Modulation of endothelin receptor expression in human vascular smooth muscle cells by interleukin-1β

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Abstract Endothelin may play a role in atherosclerosis as it causes smooth muscle cell proliferation and its levels are elevated in patients with atherosclerosis. We report that interleukin-1β is a potent inducer of endothelin receptor expression in cultured human vascular smooth muscle cells. The effect is dose- and time-dependent and is due to an increase in receptor number. Endothelin receptor mRNA levels are also elevated. Interleukin-1β is a major regulator of endothelin release so, together with its effects on endothelin receptors, it may be responsible for a generalized activation of the endothelin system in diseased vessels.

Key words: Endothelin receptor; Interleukin-1β; Smooth muscle cell; Atherosclerosis

1. Introduction

Endothelin-1 (ET) is a potent vasoconstrictor synthesized mainly by endothelial cells [1], which also mediates a number of other effects including cell proliferation. ET is a mitogen for transformed mouse fibroblasts [2,3] and rat vascular smooth muscle cells (VSMC) in monolayer cultures [4]. Its effects on VSMC proliferation were particularly potent in organ cultures of rat arterial segments [5]. It also enhances restenosis and intimal hyperplasia after balloon catheter injury of rat artery [6,7], a widely used animal model in which the arterial wall is denuded of its endothelium, showing that ET is mitogenic for VSMC in vivo. In addition to its mitogenic effects on VSMC, ET can also cause vessels to become prone to vasospasm [8], depriving tissue of blood supply. Levels of ET are elevated in the plasma of patients with atherosclerosis and in the atherosclerotic plaques themselves [9,10] and some evidence suggests that ET receptors are more abundant in plaques than in normal vascular wall tissue [11]. This suggests that ET and its receptors may play some role in the pathogenesis of atherosclerosis and that the regulation of expression of these molecules is therefore important to an understanding of this condition. ET receptor expression in cultured rat VSMC is enhanced by cAMP [12] and reduced by dexamethasone [13], and is modulated by certain growth factors in human VSMC [14]. We have tested three cytokines, interleukin-1β (IL-1β), transforming growth factor-β (TGF-β), and tumour necrosis factor-α (TNF-α) for their possible regulatory effects on ET_A subtype-receptor (ET_A-R) expression. All of these cytokines are present in atherosclerotic plaques [15] though they are not normally present in healthy artery. Initial results showed that IL-1β elevated [^{125}I]ET binding to all VSMC lines tested and so our study concentrated on this cytokine.

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2. Materials and methods

2.1. Cell culture

Four human VSMC lines designated A, B, C and D were isolated from human aorta obtained during transplant operations as previously described [16], and cultured in Dulbecco's modified Eagle's medium, DMEM, (Sigma, Poole, UK) supplemented with 10% v/v heat-inactivated foetal calf serum (FCS) (Seralab, Crawley Down, UK), 100 units/ml penicillin, 100 μg/ml streptomycin and 10 mM HEPES (all from Sigma).

2.2. Receptor binding assays

For [^{125}I]ET binding experiments, cells were plated into 48-well clusters (Costar, High Wycombe, UK) at a density of 4×10^4 cells per well, incubated in culture medium for three days, then growth-arrested for 48 h in growth-arrest medium (DMEM/0.5%FCS) prior to the assay. Agonists were added 24 h before the assay except for time course experiments in which cells were pre-treated with IL-1β for between 2 and 48 h. Radio-receptor assays were done by washing the cells once with binding buffer (Earle's balanced salts (EBSS) plus 0.2% bovine serum albumin (Sigma) and 10 mM HEPES), then incubating for 90 min in binding buffer plus [^{125}I]ET (NEN, USA; 2200 Ci/mmol) at a concentration of 80 pM at 37°C in a 5% CO_2 incubator. Non-specific binding was measured by adding excess unlabelled ET (Bachem, Torrance, CA, USA) to a final concentration of 1μM. After incubation, the wells were washed once with binding buffer, then with 1 M NaOH to solubilize the bound radioactivity. For Scatchard analysis the concentration of radiolabel was varied over a range of 9–285 pM.

