Differential localization of endothelin ET_A and ET_B binding sites in human placenta

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1 The localization and differential distribution of endothelin (ET) receptor subtypes (ET_A and ET_B) was investigated in sections of human placenta by use of quantitative *in vitro* autoradiography and receptor selective ligands.

2 Specific, high density [¹²⁵I]-ET-1 binding sites were localized to the decidua and foetal membranes as well as to arteries and veins in the chorionic plate and throughout the villous tree. Moderate to low density binding was found in the extravillous and villous trophoblast respectively.

3 [¹²⁵I]-ET-1 binding sites exhibited a rank order of inhibition by unlabelled peptide sequences (ET-1 > ET-3 > [Ala^{3,11,18}Nle⁷]-ET-1 > BQ123 ≥ sarafotoxin 6c). However, in contrast to the monophasic inhibition curve of ET-1, the other sequences produced a significantly better fit to a two component inhibition curve suggesting the presence of a heterogeneous population of ET binding sites.

4 ET_A and ET_B receptors were distinguished by competitive inhibition of [¹²⁵I]-ET-1 binding with increasing concentrations of unlabelled ET-3, [Ala^{3,11,18}Nle⁷]-ET-1, sarafotoxin 6c and BQ123 and by incubating sections with the ET_B agonist, [¹²⁵I]-BQ3020. ET receptor subtypes exhibited a differential distribution in the placenta. ET_A type binding sites predominated (~80% of the total) on veins and arteries in the chorionic plate. Veins in stem villi, blood vessels in distal regions of the villous tree and decidual cells displayed a high density (~60–70% of the total) of the ET_B receptor subtype.

5 No difference was detected in either the relative density of [¹²⁵I]-ET-1 binding sites or the proportion of ET_A to ET_B sites in placentae from pregnancies complicated by pre-eclampsia compared with normal term controls.

6 ET may have a local autocrine or paracrine role in the placenta, acting via specific receptors to influence foetoplacental blood flow and other aspects of placental function.

Keywords: Endothelin; ET-receptor subtypes; *in vitro* autoradiography; placenta

Introduction

Due to the lack of autonomic innervation, the regulation of foetoplacental blood flow is dependent on locally released or circulating vasoactive substances (Reilly & Russell, 1977). The precise mediators are not known, however, a number of vasoactive substances have been suggested including the 21 amino acid peptide, endothelin (Howard, 1987; Wilkes *et al.*, 1990). Endothelin (ET) has been shown to be a potent vasoconstrictor of the human isolated foetoplacental cotyledon (Wilkes *et al.*, 1990) and foetoplacental blood vessels (MacLean *et al.*, 1992) and has the potential to influence placental blood flow either directly or in concert with other vasoactive agents including thromboxane A₂, prostaglandin F_{2α}, prostacyclin, nitric oxide and atrial natriuretic peptide (De Nucci *et al.*, 1988; Hu *et al.*, 1988; Rae *et al.*, 1989; Wilkes *et al.*, 1990; Cameron *et al.*, 1991). Circulating ET levels have been shown to increase during pregnancy and labour (Mastrogianis *et al.*, 1991); however, conflicting evidence exists concerning the possible role of ET in the pathogenesis of pre-eclampsia. While some reports have indicated a further elevation of plasma ET levels in this condition (Nova *et al.*, 1991; Clark *et al.*, 1992), more recent studies have not found a significant difference in plasma ET levels between pre-eclamptic and normal pregnancies (Benigni *et al.*, 1992) nor any correlation between plasma ET-1 concentration, blood pressure or renal function during tox-aemic pregnancy (Otani *et al.*, 1991).

Three ET isoforms ET-1, ET-2 and ET-3 have been isolated and the mRNA encoding ET-1 and ET-3 detected in

placental tissues (Sunnergren *et al.*, 1989; Onda *et al.*, 1990; Benigni *et al.*, 1991). Endothelin-like immunoreactivity has also been localized to vascular and avascular regions of the placenta including the endothelium of stem villous blood vessels, decidual cells and the epithelium of the amniotic membrane (Hemsén *et al.*, 1991; van Pappendorp *et al.*, 1991). High affinity binding sites for [¹²⁵I]-ET have been identified in whole placental membrane preparations by affinity cross-linking (Fischli *et al.*, 1989; Nakajo *et al.*, 1989; Wada *et al.*, 1990), whilst the use of molecular cloning techniques has demonstrated the expression of two ET-receptor subtypes in placental tissues which display distinct affinities for ET isoforms (Adachi *et al.*, 1991; Ogawa *et al.*, 1991). The selective (ET_A) subtype exhibits a high affinity for ET-1 and ET-2, and ~1000 fold lower affinity for ET-3, whereas the non-selective (ET_B) receptor subtype binds all three isoforms with a similar affinity. A number of ET_A and ET_B selective peptide sequences have been identified, including the cyclic pentapeptide BQ123, a potent ET_A antagonist (Ihara *et al.*, 1991), and BQ3020, an alanine substituted truncated ET-1 analogue which is a potent ET_B agonist (Saeki *et al.*, 1991; Ihara *et al.*, 1992). ET_B type binding sites may also be distinguished on the basis of their high affinity for the agonists, sarafotoxin 6c (Williams *et al.*, 1991) and [Ala^{3,11,18}Nle⁷]-ET-1 (Hunt *et al.*, 1991).

