Endothelin receptor subtypes in the microvillous trophoblastic membrane of early gestation and term human placentas

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Abstract

The ^{125}I -labeled endothelin-1 (^{125}I ET-1) binding sites in microvillous membranes from early gestation and term human placentas were investigated. The K_{d} s for ^{125}I ET-1 binding to early gestation (68 ± 15 pmol/l) and term (45 ± 8 pmol/l) microvilli ($n = 4$) were not significantly different. The density of binding sites decreased significantly, from 243 ± 80 fmol/mg protein in early gestation microvilli to 54 ± 10 fmol/mg protein in term microvilli. The endothelin (ET) receptor (ET-R) subtype profiles were determined by competition binding studies with unlabeled ET-1, ET-3, and selective agonists and antagonists for ET_{A} -R and ET_{B} -R. In early gestation placental microvilli, we observed the presence of 72% ET_{B} -R, (mainly $\text{ET}_{\text{B}2}$ -R subtype), and 28% ET_{A} -R. Only ET_{B} -R (mainly the $\text{ET}_{\text{B}2}$ -R subtype) was present in term placental microvilli. We suggest that the ET_{B} -R on the placental microvillous membrane is involved in specific trophoblastic functions and may play a major role in ET clearance by modulating the amounts of ETs in the maternal intervillous blood space.

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Introduction

Three endothelins (ETs), ET-1, ET-2 and ET-3, each 21 amino acid residues long and encoded by three separate genes, are found in the human genome (1). Although ET-1 was first described as a potent vasoactive peptide, subsequent studies have revealed that the ETs have many physiological and pathophysiological effects. ETs are pleiotropic factors controlling several aspects of cell function, such as ion fluxes, cell-to-cell communication, cell migration, neural transmission and the release of hormones and cytokines in neuroendocrine and immune systems. Like several other vasoactive substances, ETs also have growth factor properties (2). These diverse actions are partly attributed to the existence of many subtypes of ET receptors (ET-Rs) whose cellular distribution and functions are regulated. Sequence analysis of mammalian cDNA clones indicates that there are two distinct ET-R subtypes, ET_{A} -receptors (ET_{A} -R) and ET_{B} -receptors (ET_{B} -R), which belong to the superfamily of receptors with seven transmembrane domains that are coupled to heterotrimeric G proteins. ET_{A} -R is selective for ET-1 and ET-2, whereas ET_{B} -R binds to ET-1, ET-2 and ET-3 with equal affinity (3, 4). An additional subtype (ET_{C} -R), which is present in non-mammalian species and selectively binds ET-3, has been cloned (5). The functional significance of the ET-R subtypes remains a topic for considerable speculation. In vascular smooth muscle, ET_{A} -R and ET_{B} -R generally coexist. They mediate contraction and, as in other

cell types, help control proliferation (6, 7). ET_{B} -R is probably the only receptor on the vascular endothelium, where it mediates relaxation of the neighboring muscle layer via the release of relaxing and antiproliferative factors such as nitric oxide and prostacyclin (8). ET_{B} -R is also the predominant subtype in epithelial and neural tissues.

In human placenta, ET-Rs were initially identified in crude membrane fractions (9, 10). They appear to be most abundant in first-trimester placentas, and their concentration gradually decreases towards term (11). The human placenta is a very complex endocrine organ and the ET system in each placental cell seems to develop and change during gestation (12). Specific high affinity ET-1 binding sites have been shown on fetoplacental vessels, trophoblasts and plasma membranes purified from the syncytiotrophoblast of term placentas (13–15). Transport through the microvillous membrane is the first step in placental transfer. This trophoblastic membrane, facing the maternal intervillous blood space where high concentrations of immunoreactive ETs (ir ETs) were found (16), is likely to play a major role in exchanges between maternal and fetal circulations.

