Secretion of endothelin-1 and endothelin-3 by human cultured vascular smooth muscle cells

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1 It is generally accepted that endothelial cells secrete endothelin (ET) to the underlying media which mediates the contractile effects of ET. However, there is some evidence that animal vascular smooth muscle cells (VSMCs) also secrete ET. We cultured VSMCs from human vessels representative of a number of different vascular beds to determine whether human VSMCs endogenously secrete ET.

2 VSMCs explanted from adult arterial vessels secrete picomolar quantities of immunoreactive mature ET: coronary artery 226.6 ± 58.8 pM/ 10^6 cells ($n = 7$), thoracic aorta 169.5 ± 105.4 pM/ 10^6 cells ($n = 3$), left internal mammary artery 102.4 ± 23.1 pM/ 10^6 cells ($n = 3$) and saphenous vein 69.4 ± 19.9 pM/ 10^6 cells ($n = 3$), as well as from umbilical vein (HUVSMCs) 38.3 ± 4.3 pM/ 10^6 cells ($n = 3$). Secretion of immunoreactive big ET-1 was also detected: coronary artery 249.1 ± 59.4 pM/ 10^6 cells ($n = 7$), thoracic aorta 120.0 ± 13.4 pM/ 10^6 cells ($n = 3$), left internal mammary artery 170.0 ± 68.2 pM/ 10^6 cells ($n = 3$), saphenous vein 105.1 ± 30.7 pM/ 10^6 cells ($n = 3$) and from umbilical vein 146.3 ± 7.4 pM/ 10^6 cells ($n = 3$). Comparable, intracellular levels of immunoreactive big ET-1 and mature ET were also detected in cultured VSMCs.

3 Since enzyme-dispersed VSMCs are thought to be more differentiated and more closely resemble their *in vivo* counterparts, and these enzyme-dispersed VSMCs from human umbilical vein (HUVSMCs) also secreted the greatest levels of immunoreactive peptides, they were characterized further. Reverse transcription-polymerase chain reaction assay demonstrated that HUVSMCs express ET-1 mRNA. High performance liquid chromatography coupled to radioimmunoassay revealed that HUVSMCs secrete ET-1 and ET-3, in addition to big ET-1. However, levels of ET are not altered by 100 μ M phosphoramidon, an inhibitor of metalloproteases or by 100 μ M pepstatin A, an aspartyl protease inhibitor.

4 In concordance, K_D and B_{max} values for [125 I]-ET-1 saturation binding are not altered in HUVSMC cultures incubated for 24 h with 100 μ M phosphoramidon (431 ± 218 pM and 31.1 ± 12.7 fmol mg^{-1} ; mean $\pm$ s.e.mean, $n = 3$) or 100 μ M pepstatin A (381 ± 169 pM and 19.9 ± 7.8 fmol mg^{-1} , $n = 3$) as compared to controls (355 ± 99 pM and 33.3 ± 9.3 fmol mg^{-1} ; $n = 3$). This observation indicates the absence of an autocrine 'unmasking' effect for ET receptors.

5 HUVSMCs synthesize and secrete immunoreactive ET-1, ET-3 and big ET-1, and possess intracellular levels of immunoreactive mature ET and big ET-1. There is some evidence of common cellular mechanisms between growth factors and vasoconstrictor peptides, suggesting a close relationship between contraction and proliferation. Since the development of various vascular pathologies such as atherosclerosis, hypertension and after vessel injury has been attributed to alterations in the normal growth pattern of VSMCs, the role of ET in these diseases may be of significance.