2.3. Preparation of RNA

Total RNA was prepared from 75 cm^2 flasks of cultured cells by lysis with guanidine isothiocyanate buffer followed by centrifugation over a CsCl cushion [17]. RNA was found to be intact by electrophoresis on agarose/formaldehyde gels and concentrations were estimated by measuring absorbance at 260 nm.

2.4. RT/PCR

2.4.1. cDNA synthesis. For each reaction, 1 μg of RNA was incubated at 37°C in 20 μl RT buffer (Promega, Southampton, UK) containing RNAsin, 0.1 μg random hexamer primers and 2 units of MMLV reverse transcriptase (all from Promega). After 1 h, the reaction was terminated by heating to 94°C for 5 min. The volume was then diluted to 100 μl with double-distilled water and 10 μl of this solution was used for the PCR.

2.4.2. Polymerase chain reaction. Reactions were carried out in a thermal cycler (Cetus-Perkin Elmer, USA) in a final volume of 50 μl of Taq buffer (Promega) containing dNTPs at 0.2 mM each, 200 ng of each primer and 2.5 units of Taq polymerase (Promega). Each cycle consisted of 1.5 min denaturation at 94°C, 1 min annealing at 62°C and 1.5 min primer extension at 72°C. Two sets of primers were used, both of which have been used and reported elsewhere, one set specific for ET_A-R [18] and the other set specific for glyceraldehyde-3-phosphate dehydrogenase (G-3-PDH) as a control for the cDNA synthesis reactions [19]. The sequences are: ET_A forward: CCTTTTGATCACAAT-

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GACTTT (bp 439–459); ET_A reverse: TTTGAT GTGGCATTGAG-CATACAG (bp 737–714); G-3-PDH forward: TGAAGGTCGGAG-TCA ACGGATTTGGT (bp 71–96); G-3-PDH reverse: CATGTGG-GCCATGAGGTCCACCAC (bp 1053–1030). Reactions primed with ET_A-R specific oligos were incubated for 33 cycles and those primed with G-3-PDH oligos were given 28 cycles. After PCR, 10μl aliquots of each reaction mixture were electrophoresed on a 2% agarose gel and stained with ethidium bromide.

2.5. Cell proliferation assays

Cell proliferation in response to agonists was estimated by cell counting and by [^{3}H]thymidine incorporation. For cell counting assays, cells were plated in 24-well clusters (Falcon, Oxford, UK) at 4×10^4 per well, cultured in DMEM/10% FCS for 48 h then growth arrested for 48 h before the addition of ET. IL-1β or forskolin were added for the final 24 h of the growth arrest period. The cells were then washed with growth-arrest medium and incubated with 1 nM ET in the same medium for 3 days or, for control cells, in growth arrest medium only. The cells were then harvested from each well using trypsin/EDTA and counted using a haemocytometer.

2.6 [^{3}H]Thymidine incorporation assays

Cells were plated into 96-well clusters (Falcon) at 1×10^4 cells per well, cultured for 3 days in DMEM/10% FCS then growth-arrested for 48 h. IL-1β was added for the last 24 h of the growth arrest period. The cells were then stimulated for 24 h with agonists in serum-free medium followed by a further 24 h with the addition of 1 μCi/well [^{3}H]thymidine (5 Ci/mmol) (Amersham, Bucks, UK). The cells were then trypsin/EDTA-treated and the DNA harvested using a cell harvester (Brandel, MD, USA) as described previously [16].