The present study was designed to determine the ET-R subtypes of microvilli prepared from early gestation and term human placentas. For this purpose, the selective ET_{B} -R agonists sarafotoxin 6c (S6c) (17) and IRL 1620 (18), the selective ET_{B} -R antagonists BQ 788 (19), IRL 2500 (20) and IRL 1038 (21), and the selective ET_{A} -R

antagonist BQ 123 (22) were used as tools to define the ET-R profiles.

Materials and methods

Placentas

Term placentas were all obtained immediately after elective cesarean section from healthy mothers. The cesarean section was performed before labor under thiopental–sodium succinylcholine anesthesia in the 39th week of pregnancy because of diagnosed cephalopelvic disproportion. No preoperative medication except atropine was given. Early gestation placentas were obtained from healthy mothers undergoing legal abortion by vacuum curetage between 8 and 10 weeks of amenorrhea.

Placental microvilli preparation

Placental microvilli were isolated by cold saline extraction and centrifugations using the modification of the method of Smith *et al.* (23) described by Alsat *et al.* (24). Briefly, the villous tissue was cut into small fragments and placed in 0.1 mol/l CaCl_2 to remove blood. All subsequent procedures were performed at 4 °C. The tissue was washed with isotonic Dulbecco's PBS pH 7.4, gently stirred for 30 min in the same buffer containing 10 µg/ml aprotinin, 1 µg/ml leupeptin and 25 µg/ml pepabloc, and the suspension filtered through a single thickness of cheesecloth. The filtrate was centrifuged at 800 *g* for 10 min to remove blood and cell debris and the resulting supernatant was centrifuged at 2500 *g* for 15 min. The microvilli were sedimented from this second supernatant by centrifugation at 50 000 *g* for 30 min. The pellet was suspended in 50 mmol/l Tris–HCl buffer pH 7.4, frozen in liquid nitrogen and stored at –80 °C.

The purity of the membrane preparations was checked by assaying marker enzyme activity and by electron microscopy using previously established procedures (25). Assay of the specific microvillous membrane enzyme, alkaline phosphatase, showed a 25-fold enrichment for early gestation microvillous preparations and a 17-fold enrichment for term microvilli. The purity of placental microvilli was also checked by the lack of adenyl cyclase activity in both the early gestation and term membrane preparations and by ultrastructural studies (data not shown).

Binding assay

Binding studies were performed as previously described (15). Briefly, aliquots of membranes (10 µg protein, in a final volume of 250 µl) were incubated for 60 min at 37 °C with 2–300 pmol/l ^{125}I -labeled ET-1 (^{125}I ET-1) for saturation studies. Specific binding of ^{125}I ET-1 was defined as total binding minus binding not displaced by

1 µmol/l unlabeled ET-1. At ligand concentrations near the dissociation constant (K_d), the non-specific binding was less than 10–15% of the total binding in the early gestation and in the term membrane preparations. Competition experiments were carried out using a fixed concentration of ^{125}I ET-1 (30 pmol/l) and increasing concentrations of competing agents. All binding assays were performed at protein concentrations within the linear range.

The results are expressed, for each stage of pregnancy, as the means $\pm$ S.E.M. of three to six separate experiments each on different placentas, performed singly for early gestation and in duplicate for term placentas. The *n* values in this paper refer to the number of independent membrane preparations from distinct human placentas. Radioligand binding data were analyzed using the Inplot Computer program (Graph-Pad Software, San Diego, CA, USA). The inhibition constant (K_i) was calculated, for individual competition curves, by the equation of Cheng & Prusoff (26), and K_d was determined by Scatchard analysis. Data were also subjected to non-linear least-square curve fitting using EBDA-LIGAND (Biosoft, Cambridge, UK) for simultaneous analysis of replicated competition curves. This enabled us to verify that the mean K_i values for each competition were the same as that averaged from the calculation made on individual curves, and that when applicable a two binding site model provided a significantly better fit, according to the F test, than a one binding site model (27). The protein concentration was determined by the method of Lowry using BSA as standard. Statistical differences were determined using ANOVA and Student's *t*-test for multiple comparisons. Significance was set at $P < 0.05$.