Keywords: Human vascular smooth muscle; endothelin secretion; endothelin converting enzyme; phosphoramidon; pepstatin A

Introduction

The endothelins (ET-1, ET-2 and ET-3) are 21 amino acid peptides with potent and protracted vasoconstrictor activity (Yanagisawa *et al.*, 1988) which are all expressed in the human cardiovascular system (Plumpton *et al.*, 1993). Endothelins were first isolated from cultures of porcine endothelial cells (Yanagisawa *et al.*, 1988) but endothelins and their precursor have since been shown to be secreted from human endothelial cells, renal epithelial cell lines, endometrial cells, bronchial epithelial cells, and pleural mesothelial cells (Huggins *et al.*, 1993, for review). In the vasculature, it has been postulated that ET-1 and its precursor, big ET-1, are predominantly secreted by endothelial cells to the underlying media. However, it has been observed that rat and rabbit vascular smooth muscle cells (VSMCs) also secrete various isoforms of mature ET (Kanse *et al.*, 1991). The induction of ET mRNA and peptide was also demonstrated in human omental VSMCs (Resink *et al.*, 1990).

Mature ETs are generated from big ET precursors by unusual proteolytic processing between Trp 73 and Val 74

with the possible involvement of an 'endothelin-converting enzyme' (ECE). Numerous conflicting theories have arisen regarding the identity of the ECE(s), but there is substantial evidence that the enzyme can be inhibited by phosphoramidon, a metalloproteinase inhibitor. Phosphoramidon blocks both exogenous and endogenous big ET-1 conversion in bovine aortic endothelial cells (Corder *et al.*, 1993) while phosphoramidon studies in rat *in vitro* provides evidence that big ET-1 conversion occurs outside the vessel lumen (Corder & Vane, 1994). The ECE in bovine and porcine smooth muscle also appears to be sensitive to phosphoramidon (Hioki *et al.*, 1991; Matsumura *et al.*, 1990; 1991; Tsukahara *et al.*, 1993). More recently, Murphy *et al.* (1994) compared the ECE potential of two phosphoramidon-sensitive peptidases, endopeptidase-24.11 and bacterial thermolysin. *In vivo* studies also favour a phosphoramidon-sensitive protease as the putative ECE. McMahon *et al.* (1991) demonstrated that phosphoramidon prevents the pressor activity of big ET-1 in both Wistar-Kyoto and spontaneously hypertensive rats. Lastly, big ET-1 conversion was shown to be inhibited by 12 mM phosphoramidon in human umbilical vein endothelial cells (HUVECs) which led to increased specific binding of radiolabelled ET-1 (Fujitani *et al.*, 1992). Thus, it has been

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hypothesized that autocrine ET secretion 'masks' ET receptors, so that inhibition of big ET-1 conversion would reveal more ET-1 receptors in binding studies.

Since it is hypothesized that ET secretion from the endothelium to the underlying media elicits the contractile effects of ET, the aim of our study was to determine whether endogenous secretion of ET occurs in VSMCs cultured from human vessels susceptible to abnormal VSMC function. Vessels representative of a number of different vascular beds were cultured to determine ET secretion in both adult and neonates, whose developing vasculature may be expressing a plethora of genes including the gene for ET, and from both arteries and veins, where the former requires stronger tonic contractions. VSMCs were cultured using the explant method or by enzyme dispersion. Since enzyme-dispersed HUVMSCs produced the highest levels of peptides, and are thought to resemble more closely their *in vivo* counterpart, these were further characterized: ET-1 messenger RNA was detected in HUVMSCs using reverse transcription and polymerase chain reaction. High performance liquid chromatography (h.p.l.c.) coupled to radioimmunoassay (RIA) was used to identify the mature ET isoform secreted, and levels of mature ET secretion were determined in the presence and absence of phosphoramidon, or pepstatin A. Finally, the effect of phosphoramidon and pepstatin A on K_D and B_{max} values for ET-1 binding were determined. A preliminary account of this work has been presented to the British Pharmacological Society (Yu & Davenport, 1994).

Methods

Tissue collection

Untraumatized human umbilical veins were collected within 24–48 h post-parturition from the Rosie Maternity Hospital, Cambridge. Epicardial coronary arteries and thoracic aortae were obtained from heart transplant patients, while left internal mammary arteries and saphenous vein were obtained from patients undergoing bypass surgery, at Papworth Hospital, Cambridge.