In view of the relatively high level expression of ET and ET-receptor subtypes in the human placenta, it may be postulated that endothelin plays a local regulatory role in this organ. In the present studies, we have employed the technique of quantitative *in vitro* autoradiography and used receptor selective ligands to localize ET binding sites in human

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placental tissues and to determine the differential distribution of receptor subtypes.

Methods

Tissues

Placentae were obtained from normal term pregnancies, immediately following transvaginal delivery or elective caesarean section, and from cases complicated by pre-eclampsia following caesarean section (Table 1). Pregnancies in the latter group were characterized by proteinuria, a sustained rise (> 30 mmHg) in the maternal diastolic blood pressure from the initial measurement made in the first half of the pregnancy and the absence of systemic disease or pre-existing hypertension. Four of the pre-eclamptic pregnancies were further complicated by (intrauterine) foetal growth retardation, the birth weight being below the 10th centile for gestational age. Tissue samples were taken through the whole placenta to include the chorionic plate, the placental villi, basal plate (decidua) and foetal membranes. The tissues were placed on cork mats, surrounded in mounting medium (Tissue-TekTM, Miles Inc., Elkhart, Indiana, U.S.A.) and frozen in melting dichlorodifluoromethane (Arcton-12, I.C.I., Cheshire) suspended in liquid nitrogen.

In vitro autoradiography

Cryostat sections (10 μ m thick) were cut at -25°C , thaw mounted onto Vectabond (Vector Laboratories, Peterborough)-treated glass slides and stored at -20°C . Sections were preincubated at room temperature for 15 min in 50 mM Tris HCl buffer (pH 7.2) containing, NaCl 100 mM, MgCl_2 5 mM and bacitracin 28 μ M and then incubated in fresh buffer containing bovine serum albumin 0.5%, phosphoramidon 1 μ M and 3-([^{125}I]-iodotyrosyl)-ET-1 150 pM, for up to 180 min at 20°C . The slices were then washed twice for 5 min in fresh buffer at 4°C , rinsed briefly in distilled water and dried under a stream of cold air. Non-specific binding was assessed by incubating serial sections in the presence of 10^{-6} M unlabelled ET-1. Depletion of ligand binding capacity was assessed by reapplying the incubation medium to fresh sections, under identical conditions. In order to distinguish ET_A and ET_B receptor subtypes in the placenta, sections were co-incubated with 150 pM [^{125}I]-ET-1 in the presence of either the ET_A selective antagonist, BQ123 (10^{-6} M– 10^{-8} M) or the ET_B selective agonist, [$\text{Ala}^{3,11,18}\text{Nle}^7$]-ET-1 (10^{-7} M). Further localization of the ET_B receptor subtype was provided by incubating sections with the ET_B agonist [^{125}I]-BQ3020 (150 pM) for 120 min at 20°C . The specificity of binding was also investigated by co-incubation of the ligands with 1 μ M of unrelated peptides including vasoactive intestinal polypeptide (VIP), calcitonin gene-related peptide (CGRP), atrial natriuretic peptide (ANP), neuropeptide tyrosine (NPY) and

substance P. Macroautoradiographic images were produced by exposing the dry labelled sections to Hyperfilm- ^3H together with [^{125}I]-microscales (Amersham, Bucks) for 18 h at 4°C . The autoradiographic film was developed in Kodak D-19 developer for 5 min, fixed in Amfix and rinsed in running water for at least 30 min.