Chemicals

^{125}I ET-1 (specific activity 2000 Ci/mmol) was purchased from Amersham International (Bucks, UK). ET-1, ET-3, S6c, BQ 123 [cyclo (D-Trp-D-Asp-Pro-D-Val-Leu)], BQ 788 [N-*cis*-2, 6-dimethyl-piperidinocarbonyl-L-γ-methyleucyl-D-1 methoxycarbonyl-tryptophanyl-D-norleucine], IRL 2500 [N-(3,5-dimethyl benzoyl)-N-(methyl)-(D)-(4-phenylphenyl)-alanyl-L-tryptophan], IRL 1620 [succinyl-(Glu⁹,Ala^{11,15}) endothelin-1 (8–21)] and IRL 1038 [(cys¹¹-cys¹⁵) endothelin-1(11–21)] were all obtained from Neosystem (Strasbourg, France). Other drugs and chemicals used were of the highest quality available from Sigma Chemicals (St Louis, MO, USA).

Results

Equilibrium studies

^{125}I ET-1 bound to microvillous membranes from early gestation and term placentas in a specific, saturable fashion and with high affinity (Fig. 1). Scatchard plots of

Figure 1 Scatchard analysis of [125 I]ET-1 binding to term (●) and early gestation (○) microvilli. Membranes were incubated with the indicated concentrations of [125 I]ET-1 (2–300 pmol/l) in the presence (non-specific binding) or absence (total binding) of 1 μ mol/l unlabeled ET-1. The data shown represent typical experiments performed in duplicate ($n = 4$).

the specific binding from saturation binding experiments were linear, indicating a single class of high affinity binding sites. The apparent K_d values for early gestation (68 ± 15 pmol/l) and term microvillous membranes (45 ± 8 pmol/l) ($n = 4$) were not significantly different. The density of ET-R (B_{max}) in term microvillous preparations was 54 ± 10 fmol/mg protein. The values of B_{max} for early gestation microvilli varied with the microvillous preparation. The mean value of B_{max} for early gestation was 243 ± 80 fmol/mg protein, which was five times greater than that for term microvilli.

Competition binding experiments

The profiles of ET-R subtypes in microvillous preparations were determined by measuring the binding of [125 I]ET-1 in the presence of ET-R agonists and antagonists. In early gestation microvilli, ET-1, ET-3, S6c and IRL 1620 were all potent competitive inhibitors of [125 I]ET-1 binding (Fig. 2A). ET-1 and ET-3 totally inhibited binding. A monophasic curve was obtained for ET-1 with a K_i value of 35 ± 5 pmol/l. Two K_i values were determined for ET-3: one for high affinity sites ($K_i = 28 \pm 3$ pmol) and one for low affinity sites ($K_i = 4.6 \pm 1.4$ nmol/l). The high affinity sites, which represented 72% of the total receptor population, had a K_i similar to that of ET-1 and were characteristic of the 'non-selective' or ET_B-R subtype (4). The other sites, which represented 28% of total receptors, had a much lower affinity for ET-3 than for ET-1, typical of the ET_A-R subtype (3). These data were in agreement with the finding that the selective ET_B-R agonists S6c and IRL 1620 displaced only 80% of [125 I]ET-1, even up to 1 μ mol/l (Fig. 2A). However, while the IRL 1620 displacement curve was monophasic (Hill coefficient close to unity) with a K_i value of 290 ± 50 pmol/l, S6c displaced the binding with two affinities: a high affinity

Figure 2 Displacement of [125 I]ET-1 from early gestation microvilli by (A) unlabeled agonists ET-1 (○), ET-3 (●), S6c (▲) and IRL 1620 (□), and (B) by unlabeled antagonists BQ 788 (△), IRL 2500 (half-filled squares), IRL 1038 (■) and BQ 123 (◇). Membranes were incubated with [125 I]ET-1 (30 pmol/l) and increasing concentrations of unlabeled ligands. Results are expressed as percentages of control (specific binding in the absence of unlabeled ligand). Non-specific binding, determined in the presence of 1 μ mol/l ET-1, was subtracted from the total binding. Each point is the mean $\pm$ S.E.M. of three to six experiments from different placentas.