3. Results

3.1. ET-1 binding experiments

Up to four different human VSMC lines designated A, B, C and D were used to investigate the effects of pre-treatment with three cytokines: IL-1β, TGF-β and TNF-α on levels of [^{125}I]ET binding to cultured cells. As a positive control 20 μM forskolin, which is known to increase levels of ET receptor mRNA [12] increased [^{125}I]ET binding in all of the cell lines tested between 1.3- and 2.1-fold (data not shown). In all of these lines IL-1β was found to increase the amount of [^{125}I]ET which bound to the cells by 2.3- to 5.3-fold (Table 1), or around double the range of values found for forskolin. Neither TGF-β nor TNF-α were found to significantly elevate [^{125}I]ET binding in all of the cell lines tested although TNF-α increased binding quite strongly in one of them (Fig. 1). Thus, IL-1β was considered the most significant regarding ET receptor expression. [^{125}I]ET binding was found to increase within 4 h of addition of IL-1β and reached a maximum after 24 h (Fig. 2).In a separate experiment, when IL-1β was removed after 24 h of treatment and replaced with ordinary medium, [^{125}I]ET binding was found to remain elevated for at least 48 h, remaining at 3.5(± 1.1)-fold above control levels at 48 h after an initial 5.5(± 0.3)-fold increase. The effect of IL-1β pre-treatment upon [^{125}I]ET binding was dose-dependent and was detected at IL-1β concentrations as low as 10 pg/ml (Fig. 3). Scatchard analysis showed that the effect was due to an increase in the number of binding sites per cell with no significant change in K_d (Fig. 4). For cells pre-treated 24 h with 10 ng/ml IL-1β (open squares), B_{max} = 4.5 fmol/10^4 cells and K_d = 55 pM; for untreated cells (full squares) B_{max} = 0.9 fmol/10^4 cells and K_d = 31 pM.

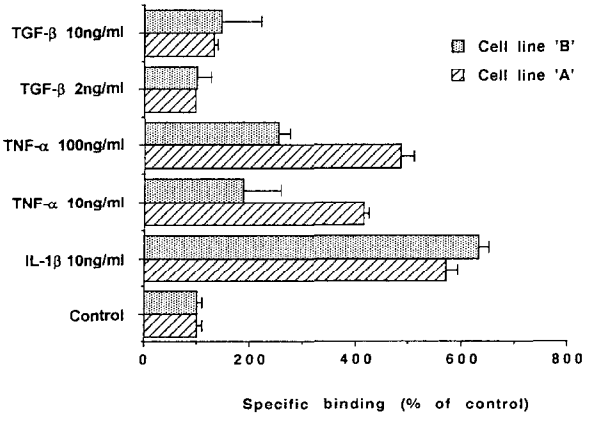

Fig. 1. Effect of IL-1β, TNF-α and TGF-β on specific [^{125}I]ET binding to cell lines A and B. Confluent monolayers of cells were transferred to growth-arrest medium 48 h before the assay. Cytokines were added 24 h prior to the assay. Each bar represents the specific binding as mean ± S.E.M. of 3 wells.

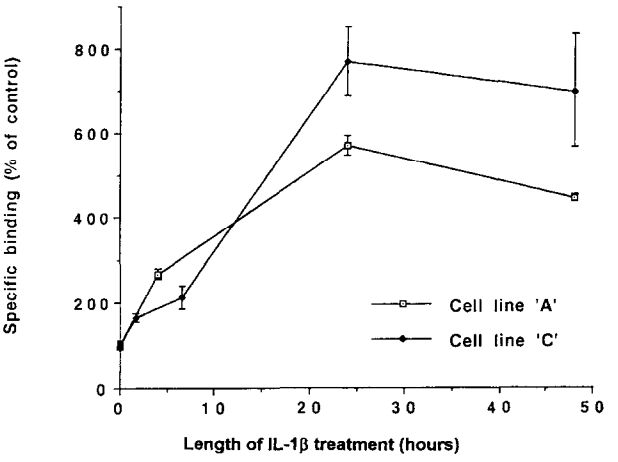

Fig. 2. Specific [^{125}I]ET binding to cell lines A and C after various lengths of IL-1β treatment. Cells were cultured in 48-well plates and growth arrested as described. 10 ng/ml of IL-1β was added between 0 and 24 h prior to the receptor binding assay. Each point represents the specific binding as mean ± S.E.M. of 3 wells.

3.2. Cell proliferation studies

In monolayer cultures ET has been shown to be mitogenic for VSMC from some sources but not all. We investigated the possibility that pre-treatment with IL-1β or with forskolin, which raises intracellular cAMP levels, followed by ET treatment would lead to cell proliferation. 24 h of pre-treatment with 10 ng/ml IL-1β or 20 μM forskolin followed by incubation with 1μM ET for 3 days did not lead to an increase in cell number. In each case cell numbers remained at between 90% and 105% of the control values. When cells were pre-treated with IL-1β for 24 h followed by 24 h treatment with 1 μM ET in the presence of [^{3}H]thymidine no increase in thymidine incorporation above that obtained with IL-1β alone was observed (data not shown).