Microautoradiography, immunohistochemistry and histochemistry

Greater anatomical resolution was obtained when labelled sections were fixed in Bouin's solution for 1 h at 4°C , washed twice in cold phosphate buffer (2–3 min), rinsed in distilled water at 4°C , dried under a stream of cold air and dipped in LM-1 emulsion (Amersham, Bucks) at 43°C . Slides were then stored in the dark at 4°C for up to 5 days, developed for 2.5 min in Kodak D-19 at 20°C , fixed, stained with haematoxylin and eosin and mounted in DPX mounting medium. Fixation of labelled sections in Bouin's solution and rapid washing at 4°C , prior to dipping dried sections in emulsion, retains regional differences in ligand binding, inhibits the dissociation of ligand and preserves tissue morphology for microscopic examination. The localization of ET binding sites to the media and endothelium of placental blood vessels was further examined by comparing the distribution of [^{125}I]-ET-1 binding sites with immunofluorescence staining in adjacent sections of placenta. Vascular smooth muscle and endothelial cells were identified with monoclonal antisera to α -smooth muscle actin (IA4, Sigma) and the endothelium associated CD31 glycoprotein (JC70A, Dako) respectively. Cryostat sections were post fixed in acetone at -20°C for 10 min, rinsed and incubated with either IA4 (1/1000) or JC70A (1/40) antibodies overnight at 4°C . Immunoreactivity was visualized by incubating sections in fluorescein isothiocyanate-labelled goat anti-mouse IgG (1/100, Miles) for 60 min at room temperature and the sections examined with an Olympus AH2 microscope. Controls included the omission of the primary antisera and replacement with non-immune serum. Differential binding of [^{125}I]-ET-1 to trophoblast and decidua cells was demonstrated by the histochemical localization of glucose-6-phosphate dehydrogenase activity in adjacent sections (Bancroft, 1975). Briefly, sections were incubated in a Tris buffer solution (pH 7.4) containing the specific substrate glucose-6-phosphate, nitro-blue tetrazolium and nicotinamide adenine dinucleotide phosphate, for 60–90 min at 37°C . Sections were subsequently fixed in 10% formol saline, washed and counterstained with 2% methyl green.

Quantification and characterization of ET binding sites

Quantitative image analysis of the film autoradiographic images was carried out by means of computer assisted microdensitometry using an IBAS 386 Image Analysis System

Table 1 Clinical features of pregnancies

Pregnancies	Birth weight (kg)	Gestational age (weeks)	Maternal age (years)	Blood pressure at birth (mmHg)	
				systolic	diastolic
<i>Normal</i>					
Transvaginal delivery ($n = 8$)	3.4 (2.9–4.0)	39 (38–40)	28 (24–32)	119 (110–127)	74 (68–80)
Caesarean section ($n = 5$)	3.6 (3.1–4.1)	40 (39–41)	28 (23–34)	120 (111–128)	75 (69–81)
<i>Pre-eclampsia</i> ($n = 6$)	1.4 (0.6–2.3)**	32 (28–36)*	28 (22–33)	149 (138–160)*	103 (96–109)**

*In 4 of the pre-eclampsia cases the pregnancies were further complicated by (intrauterine) foetal growth retardation, the birth weight being below the 10th centile for gestational age. Values are expressed as the mean and 95% confidence interval. Significant difference (** $P < 0.001$ and * $P < 0.01$) in pregnancies complicated by pre-eclampsia compared with normal-term transvaginal and caesarean deliveries.

(Kontron Elektronik, Watford, UK). The density of ligand binding (amol mm^{-2}) was determined for placental villi, decidua and blood vessels of the chorionic plate and stem villi. Specific binding was determined by subtracting the non-specific binding, obtained by incubating sections with an excess ($1 \mu\text{M}$) of unlabelled ET-1, from the total binding obtained by incubating sections with 150 pM [^{125}I]-ET-1 or [^{125}I]-BQ3020 alone. Competitive inhibition of specific [^{125}I]-ET-1 binding was examined in the presence of increasing concentrations (10^{-13} – 10^{-6} M) of unlabelled ET-1, ET-3, [$\text{Ala}^{3,11,18}\text{Nle}^7$]-ET-1 and sarafotoxin 6c (S6c). Association kinetics of ET-1 binding were examined by incubating placental sections with [^{125}I]-ET-1 (150 pM) for up to 180 min. Dissociation of the ligand was assessed by removing the incubation medium after 120 min, applying excess ($1 \mu\text{M}$) unlabelled ET-1 and reincubating for up to 300 min. The possible role of guanosine nucleotides in endothelin binding was determined by examining the effect of the non-hydrolysable analogue, guanosine 5'-O-(thiotriphosphate) (GTP- γ -S), on the association and dissociation of [^{125}I]-ET-1 binding. The non-linear curve fitting programme, GraphPad InPlot version 3.1 (GraphPad Software, San Diego, U.S.A.) was used to analyse the binding data and a four parameter logistic equation used to describe the relationship between the percentage of [^{125}I]-ET-1 bound and the concentration of ET-1, ET-3, [$\text{Ala}^{3,11,18}\text{Nle}^7$]-ET-1 and S6c in competitive inhibition experiments. A one or two component competitive binding curve was fitted to the data and the 'best fit' sums of squares compared statistically by an *F*-test. The concentration of peptide which inhibited 50% of the specific ligand binding (IC_{50}) to the high and low affinity ET binding sites was calculated for each competing peptide and the values expressed as a geometric mean with 95% confidence interval (95% CI), being derived from logarithmically transformed data.