Cell culture

Vessels were opened transversely, the endothelium removed by gently scraping the intimal surface with a scalpel blade, and cut into approximately 0.5 cm² portions. To obtain HUVMSC cultures by enzyme dispersion, the endothelium was removed from veins of untraumatized human umbilical cords by an initial enzyme dispersion with collagenase type II at 1 mg ml⁻¹ (Sigma Chemical, Dorset, U.K.). The endothelial cells (HUVECs) were cultured for subsequent comparison during immunocytochemistry and fluorescence-activated cell sorting. After the removal of endothelial cells, a subsequent enzyme dispersion was performed to obtain smooth muscle cells as previously described (Gimbrone & Cotran, 1975). All VSMC cultures were maintained in complete medium (Medium 199 (Gibco BRL, Paisley, Scotland) supplemented with 50 iu ml⁻¹ penicillin, 50 µg ml⁻¹ streptomycin, 2.5 µg ml⁻¹ amphotericin B) containing 15% heat-treated foetal calf serum (Seralab, Sussex, U.K.). Culture medium was changed two to three times per week. All cell growth occurred in a humidified incubator at 37°C in which the atmosphere was equilibrated with 5% CO₂.

Characterization of VSMC cultures

The cultured VSMCs displayed a 'hill-and-valley' morphology typical of smooth muscle cells. Since HUVMSCs were used for further studies, these cultures were further characterized by immunocytochemistry and fluorescence-activated cell sorting (FACS).

For immunocytochemical studies, HUVECs and HUVMSCs were stained for von Willebrand factor (vWF) (Dako, High Wycombe, Buckinghamshire, U.K.) and for smooth muscle α -actin (Sigma). Secondary antibodies conjugated to fluorescein isothiocyanate (FITC) or the peroxidase anti-peroxidase immune complex method, was employed to visualize the staining patterns. Propidium iodide was used to counterstain nuclei. Immunoreactive (IR) vWF was distributed on endothelial cells in a typical punctate pattern, but endothelial cells did not stain for α -actin. Conversely, IR vWF was not detectable on HUVMSCs while α -actin was distributed as a filamentous network.

FACS was used to confirm that contamination of smooth muscle cell cultures by endothelial cells was not detectable. Single cell suspensions of HUVMSCs and HUVECs were obtained by trypsinization. The cell suspensions were stained for 15 min on ice, with primary rabbit antibodies against human vWF. The cells were washed twice with PBS, centrifuged and re-suspended. The suspensions were then stained for 15 min on ice, with goat anti-rabbit secondary antibodies conjugated to fluoroisothiocyanate. Cells were analysed for fluorescence with FACSsort (Becton-Dickinson, Plymouth, U.K.). Results were further analysed using Lysis II (Becton-Dickinson) to determine that less than 1% of HUVMSCs stained for vWF.

Molecular biology

HUVMSCs were grown to confluence in gelatin-coated 75 cm² flasks (Galcon, Cowley, Oxford, U.K.). Total RNA was isolated by a guanidinium thiocyanate/isopropanol method with minor modifications (Chomczynski & Sacchi, 1987). Reverse transcription coupled with polymerase chain reaction was carried out as previously described (Nunez *et al.*, 1990). cDNA was generated using 10 µg of total RNA as template for hexanucleotide random primers, in a reaction mixture containing reverse transcription buffer (50 mM Tris-HCl pH 8.3, 10 mM MgCl₂, 75 mM KCl, 10 mM dithiothreitol, 0.1 mg ml⁻¹ BSA), deoxynucleotides, 38 u human placental ribonuclease inhibitor (Pharmacia Fine Chemicals, Hertfordshire, U.K.), and 32 u cloned murine Maloney leukaemia virus reverse transcriptase (Pharmacia Fine Chemicals). Resulting cDNA (2.5 µl volume) was amplified using two specific human ET-1 primers (sense: 5'-TGC TCC TGC TCG TCC CTG ATG GAT AAA GAG TGT GTC; anti-sense: 5'-GGT CAC ATA ACG CTC TCT GGA GGG CTT). The reaction mixture contained PCR buffer (Cambio, Cambridge, U.K.), 0.2 mM dNTPs, 25 pmol of each primer, and 1 u of cloned Taq polymerase (Cambio). PCR was performed for 40 cycles, with each cycle consisting of 2 min denaturation at 95°C, 45 s for primer annealing at 60°C and 2.5 min extension time at 72°C, with the final extension occurring for 10 min at 72°C. The PCR products were separated by electrophoresis on a 1% agarose gel and DNA fragment sizes were estimated by reference to a 1 kb DNA molecular weight standard.

