An immunohistochemical study of endothelial cell heterogeneity in the rat: observations in “en face” Häutchen preparations

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Summary. “En face” sheets of endothelium, in which cellular spatial relationships were maintained, were prepared from proximal pulmonary and femoral arteries and aortae of the Wistar rat, in order to visualise patterns of heterogeneity in populations of endothelial cells. These preparations, termed Häutchen, were immunolabelled with antibodies to angiotensin II, endothelin and Factor-VIII-related antigen, and visualised by an avidin/biotin peroxidase complex. Clusters of cells, which accounted for approximately 30% of the total endothelial cell population, and which were positively immunostained for angiotensin II, were found perpendicular to the longitudinal axis of the aorta and femoral artery. Cells in the pulmonary artery were immunonegative for angiotensin II. The majority of cells in all three vessels were immunopositive for endothelin; groups of intensely stained cells were present in both the femoral artery and aorta, but not in the pulmonary artery. Immunoreactivity to Factor-VIII-related antigen was heterogeneous, with intensely stained amorphous patches of endothelial cells present in the femoral artery and aorta. Häutchen preparations present an opportunity for the investigation of endothelial cell heterogeneity, both within and between vessels; this may provide a basis for the interpretation of the heterogeneity of endothelium-dependent responses in vessels of differing origin.

Key words: Angiotensin II – Endothelin – Factor VIII (von Willebrand factor) – Heterogeneity – Endothelium – Rat (Wistar)

It has become evident in the past few years that the control of local blood flow is effected by a dual process of transmitters released from perivascular nerves and by endothelium-derived relaxing (EDRF) factors (see Burnstock 1988; Lincoln and Burnstock 1990). The release of EDRF is consequent upon the occupation of receptors on endothelial cells by a variety of substances including acetylcholine (ACh), 5-hydroxytryptamine (5-HT), substance P (SP), and adenosine 5'-triphosphate (ATP) (Furchgott and Zawadski 1980; Furchgott 1981; De Mey et al. 1982; Cocks and Angus 1983). It is unlikely, in all except the smallest vessels, that transmitter released from perivascular nerves diffuses through the medial muscle coat and elastic tissue without degradation, to reach the endothelial cell receptor sites. The more likely source of these substances is platelets or the endothelial cells themselves.

Endothelial cells have the capacity to take up, synthesise and/or store and release mediators capable of modifying vascular tone. The active uptake of adenosine and its subsequent synthesis and storage as ATP has been reported (Pearson et al. 1978; Pearson and Gordon 1985; Catravas et al. 1988), and a sodium-dependent uptake mechanism for 5-HT has been demonstrated (Junod and Ody 1977; Robinson-White et al. 1981). Evidence also exists for the uptake of choline (Hamel et al. 1987) and angiotensin II (AgII) into endothelial cells (Ng and Vane 1967). Furthermore, the release of 5-HT, ACh, SP and ATP has been demonstrated from endothelial cells, both in culture and intact vessels during increased flow and hypoxia (Burnstock et al. 1988; Milner et al. 1989; Ralevic et al. 1990).

Immunohistochemical investigations at the ultrastructural level have localised choline acetyltransferase (ChAT), SP, 5-HT, vasopressin and AgII within subpopulations of endothelial cells (Parnavelas et al. 1985; Burnstock et al. 1988; Loesch and Burnstock 1988; Milner et al. 1989; Lincoln et al. 1990) and, recently, the presence of SP has been confirmed by high performance liquid chromatography (Linnik and Moskowitz 1989). Heterogeneity of endothelial-dependent responses between vessels of differing anatomical origin and from different species is well documented (Vanhouette and Miller 1985), and it seems likely from the immunohistochemical studies previously cited that there is hetero-
geneity in the localisation of vasoactive substances at the cellular level.

Therefore, the present project was undertaken to develop a preparation suited to the investigation of endothelial cell heterogeneity within and between vessels. Whole mounts of endothelial cell sheets, termed Häutchen preparations (Poole et al. 1958), were prepared from several vessels in the rat and were immunolabelled with antibodies to AgII and endothelin (ET), peptides possessing both vasoconstrictor and vasodilator properties dependent upon the vessel (Toda and Miyazaki 1981; Toda 1984; Katusic et al. 1984a, b; Yanagisawa et al. 1988; Warner et al. 1989). Localisation of antibody to Factor-VIII-related antigen (F VIII-RAg), generally accepted as a diagnostic marker of endothelial cells, was also carried out. These “en face” endothelial cell sheets allowed better visualisation of heterogeneity of patterns of immunolabelling than in previous studies.

Materials and methods

Twelve adult male Wistar rats were anaesthetised with sodium pentobarbitone (Sagatal 60 mg/kg i.p.), the heart exposed by a thoracotomy, the right atrium opened, and the animal perfusion-fixed via the left ventricle with 4% paraformaldehyde in 0.1 M phosphate buffer (3 x 5 min). Blood vessels were removed, cleaned and cut longitudinally. Strips of artery up to 1 cm in length were pinned out, endothelium uppermost, onto Sylgard 184 elastomer resin (BDH, Poole, UK) and immersion-fixed for a further 1 h.