Statistics

Binding data were analysed using one way analysis of variance and differences considered to be significant if the *P* value was less than 0.05. The IC_{50} values of competitive inhibition data for villi, decidua and blood vessels were used as three separate dependent variables and the competing peptides as within subject factors. To reduce the probability of significant differences arising by chance a Bonferroni correction was applied to the data (Wallenstein *et al.*, 1980).

Drugs

Drugs used were 3-([^{125}I]-iodotyrosyl)-ET-1 and [^{125}I]-labelled [$\text{Ala}^{1,3,11,15}$]-endothelin-7(16–21) ([^{125}I]-BQ3020) ($2\sim 2000 \text{ Ci mmol}^{-1}$; Amersham, Bucks.), bacitracin, GTP- γ -S, bovine serum albumin (fraction V) and phosphoramidon (Na salt)

(Sigma Chemical Co., UK). Cyclo(-D-Asp-Pro-D-Val-Leu-D-Trp) (BQ123) was a generous gift from the Banyu Pharmaceutical Co. Ltd., Japan. ET-1, ET-3, [$\text{Ala}^{3,11,18}\text{Nle}^7$]-ET-1, S6c, VIP, CGRP, ANP, NPY and substance P (Peninsula Laboratories).

Results

Specific, high density binding sites for [^{125}I]-ET-1 were localized to placental villi, decidua, foetal blood vessels in the chorionic plate and stem villi and to foetal membranes (Figure 1, Table 2). Co-incubation of [^{125}I]-ET-1 with an excess ($1 \mu\text{M}$) of unlabelled ET-1 resulted in a complete inhibition of binding to all placental sites (Figure 1) whereas co-incubation with an excess of either unlabelled ET-3, S6c, BQ123 or the ET_B receptor agonist, [$\text{Ala}^{3,11,18}\text{Nle}^7$]-ET1, produced a differential inhibition. ET-3 completely inhibited binding to the decidua and distal regions of the villous tree whilst not completely inhibiting [^{125}I]-ET-1 binding to blood vessels in the stem villi and chorionic plate (Figure 1). Coincubation with an excess of either of the ET_B selective agonists, S6c or [$\text{Ala}^{3,11,18}\text{Nle}^7$]-ET-1 (10^{-7} M) had a minimal effect on binding of [^{125}I]-ET-1 to foetal blood vessels in stem and intermediate villi, but inhibited the ligand binding to terminal villi, decidua and foetal membranes (Figure 1). In contrast, coincubation with $1 \mu\text{M}$ BQ123, significantly inhibited [^{125}I]-ET-1 binding to foetal blood vessels in the chorionic plate and stem villi, whilst only slightly reducing the density of binding to the decidua, distal regions of the villous tree and foetal membranes. The differential distribution of ET receptor subtypes in foetoplacental blood vessels, decidua and villi was further investigated using the ET_B selective ligand [^{125}I]-BQ3020. Unlike [^{125}I]-ET-1 this ligand demonstrated regional differences in the distribution of binding sites on foetoplacental arteries and veins. Arteries in the chorionic plate, intermediate and stem villi and veins in the chorionic plate exhibited relatively low binding whereas a higher density of binding sites was observed on veins in distal regions of the stem villi (Table 2). As expected from the differential inhibition of [^{125}I]-ET-1 binding by ET_B selective ligands, a relatively high density of [^{125}I]-BQ3020 binding sites was detected in the decidua, distal regions of the villous tree and foetal membranes (Table 2). Specific [^{125}I]-ET-1 binding in all regions of the placenta was unaffected by coincubation of radiolabelled ligands with an excess ($1 \mu\text{M}$) of the unrelated peptides CGRP, VIP, NPY, ANP and substance P. No significant difference was found between the relative density of vascular and villous [^{125}I]-ET-1 binding sites in placenta obtained after elective caesarean section and normal transvaginal delivery ($P > 0.365$) or between placenta from normal pregnancies and those complicated by pre-eclampsia ($P > 0.144$). Binding sites were further resolved by micro-

Table 2 Relative binding density of [^{125}I]-endothelin-1 ([^{125}I]-ET-1) and [^{125}I]-BQ3020 in human placenta

Region	Binding density (amol mm^{-2})	
	[^{125}I]-ET-1 (ET_A and ET_B sites)	[^{125}I]-BQ3020 (ET_B sites)
Chorionic plate		
Arteries ($n = 5$)	415 (289–541)	81 (60–102)
Veins ($n = 5$)	306 (190–422)	67 (23–111)
Stem villi		
Arteries ($n = 8$)	370 (312–428)	41 (28–54)
Veins ($n = 8$)	401 (290–512)	226 (148–304)*
Decidua ($n = 7$)	466 (390–542)	362 (291–433)*
Villi ($n = 11$)	252 (216–288)	153 (131–175)*

Binding density values expressed as the mean and 95% Confidence Interval (CI) for *n* separate normal term placenta, obtained following either transvaginal delivery or elective caesarean section. All sections were incubated for 120 min at 20°C with either [^{125}I]-ET-1 (150 pM) or [^{125}I]-BQ3020 (150 pM).