Reverse phase high performance liquid chromatography and radioimmunoassay

Supernatants were solid-phase extracted as previously described (Davenport *et al.*, 1990). Briefly, ethyl silica minicolumns (Amprep C2; 500 mg; Amersham International plc, Amersham) were equilibrated by washing with methanol (2 ml). Supernatants collected from confluent cultures of HUVMSCs were acidified by the addition of 2 M HCl (1:0.25 ml, v v⁻¹), clarified by centrifugation for 10 min at 10,000 g and loaded onto the column. Each column was washed with 5 ml of 0.1% (v v⁻¹) trifluoroacetic acid and eluted with 0.1% (v v⁻¹) trifluoroacetic acid in 80% methanol. The eluant was dried down under vacuum centrifugation into polypropylene tubes and retained for radioimmunoassay.

To determine secreted levels of big ET-1 and mature ET, and to determine intracellular levels, supernatants of cultures were collected and the cells were washed twice with PBS. Cells were then trypsinized to obtain estimates of cell number in a haemocytometer. Cell membranes were permeabilized with 0.1% Triton X-100. The preparations were spun at 4,000 g for 5 min and the resulting supernatants were retained. These supernatants and the culture supernatants were directly radioimmunoassayed for mature ET and big ET-1 as previously described (Plumpton *et al.*, 1992). Briefly, the mature ET antisera were raised against the C-terminus of ET-1 [15-21] and showed 100% cross-reactivity with ET-2 and ET-3. Antisera against big ET-1 was raised against the C-terminus [31-38] and showed <0.007% cross-reactivity with the mature peptides (ET-1, ET-1 and ET-1), big ET-2 and big ET-3. The sensitivity of detection of both assays was 0.4 fmol/tube. The inter- and intra-assay variations were <13% in all cases.

To determine the effect of 100 μ M phosphoramidon (Scientific Marketing Ass., Barnet, U.K.) or 100 μ M pepstatin A (Sigma) on secretion of mature ET, HUVMSCs were incubated for 12 h in the absence or presence of either inhibitor. Culture medium was collected at designated intervals and directly assayed for big ET-1 and mature ET.

Saturation binding

HUVMSCs were plated onto a collagen-coated Costar 96-well strip plate with low evaporation lid (Cambridge, MA, U.S.A.) and grown to confluence. A Titertek Microplate Washer (ICN/Flow, High Wycombe, U.K.) was used to wash the cells. Saturation binding for HUVMSCs was carried out in the absence and the presence of 100 μ M phosphoramidon or 100 μ M pepstatin A for 24 h, using [125 I]-ET-1 (Amersham International, Little Chalfont, U.K.) as previously described (Davenport *et al.*, 1993). Dissociation constants and receptor density were estimated using EBDA (McPherson, 1983) and an iterative non-linear curve-fitting programme LIGAND (Munson & Rodbard, 1980).

Protein determinations

Protein concentrations of washed cells were estimated after solubilization (0.5 M NaOH and 1% sodium dodecyl sulphate) for 5 min at room temperature. The Bio-Rad Detergent Compatible 96-well microtiter plate method (BioRad Laboratories, Hertfordshire, U.K.) was used with bovine serum albumin as a reference standard.

Statistical analysis

There was no evidence for non-normality of the data using the Shapiro and Francia probability plot correlation test. RIA data and B_{max} values were analysed by one-way analysis of variance followed by Student's *t*-test for comparison of RIA data at each time point, $P < 0.05$. Results are given as mean $\pm$ s.e.mean.

