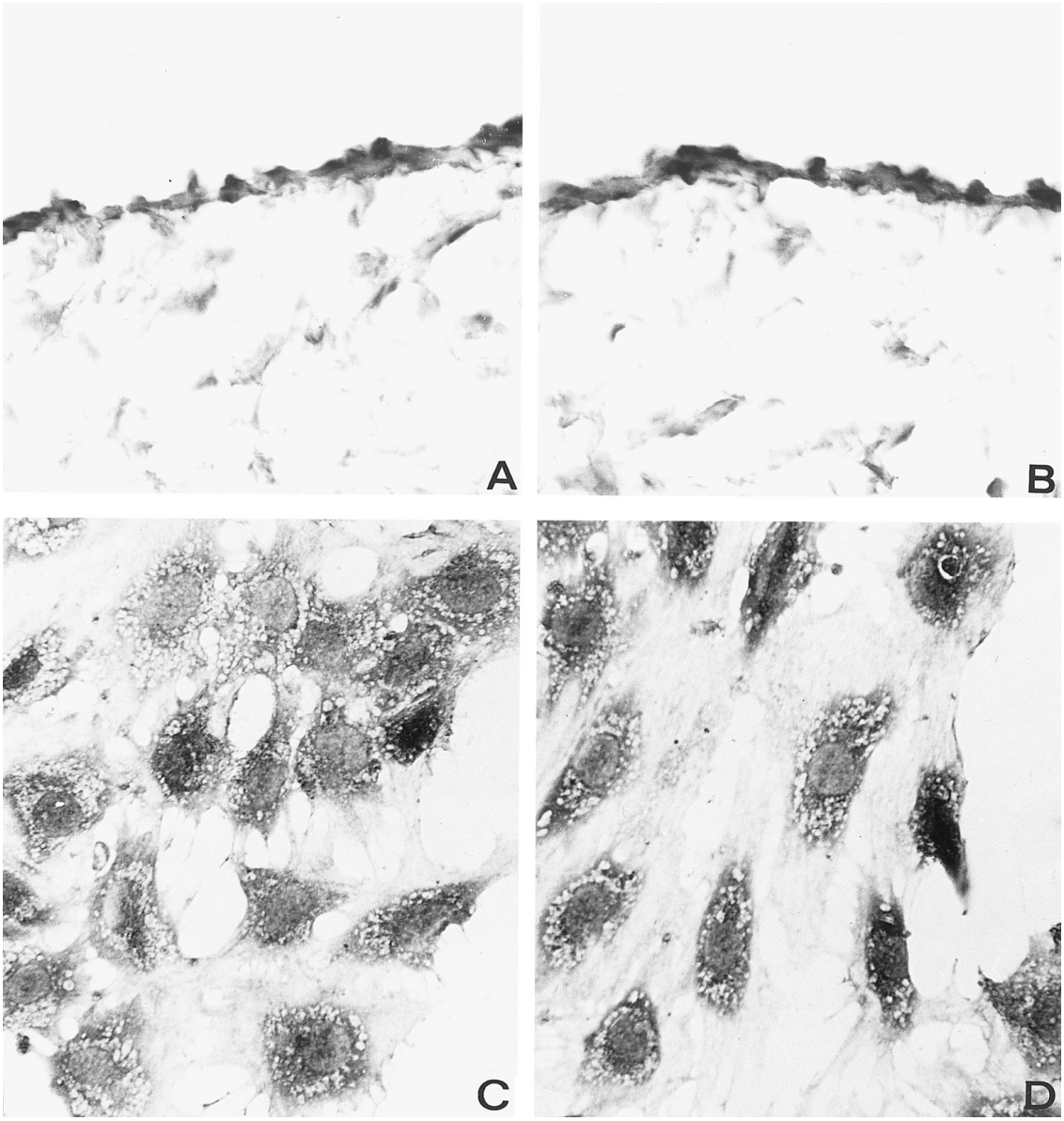

FIG. 1. (A, B) ET (A) and NOs (B) immunolocalization in endothelial cell layer of bovine lymphatic vessels. Immunoperoxidase method. Original magnification, 800×. (C, D) ET (A) and NOs (B) immunolocalization within the cytoplasm of cultured lymphatic endothelial cells. Immunoperoxidase method. Original magnification, 500×.

hibited a continuous positive pattern confined to the endothelial layer (Figs. 1A, 1B). No specific immunoreaction was seen in the muscular layer.

Cultured cells, positive to the vWf reaction, specific marker for endothelial cells, were also intensely positive to the immunostaining with ET and NOs antisera (Figs. 1C, 1D).

DISCUSSION

This study demonstrates the occurrence of both ET and constitutive endothelial NOs immunoreactivities in endothelial cells of lymphatic vessels. Endothelial cells isolated from lymphatic vessels also display such im-

munoreactivities in culture, thus suggesting that lymphatic endothelial cells maintain their capability to produce ET and NO *in vitro.*

It is of interest to note that we found specific immunostaining for ET and NOs only within endothelial cells of lymphatic vessels *in situ,* whereas both muscle cells and adventitial cells displayed no immunoreaction.

Our cytochemical data agree well with the hypothesis proposed by Ohhashi (1993) concerning the so-called ''active driving mechanism'' for lymph flow. According to this hypothesis, the lymphatic vessel has been suspected to produce vasoactive substances that modulate the vascular tone and contractile activity. Our findings suggest that lymphatic endothelial layer represents a source of powerful vasoactive molecules (e.g., ET and NO) and may play a pivotal role in self-regulation mechanisms that control the lymph flow in lymphatic vessels.

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