*Significantly greater binding site density compared to vessels in the chorionic plate and arteries in stem villi ($P < 0.01$).

Figure 1 Darkfield images of film autoradiograms demonstrating the localization of [125 I]-endothelin-1 ([125 I]-ET-1) binding sites in serial sections (A–D, E–F) of human placenta incubated in the absence (A,E) or presence of an excess of unlabelled ET-1 (B), ET-3 (C), S6c (D) or [Ala^{3,11,18}Nle⁷]-ET1 (F). Binding is completely inhibited in the presence of 1 μ M unlabelled ET-1 (B) and selectively inhibited in the decidua (open arrows) and placental villi by ET-3 (C), S6c (D) and [Ala^{3,11,18}Nle⁷]-ET-1 (F). [125 I]-ET-1 binding sites on blood vessels in the chorionic plate and stem villi (arrows) display a low affinity for ET_B receptor selective ligands. Bar = 2 mm.

scopic examination of emulsion-coated sections and comparison with immunohistochemical and histochemical preparations. Enzyme histochemistry for glucose-6-phosphate dehydrogenase discriminated between trophoblast and decidua cells, strong enzyme activity being detected in villous and extravillous trophoblast cells, as well as amnion cells, whilst decidua cells were faintly stained (Figure 2). Moderate or low level [125 I]-ET-1 binding was localized in the villous trophoblast and extravillous cytotrophoblast (X-cells) whereas decidua cells in the basal plate and those attached to the chorion in the foetal membranes exhibited a relatively high density of binding sites (Figure 2). No specific binding was observed to chorionic mesenchyme or amnion cells.

Microscopic examination of villous [125 I]-ET-1 binding demonstrated that binding sites were localized to the media in arteries, arterioles, veins and venules at all levels of the villous tree (Figure 3). Due to the limited resolution of the autoradiographic images obtained with 125 I-labelled ET-1 and the high density of binding sites on the media, a distinct receptor subtype could not be identified in the endothelium. Vascular smooth muscle and endothelial cells were, however, distinguished by immunofluorescence staining and [125 I]-ET-1 binding sites localized microscopically to the endothelial layer in foetoplacental blood vessels (Figure 3).

[125 I]-ET-1 binding sites were characterized by association, dissociation and competitive inhibition studies. The time

Figure 2 Photomicrographs of sections of decidua (A–B) and foetal membranes (C) showing the microscopic localization of glucose-6-phosphate dehydrogenase activity (A) and [125 I]-endothelin-1 ([125 I]-ET-1) binding sites (B–C). Decidual cells (dc) exhibit a high binding site density and lack enzyme activity whereas the extravillous trophoblast (X-cells) display strong enzyme staining and a moderate amount of [125 I]-ET-1 binding (A–B). Amnion epithelial cells (arrows) and underlying chorionic mesenchyme show no specific ligand binding sites (C). Box (A) corresponds to the area in an adjacent section showing the distribution of [125 I]-ET-1 binding sites (B). Bar = 50 μ m.

Figure 3 Photomicrographs of serial section of an artery in a stem villous demonstrating the microscopic localization of [125 I]-endothelin-1 ([125 I]-ET-1) binding sites (A) to the endothelium (open arrows) and media (m), which exhibit immunofluorescence staining for CD31 (B) and α -smooth muscle actin (C) respectively. Silver grains are also shown localized to the vascular endothelium (arrows) and trophoblast in a tertiary stem villous (D). T, villous trophoblast. Bar = 50 μ m.

Figure 4 Association (■) and dissociation (□) of [125 I]-endothelin-1 ([125 I]-ET-1) to decidual binding sites in the human placenta. Each point represents the mean $\pm$ s.e. mean derived from six normal-term placentae expressed as a percentage of maximum specific binding. A similar time course was observed for placental villi and vessels of the chorionic plate and stem villi.

Figure 5 Competitive inhibition curves of specific [125 I]-endothelin-1 ([125 I]-ET-1) binding to blood vessels in the chorionic plate and stem villi. Each point represents the mean $\pm$ s.e. mean derived from six separate normal-term placentae expressed as a percentage of ligand binding in the presence of increasing concentrations of unlabelled ET-1 (■), ET-3 (○), [Ala 3,11,18 Nle 7]-ET-1 (●), BQ123 (▲) and S6c (□).