Results

Secretion of big ET-1 and mature ET from explanted VSMCs

Cultures of explanted VSMCs from adult human vessels secreted comparable levels of immunoreactive ET: coronary artery 226.6 ± 58.8 pM/ 10^6 cells ($n = 7$), thoracic aorta 169.5 ± 105.4 pM/ 10^6 cells ($n = 3$), left internal mammary artery 102.4 ± 23.1 pM/ 10^6 cells ($n = 3$), and saphenous vein 69.4 ± 19.9 pM/ 10^6 cells ($n = 3$), as well as from umbilical vein (HUVMSCs) 38.3 ± 4.3 pM/ 10^6 cells ($n = 3$); each value represents mean $\pm$ s.e.mean of n different cultures. Secretion of immunoreactive big ET-1 was also detected: coronary

Figure 1 Levels of immunoreactive mature endothelin (ET) (open columns) and immunoreactive big ET-1 (hatched columns) secreted from VSMCs over a 72 h period, as determined by radioimmunoassay in (a) coronary artery, $n = 7$, (b) aorta, $n = 3$, (c) left internal mammary artery, $n = 3$, (d) saphenous vein, $n = 3$ and (e) umbilical vein, $n = 3$. Each value represents mean $\pm$ s.e.mean and is standardized to pM per 10^6 cells.

Figure 2 Intracellular levels of immunoreactive mature endothelin (ET) (open columns) and immunoreactive big ET-1 (hatched columns) in VSMCs over a 72 h period, as determined by radioimmunoassay in (a) coronary artery, $n = 7$, (b) aorta, $n = 3$, (c) left internal mammary artery, $n = 3$, (d) saphenous vein, $n = 3$ and (e) umbilical vein, $n = 3$. Each value represents mean $\pm$ s.e.mean and is standardized to pM per 10^6 cells.

artery 249.1 ± 59.4 pM/ 10^6 cells ($n = 7$), thoracic aorta 120.0 ± 13.4 pM/ 10^6 cells ($n = 3$), left internal mammary artery 170.0 ± 68.2 pM/ 10^6 cells ($n = 3$), saphenous vein 105.1 ± 30.7 pM/ 10^6 cells ($n = 3$) and umbilical vein 146.3 ± 7.4 pM/ 10^6 cells ($n = 3$); each value represents mean $\pm$ s.e.mean of n different cultures (Figure 1). The ubiquitous secretion of immunoreactive big ET-1 and mature ET from the VSMC cultures indicate that secretion was not restricted to foetal or arterial or pathophysiological SMCs. In comparison to secretion from VSMCs, endothelial cells cultured from umbilical vein secreted higher levels of ET (1469 ± 217 pM/ 10^6 cells, $n = 3$) and big ET-1 (564 ± 199 pM/ 10^6 cells, $n = 3$); each value represents mean $\pm$ s.e.mean. As demonstrated in Figure 2, intracellular irET in addition to irbig ET-1 was detected from all VSMC explant cultures by RIA: coronary artery irET: 166.6 ± 33.5 irbigET-1: 132.2 ± 44.3 pM/ 10^6 cells ($n = 7$), thoracic aorta irET: 30.0 ± 4.3 irbigET-1: 50.6 ± 7.5 pM/ 10^6 cells ($n = 3$), left internal mammary artery irET: 42.0 ± 16.8 irbigET-1: 119.2 ± 29.2 pM/ 10^6 cells ($n = 3$), saphenous vein irET: 15.2 ± 6.5 irbigET-1: 52.5 ± 6.5 pM/ 10^6 cells ($n = 3$), and umbilical vein irET: 17.8 ± 6.8 irbigET-1: 126.4 ± 20.7 pM/ 10^6 cells ($n = 3$); each value represents mean $\pm$ s.e.mean. Comparable intracellular levels of both peptides were also detected within endothelial cells cultured from umbilical vein: ET: 122 ± 35 pM/ 10^6 cells and big ET-1: 149 ± 62 pM/ 10^6 cells, $n = 3$; each value represents mean $\pm$ s.e.mean. We noted that for HUVMSCs obtained by enzyme-dispersion, levels of ET and big ET-1 were more abundant (for example $n = 1$, 657 pM/ 10^6 cells and 124 pM/ 10^6 cells, respectively), so these cultures were characterized further.