K_i value of 26 ± 9 pmol/l (63% of total receptors) and a low affinity K_i value of 47 ± 20 nmol/l (37% of total receptors), suggesting the presence of different ET_B-R subtypes. Competitive experiments with antagonists confirmed these results (Fig. 2B): the selective ET_B-R antagonists BQ 788 and IRL 2500 almost totally displaced [125 I]ET-1 with K_i values of 17 ± 7 and 22 ± 6 nmol/l respectively, whereas the selective ET_B-R antagonist IRL 1038 only partially displaced it. The ET_B-R which is IRL 1038-insensitive has been shown to be of the ET_{B2}-R subtype (28), indicating that in term microvilli, ET_B-Rs were mainly of the ET_{B2}-R subtype. The selective ET_A-R antagonist BQ 123 inhibited only 30% of [125 I]ET-1 binding, even at concentrations of

Figure 3 (A) Effects of BQ 123 (0.1 nmol/l to 1 μ mol/l) ($\diamond$) and 1 μ mol/l BQ 123 plus BQ 788 (10 pmol/l to 1 μ mol/l) ($\blacklozenge$), and (B) effects of BQ 788 (10 pmol/l to 1 μ mol/l) ($\triangle$), and 1 μ mol/l BQ 788 plus BQ 123 (1 nmol/l to 1 μ mol/l) ($\blacktriangle$) on specific [¹²⁵I]ET-1 binding to early gestation microvillous membranes. Each point is the mean $\pm$ S.E.M. of three experiments from three different placentas.

1 μ mol/l, indicating a minority of ET_A-R. We confirmed these observations by investigating the effect of simultaneously blocking ET_A-R with BQ 123 and ET_B-R with BQ 788. Micromolar concentrations of BQ 123 displaced 30% of [¹²⁵I]ET-1 (Fig. 3A), while the remaining 70% were resistant. Concomitant treatment with BQ 123 (1 μ mol/l) + BQ 788 (10 pmol/l to 1 μ mol/l) completely inhibited the BQ 123-resistant component of [¹²⁵I]ET-1 binding. Similar results were obtained when the converse experiment was performed, e.g. total displacement of bound [¹²⁵I]ET-1 by BQ 788 only in the presence of BQ 123 (Fig. 3B). These results indicate that ET_B-R accounts for 72% of the total binding sites on early gestation microvilli, while 28% are of the ET_A-R subtype.

Figure 4 Displacement of [¹²⁵I]ET-1 from term microvilli by (A) unlabeled agonists ET-1 ($\circ$), ET-3 ($\bullet$), S6c ($\blacktriangle$) and IRL 1620 ($\square$), and (B) by unlabeled antagonists BQ 788 ($\triangle$), IRL 2500 (half-filled squares), IRL 1038 ($\blacksquare$) and BQ 123 ($\diamond$). Membranes were incubated with [¹²⁵I]ET-1 (30 pmol/l) and increasing concentrations of unlabeled ligands. Results are expressed as percentages of control (specific binding in the absence of unlabeled ligand). Non-specific binding, determined in the presence of 1 μ mol/l ET-1, was subtracted from the total binding. Each point is the mean $\pm$ S.E.M. of three to six experiments from different placentas performed in duplicate.