Figure 6 Competitive inhibition curves of specific [125 I]-endothelin-1 ([125 I]-ET-1) binding to the decidua. Each point represents the mean $\pm$ s.e. mean derived from six separate normal-term placentae expressed as a percentage of ligand binding in the presence of increasing concentrations of unlabelled ET-1 (■), ET-3 (○), [Ala 3,11,18 Nle 7] (●), BQ123 (◆) and S6c (□). Competitive inhibition studies for binding to the placental villi gave very similar inhibition curves.

course of association for 150 pM [125 I]-ET-1 at 20°C binding to the decidua gave a rate constant (k_{+1}) of $2.245 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$, reaching an apparent equilibrium by 120 min, with no detectable dissociation after 5 h (Figure 4). Similar binding characteristics were observed in the placental villi and foetal blood vessels. Preincubation for 30 min and coincubation of the ligand with 10^{-5} M GTP- γ -S had no apparent effect on either the association or dissociation of [125 I]-ET-1 binding to decidua, villi or blood vessels (data not shown). Re-application of the incubation medium after 120 min to fresh sections of placenta and incubation for a further 120 min showed a $\sim 40\%$ decrease in the binding density over the total incubation period. The apparent depletion in ligand binding was not inhibited by inclusion of the enzyme inhibitors bacitracin (28 μM) and phosphoramidon (1 μM). Unlabelled peptides exhibited a similar rank order of inhibitory potency (ET-1 > ET-3 > [Ala 3,11,18 Nle 7]-ET-1 > BQ123 $\geq$ S6c) for [125 I]-ET-1 binding sites. In contrast to the single phase sigmoid curve obtained with ET-1, however, the data produced a significantly better fit to a two component than a one component inhibition curve for unlabelled ET-3 ($P < 0.05$), [Ala 3,11,18 Nle 7]-ET-1 ($P < 0.05$), BQ123 ($P < 0.001$) and S6c ($P < 0.001$) (Figures 5–6). Analysis of the inhibition curves indicated a heterogeneous population of binding sites displaying high affinity for ET-1 and a differential affinity for ET-3, [Ala 3,11,18 Nle 7]-ET-1, BQ123 and S6c (Table 3). S6c was found to be the most selective ET $_B$ -receptor ligand displaying a significantly lower inhibitory potency for ET $_A$ type binding sites than either ET-3 or [Ala 3,11,18 Nle 7]-ET-1 whilst exhibiting a high affinity for ET $_B$ type binding sites in all regions of the placenta. BQ123, on the other hand, displayed the lowest

affinity for ET $_B$ type binding sites and was equipotent with ET-1 for high affinity ET $_A$ type sites (Table 3). Placental tissues displayed a heterogeneous population of ET binding sites and exhibited marked regional differences in the relative proportion of ET $_A$ and ET $_B$ type binding sites. ET $_A$ sites predominated in proximal regions of foetal vessels in the

Table 3 Selective inhibition of specific [125 I]-endothelin-1 ([125 I]-ET-1) binding to receptor subtypes in human placenta by unlabelled peptide sequences

Region	ET-1 ^a IC ₅₀ (nM)	Receptor subtype	BQ123 IC ₅₀ (nM)	ET-3 IC ₅₀ (nM)	[Ala 3,11,18 Nle 7]-ET-1 IC ₅₀ (nM)	S6c IC ₅₀ (nM)
Foetal vessels†	0.67 (0.44–1.01)	ET $_B$	2400 (1500–3900)*	0.19 (0.08–0.41)	0.16 (0.01–1.82)	0.29 (0.09–0.89)
		ET $_A$	0.41 (0.20–0.85)	96 (18.2–512.9)	149 (94.0–237.1)	7311 (3199–16749)*
Villi	0.45 (0.32–0.63)	ET $_B$	3300 (1300–8300)*	0.42 (0.30–0.61)	0.97 (0.72–1.31)	1.02 (0.66–1.58)
		ET $_A$	0.21 (0.09–0.51)	104 (49.0–218.8)	128 (69.8–233.9)	3785 (1230–11455)*
Decidua	0.90 (0.51–1.61)	ET $_B$	5300 (2080–13500)*	0.96 (0.54–1.70)	2.37 (1.49–3.78)	1.62 (0.97–2.71)
		ET $_A$	0.29 (0.07–1.14)	219 (81.3–588.8)	105 (73.6–150.3)	1954 (822–4645)*

IC₅₀ values derived from non-linear regression analysis of inhibition binding data fitted to a one (ET-1) or two component inhibition curve. Data are expressed as the geometric mean and 95% Confidence Interval (CI) for six separate placentae.

†Blood vessels in the chorionic plate and proximal stem villi.

*Significantly lower ($P < 0.01$) inhibitory potency of the receptor selective ligands S6c and BQ123 for ET $_A$ and ET $_B$ binding sites respectively, compared with the other unlabelled peptide sequences. ^aInhibition of total [125 I]-ET-1 binding to ET $_A$ and ET $_B$ subtypes.