Figure 3 RNA extracted from two separate cultures of human umbilical vein smooth muscle cells was reverse transcribed and amplified by polymerase chain reaction using primers specific for endothelin-1 (ET-1). Product were separated on a 1% agarose gel. Arrowheads in lanes 2 and 3 indicate bands of cDNA from two separate cultures amplified for ET-1, at the expected fragment size (461 base pairs) in comparison to the 1 kb ladder in lane 1. Low molecular weight bands represent primer dimerisation and have been observed previously (Nunez *et al.*, 1990).

Characterization of enzyme-dispersed HUVMSCs

Presence of ET-1 mRNA HUVMSCs expressed RNA for ET-1. Figure 3 indicates bands of ET-1 cDNA from two different HUVMSC cultures, at the expected fragment size (461 base pairs) in comparison to the 1 kb ladder in lane 1. The presence of low molecular weight bands represent dimerisation of primers and has been observed previously (Nunez *et al.*, 1990).

H.p.l.c.-RIA to identify ET isoforms HUVMSCs obtained by enzyme dispersion secreted significant amounts of both mature ET and big ET-1 in a time-dependent manner. Levels of mature irET were nearly double that of big ET-1 by 12 h. High performance liquid chromatography coupled to RIA resolved ET-1, ET-2, ET-3 and big ET-1 with baseline separation, and was validated using both u.v. (214 nm) and RIA detection (10^{-5} M and 10^{-8} M mixtures, respectively) on separate, but batch matched, C_{18} columns. Blank runs showed no evidence of carryover of immunoreactivity from either standard or sample runs. Retention times obtained from u.v. and RIA columns were identical (Figure 4, a and b). ET-3, ET-1 and big ET-1 were detected in supernatants from three separate HUVMSC cultures. Figure 4c demonstrates an example of immunoreactivity from HUVMSC

Figure 4 Identification of endothelin (ET) isoforms present in supernatants of enzyme-dispersed human umbilical vein smooth muscle cells (HUVMSCs) using high performance liquid chromatography coupled to radioimmunoassay (RIA). Standard runs on (a) u.v. column (214 nm) and (b) RIA column demonstrate the resolution of mature peptides ET-3, ET-1 and ET-2 respectively, followed by big ET-1. (c) Retention times for supernatants from a representative culture of collagenased HUVMSCs ($n = 3$) indicate peaks co-eluting with authentic ET-3, ET-1 and big ET-1.

Table 1 Levels of immunoreactive big endothelin-1 (ET-1) and immunoreactive mature ET detected by radioimmunoassay in supernatant of collagenase-dispersed human umbilical vein smooth cells at 4 h intervals over a 12 h incubation period

Time (h)	Control		+ Phosphoramidon	
	irbig ET-1	irET	irbig ET-1	irET
4	392.9 ± 56.7	470.7 ± 59.0	493.0 ± 78.4	472.5 ± 91.4
8	750.5 ± 64.3	1023.4 ± 132.6	1024.5 ± 106.3	1013.3 ± 193.3
12	991.4 ± 145.6	1699.7 ± 182.4	1288.6 ± 132.6	1205.0 ± 186.2

Each value is expressed as picomol litre⁻¹ and represents the mean ± s.e.mean of 3 different cultures with triplicate wells measured for each time-point. In comparison to controls, 100 µM phosphoramidon does not significantly affect secretion of either peptide ($P > 0.05$).