3.3. Estimation of ET_A-R mRNA

In preliminary binding studies with ET isopeptides we established that ET_A-R is the predominant receptor subtype in these cells (data not shown). In order to assess whether elevated ET

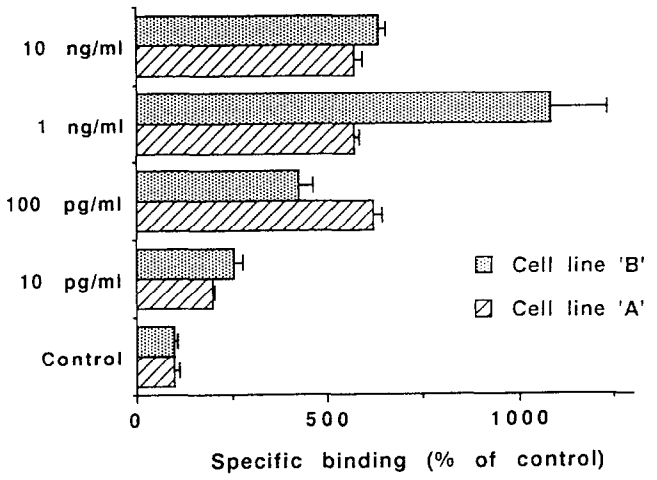

Fig. 3. Dose-dependent effect of IL-1β on elevation of [^{125}I]ET binding to cell lines A and B. Growth-arrested HVSMC were treated for 24 h with a range of concentrations of IL-1β from 10 pg/ml to 10 ng/ml before the receptor binding assay. Each bar represents the specific binding as mean ± S.E.M. of 3 wells.

binding in response to IL-1β exposure was accompannied by an increased abundance of ET receptor mRNA, we prepared RNA from IL-1β treated and non-treated cultured human VSMC and performed RT/PCR using primers specific for the ET_A-R subtype of ET receptors. Our results show that there is more ET_A-R mRNA in cells treated with IL-1β than in non-treated cells (Fig. 5A). The amount of mRNA was elevated by 4 h treatment and remained elevated after 24 h. Forskolin, previously shown to increase ET_A-R mRNA in rat VSMC [12] was also found to elevate ET_A-R mRNA above control levels in our human VSMC. Increasing amounts of cDNA in the PCR reaction gave a proportionally higher amount of product indicating that under these conditions, the amplification was semi-quantitative. No product was seen in the absence of cDNA (data not shown). The same cDNA samples were also used in reactions primed with oligonucleotides specific for glyceraldehyde-3-phosphate dehydrogenase, an enzyme whose mRNA is thought to remain constant in the cell under most circumstances, as an indication that the amount of RNA used in each reverse transcription reaction was the same (Fig. 5B).

4. Discussion

It is reasonable to hypothesize that increased expression of endothelin receptors may be involved in the pathogenesis of cardiovascular diseases such as atherosclerosis. Some preliminary in vitro data suggests that ET receptors are elevated in atherosclerotic plaques [11]. We have sought to identify the factors which may be responsible for signalling such an elevation in ET receptor expression. A similar approach has been taken before but using rat VSMC [12,13,21]. Human VSMC have been used in only one study [14] but no details of the number of lines used was provided. In our experiences, human VSMC lines demonstrate large variations with respect to expression of receptors and response to mediators. Hence in this study we used various potential regulators of the endothelin system on at least four independantly established human VSMC lines.

Table 1
Specific binding of [^{125}I]ET to four human VSMC lines. Cells were cultured in 48-well plates as described in section 2 and treated with 10 ng/ml of IL-1β for 24 h prior to assay. Each figure shows the specific binding as the mean of three individual wells with S.E.M. in brackets.