Figure 7 Relative proportion of ET_A to ET_B binding sites in the normal-term human placenta calculated from the competitive inhibition of 150 pM [¹²⁵I]-endothelin-1 ([¹²⁵I]-ET-1) binding by S6c (hatched columns) and BQ123 (open columns), after fitting a two component competitive binding curve to the data. Each column represents the mean $\pm$ s.e. mean derived from six placentae. ‡Blood vessels in the chorionic plate and proximal stem villi.

chorionic plate and stem villi whereas ET_B sites were concentrated on intermediate and terminal villi, the decidua and foetal membranes. The relative proportion of ET-receptor subtypes was calculated by fitting a two component binding curve to the S6c and BQ123 inhibition data and indicates that in the decidua and terminal villi 60–70% of the total [¹²⁵I]-ET-1 binding sites are of the ET_B subtype while in vessels in the chorionic plate and stem villi the proportion is less than 20% (Figure 7).

Discussion

Two populations of specific, high affinity ET binding sites were demonstrated in human placental sections by the use of selective agonist and antagonist sequences, displaying differential, tissue-specific distribution patterns. This corresponds with the recent molecular cloning of nucleotide sequences from human placental cDNA libraries encoding both ET_A and ET_B receptor subtypes (Ogawa *et al.*, 1991; Adachi *et al.*, 1991). These latter studies did not, however, determine the cellular localization of ET receptor subtype expression.

The decidua and blood vessels in placental villi contained a high proportion of ET_B type binding sites whereas ET_A sites predominated in vessels in the chorionic plate. A differential distribution of ET receptor subtypes has also been demonstrated in the human foetal heart (Wharton *et al.*, 1991) and porcine pulmonary tissues (Nakamichi *et al.*, 1991). The present investigation of placental [¹²⁵I]-ET-1 and [¹²⁵I]-BQ 3020 binding sites further indicates that the proportion of ET_A/ET_B receptors varies between different regions of the placental vascular bed. In particular, arteries in stem villi and chorionic plate, as well as veins in the chorionic plate, contain a high proportion of ET_A sites whereas veins in stem villi and vessels in distal regions of the villous tree possess mainly ET_B sites. The current findings support and extend the results obtained by ligand binding to membrane preparations (Robaut *et al.*, 1991) whilst the localization of [¹²⁵I]-ET-1 binding sites to vessels which also contain ET-like immunoreactive endothelial cells (Hemsén *et al.*, 1990; van Papendorp *et al.*, 1991) suggests a local autocrine or paracrine role for ET in the foetoplacental vasculature. The combined microautoradiographic and immunohistochemical observations suggest that [¹²⁵I]-ET isoforms bind to both endothelial and smooth muscle cells. Furthermore, it was apparent from quantitative analysis of film autoradiographs that both ET_A and ET_B type binding sites are localized to the media in foetoplacental blood vessels. ET_B receptors have previously been considered only to occur on endothelial cells and may mediate the transient vasodilator effect of ET via the release of nitric

oxide (Takayanagi *et al.*, 1991; Namiki *et al.*, 1992). The present observations are, however, consistent with recent reports which demonstrate that both ET_B and ET_A receptors occur on vascular smooth muscle cells and mediate the ET-induced vasopressor response (Ihara *et al.*, 1991; Fukurodo *et al.*, 1992; Moreland *et al.*, 1992; Panek *et al.*, 1992). Studies on mammalian isolated blood vessels indicate that ET_A receptors mediate the ET-induced vasoconstriction of arterial smooth muscle whereas ET_B receptors are responsible for the contractile response in veins (Moreland *et al.*, 1992). [¹²⁵I]-ET-1 binding sites on veins and arteries in the chorionic plate display a similar relative density and affinity for ET isoforms, as ascertained by autoradiographic and membrane binding techniques (Robaut *et al.*, 1991), but it has been reported that the veins in the chorionic plate are some ten times more sensitive to ET-1 than the corresponding arteries (MacLean *et al.*, 1992). It is uncertain to what extent regional variation in the relative density of ET receptor subtypes may influence the vasopressor response of different foetoplacental blood vessels, but this may also be dependent on factors such as differences in the transduction mechanisms of the two receptors (Aramori & Nakanishi, 1992).