Table 2 [¹²⁵I]-endothelin-1 ([¹²⁵I]ET-1) saturation binding of enzyme-dispersed human umbilical vein smooth muscle cells incubated with control medium, or 100 µM phosphoramidon, or 100 µM pepstatin A for 24 h

	n	K _D [pM]	B _{max} (fmol mg ⁻¹)
Control	6	355 ± 99	33.3 ± 9.3
Phosphoramidon	3	431 ± 218	31.1 ± 12.7
Pepstatin A	3	381 ± 169	19.9 ± 7.8

Each value represents the mean ± s.e.mean of 3–6 different cultures. In comparison to controls, 100 µM phosphoramidon or 100 µM pepstatin A did not significantly affect the dissociation constant or receptor density of human umbilical vein smooth muscle cells ($P > 0.05$). The latter observation indicates the absence of an 'unmasking' effect for autocrine ET receptors, which supports the observation that neither phosphoramidon nor pepstatin A affected the conversion of big ET-1.

supernatant co-eluting with authentic ET-3, ET-1 and big ET-1.

Effect of phosphoramidon or pepstatin A on ET secretion

Radioimmunoassay demonstrated that secretion of immunoreactive ET was not inhibited by 12 h incubation with 100 µM phosphoramidon ($n = 3$ different cultures, triplicate wells for each time-point, protein = 75–82 µg per well) (Table 1). Similarly, ET secretion was not affected after incubation with 100 µM pepstatin A for up to 12 h when assayed at time intervals parallel to those of phosphoramidon. For example, at 12 h control cultures secreted 387 ± 57 pM (mean ± s.e.mean of $n = 3$ different cultures) irET, while cultures incubated with 100 µM pepstatin A secreted 352 ± 58 pM (mean ± s.e.mean of $n = 3$ different cultures) irET.

Saturation binding for ET-1 receptors and effect of phosphoramidon or pepstatin A

Saturation binding studies with [¹²⁵I]-ET-1 indicated that HUVSMCs possess a single population of receptors for ET-1 with a dissociation constant in the picomolar range. K_D and B_{max} values for ET-1 binding were not significantly altered in HUVSMC cultures incubated for 24 h with 100 µM phosphoramidon or 100 µM pepstatin A as compared to controls (Table 2). These results are in concordance with the observations that neither phosphoramidon nor pepstatin A have any effect on ET secretion, indicating that the putative ECE inhibitors failed to 'unmask' ET binding sites.

Discussion

We have shown by specific and sensitive RIAs that cultured human VSMCs are able to synthesise endogenously and secrete immunoreactive mature ET and big ET-1. VSMCs cultured from both adult and neonatal vessels, in addition to cells from both arteries and veins, were able to express ET peptides. There was however, a difference in levels of peptide secretion between VSMCs obtained by the explant method and those obtained by enzyme dispersion. The latter is proposed by Chamley-Campbell *et al.* (1979) to be more

differentiated and more closely resemble their *in vivo* counterparts.

Parallel to the findings of Kanse *et al.* (1991) who used cultures of rabbit VSMCs, we have shown in this study that HUVSMCs secrete ET-1, ET-3 and big ET-1. In contrast, messenger RNA for ET-1 and ET-2 only, was detected by reverse transcription-polymerase chain reaction in endothelial cells (O'Reilly *et al.*, 1993). It is thought that expression of ET-1 peptide only, occurs from endothelial cells (Chabrier *et al.*, 1993). However, to exclude the possibility of contamination by endothelial cells, cultures were characterized as smooth muscle by three methods: (a) their 'hill and valley' morphology typical of smooth muscle cell growth; (b) positive staining with antibodies against α -actin and concomitant absence of staining for vWF; (c) confirmation of the absence of vWF by fluorescence activated cell sorting.