Tissues were dehydrated and rehydrated to improve penetration of immunoreagents. Endogenous peroxidases were blocked with 0.3% H2O2 in methanol for 30 min. The samples were washed in 0.1 M phosphate buffer (20 min) and pre-incubated with normal goat serum (NGS) (1:50 in phosphate-buffered saline [PBS]+ 0.1% bovine serum albumin, globulin free) for 30 min to block non-specific binding of IgGs. Specimens were drained and incubated with the following primary antibodies: rabbit anti-AgII (Peninsula Labs., St. Helens, UK) at a dilution of 1:200 for 1 h at RT; rabbit anti-human endothelin (ET) 1 (Cambridge Research Biochemicals, Harston, Cambridge, UK) at a dilution of 1:200 for 1 h at RT or 1:1000 at 4°C overnight, and rabbit anti-human F VIII-RAg (Sera-Labs., Crawley Down, UK) at a dilution of 1:200 for 1 h at RT or 1:500 at 4°C overnight.

After washing with 0.1 M phosphate buffer (10 min), tissues were incubated with biotinylated secondary antibody for 30 min (VectaStain Elite ABC, Vector Labs., Peterborough, UK), washed again (10 min) and incubated for 30 min with the ABC horseradish peroxidase complex. Tissues were washed in 0.1 M phosphate buffer (15 min), then in 0.05 M TRIS buffer pH 7.6 (10 min), and incubated in 0.05% 3,3-diaminobenzidine tetra-hydrochloride substrate (DAB) in 0.05 M TRIS buffer (17 μl 30% H2O2 and 1 ml 8% NiCl2 added to 100 ml TRIS buffer). After washing in 0.05 M TRIS buffer (3 x 5 min), the tissues were dehydrated for Häutchen preparation.

Immunocytochemical controls

The specificity of the AgII antiserum was tested by pre-incubation with 10-5 M AgII (Peninsula Labs.). Preabsorption of the endothelin antibody was achieved with ET 1, 2 or 3. Pre-incubation with 10-5 and 10-3 M ET abolished nearly all immunostaining. Antisera to AgII, ET1 and F VIII-RAg were also replaced with normal goat and rabbit serum.

Häutchen preparation

The Häutchen procedure was essentially that described by Poole et al. (1958). The dehydrated vessels were removed from absolute alcohol and allowed to dry slightly. One drop of 4% pyroxylin (Agar Scientific, Stansted, Essex) in 50% methanol/ether was placed onto the endothelium and the vessel everted, endothelium down, onto a glass slide coated with 8% pyroxylin in 50% methanol/ether. Even pressure was applied with the thumb to adhere the vessel to the slide. Vessels were air-dried for 30 min, then immersed in 30% methanol for 1 h, removed and the muscle layers peeled away using fine forceps, leaving a sheet of endothelial cells embedded in the pyroxylin layer. Endothelial cells were mounted and photographed on an Olbrich 4000 high-definition microscope (Bayross International, Tokyo, Japan).

The morphological integrity and the maintenance of cellular relationships within Häutchen preparations of endothelial cell sheets were monitored using a silver-based stain that outlined cell borders (Nakatsu et al. 1988); haematoxylin was used as a nuclear stain.

Results

Morphology of the endothelium

Endothelial cell sheets from each vessel were consistent in appearance following perfusion-fixation (Fig. 1). However, the shape index of the endothelial cells (ratio of cell length to cell width) varied between the vessels. Endothelial cells from the pulmonary artery were less elongated in profile than those from either the aorta or the femoral artery. All cells were uninucleate and very few esquamated cells or denuded areas were visible. Intercellular junctions were uniform.

Angiotensin II

The pattern of immunolabelling of endothelial cells with antiserum to AgII was consistent with the results illustrated in Fig. 2. Analysis revealed three gradations of classification of immunostaining. One cellular population appeared to show little or no staining, another had staining confined to the perinuclear region, whereas a third showed staining extended on either one or both sides of the nucleus to produce cytoplasmic tails. When nuclei of the three cellular populations were differentially marked on a transparent overlay, which covered a montage of randomly generated micrographs, patterns of immunoreactive cells emerged. The patterns are represented diagrammatically in Fig. 3.

 Bands or clusters of cells with similar levels of immunoreactivity were apparent, usually occurring perpendicular to the longitudinal axis of the vessel, i.e. perpendicular to the blood flow. Analysis of 1660 endothelial cells from 5 rats, from randomly generated micrographs revealed that cells from the third category, i.e. with cytoplasmic tails of immunostaining, constituted 33% of the total endothelial cell population. Very occasionally, intensely immunoreactive cells, such as the one shown in Fig. 4 were observed.

In the femoral artery, immunoreactive cells with cytoplasmic tails accounted for a similar proportion of...