The relationship between ET and other vasoactive peptides in the placenta, has not been determined but binding sites for [¹²⁵I]-ET-1, [¹²⁵I]-ANP-(1-28) (Salas *et al.*, 1991) and [¹²⁵I]-[Sar¹Ile⁸]-angiotensin II (unpublished observations) have been localized to foetoplacental blood vessels and both ANP and angiotensin II modulate placental blood flow (McQueen *et al.*, 1990a) and ET-1 secretion from umbilical endothelial cells (Kohno *et al.*, 1991). Elevated levels of circulating ANP and the activation of the foetal renin-angiotensin system have been associated with a reduction in the affinity or density of ANP and angiotensin II receptors in placentae from pregnancies complicated by pre-eclampsia and intrauterine growth retardation (IUGR) (McQueen *et al.*, 1990b; Malee *et al.*, 1992). The role of ET in pre-eclampsia is unclear, however, as contradictory reports exist concerning circulating ET levels in normotensive patients and those exhibiting this condition. Some reports have shown that prepro-ET-1 gene expression and plasma ET-1 levels are similar in both normal pregnancy and pre-eclampsia (Benigni *et al.*, 1992; Otani *et al.*, 1991), whilst others have reported elevated maternal plasma ET-1 levels in pre-eclampsia implicating ET in the impaired renal function and placental blood flow associated with the disease (Mastrogianis *et al.*, 1991; Nova *et al.*, 1991; Clark *et al.*, 1992). In the present study, we were unable to detect a difference in either the relative density or proportion of vascular and villous ET receptor subtypes in placentae from pregnancies exhibiting pre-eclampsia compared with normal-term controls. The possibility that endogenously bound ligand influenced the demonstration of ET binding cannot be excluded, but these observations suggest that a modulation of placental [¹²⁵I]-ET-1 binding site density does not occur in pregnancies complicated by pre-eclampsia.

A high density of [¹²⁵I]-ET-1 binding sites was also found on non-vascular tissues in the placenta, suggesting that ET may affect other aspects of placental function. These sites predominated on decidual cells, occurring to a lesser extent on the extravillous trophoblast and were mainly of the ET_B subtype. The localization of [¹²⁵I]-ET-1 binding sites and ET-like immunoreactivity to human decidual cells (van Papendorp *et al.*, 1991) suggests a local function. ET is considered to be a putative growth factor and stimulates mitogenesis in human placental fibroblasts (Fant *et al.*, 1992) which implies a potential role as a growth factor in the placenta.

Analysis of [¹²⁵I]-ET-1 binding to placental sections at 20°C indicated an association constant (K_{+1}) of $2.245 \times 10^8 \text{ M}^{-1} \text{ min}^{-1}$, but the lack of any detectable dissociation precluded the calculation of a dissociation constant from kinetic data. Binding of [¹²⁵I]-ET-1 to placental sections was apparently irreversible with no significant dissociation being found up to 5 h after addition of an excess of unlabelled ligand. Estimating the equilibrium dissociation constant from saturation

data obtained under these conditions was therefore considered inappropriate. Nonetheless, the ligands used, inhibited binding in a competitive manner and displayed selective affinity for ET receptor subtypes. Cloning of cDNA sequences encoding ET receptors indicates that they are members of the G-protein coupled family of receptors (Ogawa *et al.*, 1991). However, in contrast to other G-protein coupled receptors, the binding affinity of ET receptors is not necessarily reduced in the presence of non-hydrolysable GTP analogues. In the present study, GTP- γ -S elicited no effect on [¹²⁵I]-ET-1 binding to placental sections. Previous studies have shown that GTP- γ -S did not inhibit [¹²⁵I]-ET-1 binding to membrane preparations of human placenta (Wilkes *et al.*, 1990) or tissues such as porcine cerebellum (Ihara *et al.*, 1992), whereas an inhibitory effect was demonstrated in human bronchial smooth muscle (Mattoli *et al.*, 1991). It is apparent that the interaction of ET receptors and G-proteins is complex (Sokolovsky, 1991) and variations between tissues may reflect differences in the distribution of ET receptor subtypes and their coupling to distinct G-proteins (Aramori & Nakanishi, 1992). Although an apparent reduction in ligand binding activity was observed after two consecutive incubation periods of 120 min each, it is unlikely that even a

40% depletion would substantially affect the IC₅₀ values derived from the competitive inhibition studies. ET isoforms are substrates for neutral endopeptidase (Vijayaraghavan *et al.*, 1990), but the inclusion of the specific and non-specific enzyme inhibitors, phosphoramidon and bacitracin, had no detectable effect on [¹²⁵I]-ET-1 binding density. Possible reasons for the apparent loss in binding activity, other than enzymatic degradation, may include the adherence of ligand to the glass slides, inactivation of constituents in the buffer and release of inhibitory factors from tissue sections.

In conclusion, the localization of [¹²⁵I]-ET-1 binding sites in human placenta suggests that ET may play a local role in the modulation of foetoplacental blood flow as well as in other aspects of placental function. The action of ET may be dependent on the relative proportion of ET_A and ET_B receptor subtypes which varies in different regions of the placenta. The significance of ET and ET receptors in the pathophysiology of pre-eclampsia remains to be established.

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