The mechanism of ET synthesis was investigated using both phosphoramidon, an inhibitor of metalloproteinases and pepstatin A, an aspartyl protease inhibitor, both of which have been widely used to inhibit the putative endothelin converting enzymes (ECE). We have shown that neither phosphoramidon nor pepstatin A affect endogenous conversion of big ET-1 in HUVSMCs. Consequently, there was no change in receptor density which would have been observed by the 'unmasking' of ET receptors. In cultures of porcine aortic endothelial cells, Ikegawa *et al.* (1991) and Matsumura *et al.* (1992) demonstrated that conversion of both endogenous big ET-1 and exogenously applied big ET-1, is inhibited by phosphoramidon. In HUVECs, conversion of endogenous big ET-1 is also inhibited by phosphoramidon (Plumpton *et al.*, 1994). In contrast, whilst in VSMCs from porcine aorta, Ikegawa *et al.* (1990) showed that phosphoramidon inhibits conversion of exogenous big ET-1, it does not affect endogenous ET-1, production. It appears then, that VSMCs possess an ECE for endogenous ET-1 production which is phosphoramidon-insensitive, while the enzyme for exogenous big ET-1 cleavage is sensitive to phosphoramidon. This is in agreement with our findings. However, we cannot exclude the possibility that the ECE in HUVSMCs is intracellularly located and simply inaccessible to phosphoramidon, since phosphoramidon is a phosphorylated sugar derivative and thought unlikely to enter cells efficiently (Turner *et al.*, 1993). The presence of intracellular immunoreactive ET is of interest and may represent newly generated ET en route to the plasma membrane for secretion.

The precise physiological or pathophysiological significance of ET secretion from human VSMCs is not yet established. In undiseased, intact vessels, such as saphenous vein and left internal mammary artery, we previously employed immunocytochemistry to localize mature ET, big ET-1 and big ET-2 to the cytoplasm of endothelial cells with no detectable staining in medial smooth muscle cells (Howard *et al.*, 1992) suggesting very low levels of ET synthesis, if any, in normal VSMCs. However, Lerman *et al.* (1991) demonstrated the presence of irET in medial VSMCs of atherosclerotic plaques. Since proliferating VSMCs *in vitro* develop cytoskeletal features similar to those observed in normal foetal, or pathological VSMCs (Skalli *et al.*, 1986; Campbell & Campbell, 1990), the use of cultured VSMCs may prove a useful model to study the pathogenesis and progression of vascular diseases. In view of the absence of ET in the media of normal vessels and the presence of ET in atheromatous VSMCs, our report that cultured human VSMCs synthesize and secrete ET suggests that under pathophysiological conditions, VSMCs may 'induce' ET synthesis.

Development of vascular pathologies in established hypertension, arteriosclerosis, and after vessel injury has been attributed to alterations in the normal growth pattern of VSMCs (Folkow, 1990). A close relationship between VSMC proliferation and contraction has been suggested on the basis of recent observations that growth factors and vasoconstrictor peptides exhibit several common cellular mechanisms of action (Berk & Alexander, 1989). However, most vascular pathologies are accompanied by alterations in VSMC function such as hyperplasia, and it is difficult to elucidate whether the induction of ET secretion enhances VSMC growth, or whether ET induction is secondary to VSMC proliferation. It is clear, however, that rapidly proliferating

VSMCs also secrete ET. The proliferation of VSMCs and induction of ET could be attributed to the selection of a specific population of cells, or due to a loss of inhibitory factors for ET induction and/or proliferation from the overlying endothelium such as heparin/heparan sulphates, TGF- β , nitric oxide and prostacyclin. Increases in blood flow and shear stress are also known to downregulate induction of preproET-1 mRNA (Yanagisawa *et al.*, 1988) and VSMC proliferation. The absence of flow and shear stress in culture conditions may serve to upregulate ET synthesis from VSMCs and enhance their proliferative rate. The isolation of blood vessels in itself, stimulates a growth response, possibly due to longitudinal and radial collapse of the vascular wall upon withdrawal of transmural pressure (De Mey *et al.*, 1993) which may also contribute to the induction of ET. Regardless of the mechanism, the induction of ET from proliferating VSMCs may serve as a useful model to study the pathogenesis and progression of vascular pathologies whose hallmark is abnormal VSMC phenotype, growth and function.

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