	Specific [^{125}I]ET bound (c.p.m./10^4 cells)		
Cell line	Control	IL-1β treated	Ratio of treated/control
A	162 (68)	612 (63)	3.8
B	2846 (102)	6841 (428)	2.4
C	681 (62)	3610 (73)	5.3
D	1083 (44)	2536 (67)	2.3

In all our cell lines forskolin up-regulated [^{125}I]ET binding consistent with previous observations [12]. However, the response to cytokines was more variable. TGF-β either had no effect or slightly down-regulated binding in some cells, whereas TNF-α strongly up-regulated binding in one cell line only, with smaller effects on others. IL-1β was the only cytokine to consistently up-regulate [^{125}I]ET binding in this system in all cell lines tested and hence this observation was examined in more detail. The effect of IL-1β was dose and time dependent but could not be reversed after the removal of the IL-1β stimulus. This may be due to IL-1β remaining bound to the receptors after the cells were washed. The time course of the increase in binding and the fact that the ET_A-R mRNA levels are elevated for up to 24 h after the initial stimulus suggests the effect is due to increased receptor synthesis and subsequently increased receptor expression on the cell surface.

One of the consequences of increased receptor expression might be an increased mitogenic response to exogenous ET but this was not observed, either as an increase in cell number or in [^{3}H]thymidine incorporation. As we [20] and others have observed that the mitogenic effect of ET in monolayer cultures is weak or non-existent, this effect was not too surprising. However, this experiment could be tested in the organotypic cultures of vessels where the mitogenic potency of ET is very high.

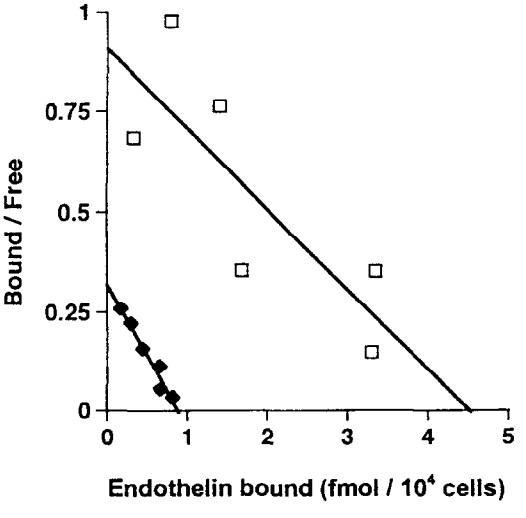

Fig. 4. Scatchard plot of ET binding to HVSMC line C. Open squares = cells pre-treated 24 h with 10 ng/ml IL-1β; full squares = untreated cells.

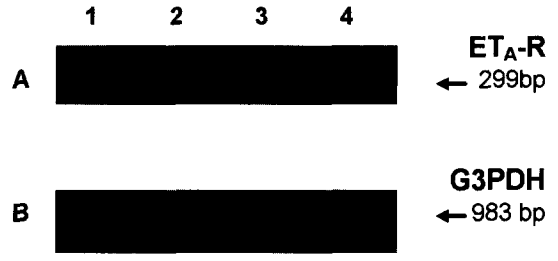

Fig. 5. Analysis of ET_A-R mRNA in control and IL-1β treated human VSMC. RT/PCR was performed as described in section 2. PCR products derived from RNA prepared from control HVSMC (lane 1); 4 h 10 ng/ml IL-1β-treated cells (lane 2); 24 h IL-1β-treated cells (lane 3); 24 h, 20 μM forskolin-treated cells (lane 4). PCR-products are shown from reactions using: (a) ET_A-R specific primers giving a product of predicted size of 299 bp; (b) G-3-PDH specific primers giving a product of predicted size 923 bp. Only one band was obtained in each reaction so only the relevant part of each gel is shown. Similar results were obtained with two cell lines.

IL-1β is a pro-inflammatory cytokine which is elevated in the vessel wall in atherosclerosis [22]. It is a potent inducer of ET in human umbilical vein endothelial cells (HUVEC) [23] of the vessel wall and the current study shows that it also elevates ET receptors on the smooth muscle making it the only cytokine known to have this dual effect. In the 'response to injury' hypothesis of atherosclerosis [15], injury to the endothelium is the initiating event. The increased expression of IL-1β in this situation may well, via elevation of ET and its receptor, induce VSMC proliferation and also make the vessel wall more prone to vasospastic events.

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