In term microvilli, [¹²⁵I] ET-1 binding was inhibited by ET-1, ET-3, and the ET_B-R agonists S6c and IRL 1620 in a monophasic manner (Hill coefficient close to unity), with K_i values of 30 ± 4 , 37 ± 6 , 48 ± 8 and 85 ± 7 pmol/l respectively (Fig. 4A). ET-1, ET-3 and S6c had superimposable competition curves, demonstrating that the main ET-R subtype in term microvillous membranes was ET_B-R. We confirmed this by competition binding experiments using subtype-selective antagonists (Fig. 4B). BQ 788 and IRL 2500, two selective ET_B-R antagonists, displaced all the [¹²⁵I]ET-1 in a monophasic fashion with K_i values of 4.1 ± 0.7 and 1.6 ± 0.1 nmol/l. In contrast, the selective ET_B-R

antagonist IRL 1038 reduced binding by only $24 \pm 9\%$. Consequently, the ET_{B2}-R subtype which was IRL 1038-insensitive seemed to be mainly of the ET_B-R subtype in term gestation microvilli. The specific ET_A-R antagonist BQ 123 had very little effect at concentrations up to $1 \mu\text{mol/l}$.

We examined the possibility that G protein coupling may modulate the binding of [¹²⁵I]ET-1 to the two microvillous membrane preparations in binding experiments with and without guanine nucleotides. Incubation of the ligand with 1 mmol/l GTP γ S had no apparent effect on [¹²⁵I]ET-1 binding to either early gestation or term microvilli (data not shown).

Discussion

The present study demonstrates that specific and high affinity binding sites for [¹²⁵I]ET-1 are expressed on microvillous membranes isolated from early gestation and term human placentas. Scatchard analysis of [¹²⁵I]ET-1 binding indicated that binding affinities for early gestation and term membrane preparations are not significantly different, and that the concentration of ET-Rs on early microvilli is five times greater than that on term microvilli.

In early gestation placenta, competitive experiments demonstrated the presence of a majority of ET_B-R (72% of total receptors), with a predominance of the ET_{B2}-R subtype. ET_A-R represented 28% of total receptors. In term placenta, we observed that only ET_B-R, with a majority being of the ET_{B2}-R subtype, is present on microvillous membranes.

All seven transmembrane cell surface receptors were coupled to G proteins, and guanine nucleotides generally inhibited agonist binding. But we found that a non-hydrolyzable GTP analog (GTP γ S) has no effect on the binding of [¹²⁵I]ET-1 to placental microvillous membranes. This is in accordance with studies demonstrating that GTP γ S does not inhibit [¹²⁵I]ET-1 binding to crude membrane fractions of human placenta (10, 11). In contrast to other G protein coupled receptors, the binding affinity of ET-Rs is not necessarily reduced by guanine nucleotides.

The human placenta has a non-uniform population of ET binding sites, with regional differences in the relative proportions of ET_A-R and ET_B-R subtypes. In term placentas, ET_A-R predominates in the proximal regions of vessels in the chorionic plate and stem villi, whereas ET_B-R is concentrated on intermediate and terminal villi and on the decidua (14, 29). The syncytiotrophoblast, which lines the intervillous space containing maternal blood, can be compared with a fetal endothelial layer. As for other endothelial cells which express only ET_B-R, the term microvillous membrane also bears only ET_B-R. Both the placenta and the trophoblast contain mainly the ET_B-R subtype during the first trimester (30). ET_B-R subtype is also present at higher density on extravillous trophoblast,

suggesting that ET_B-R is involved in regulating the invasion of the extravillous trophoblast (31).

We found that there are five times more ET-Rs on early gestation microvillous placental membranes than on membranes from term placentas. This is compatible with the results obtained in whole placental membranes showing a decrease in the ET-R concentration from the first to the third trimester of pregnancy (13, 14). The term placenta appears to be an important source of ETs. The preproET-1 and ET-3 genes are expressed in the placental villi (32, 33), in syncytiotrophoblasts in culture (34), and, for the preproET-1, in cytotrophoblasts (35). The preterm placenta expressed little preproET-1, but the term placenta had significantly more, suggesting that the concentration of preproET-1 mRNA in human placenta changes during development (36). IrETs have been detected in the syncytiotrophoblast layer of term human placental villi and in the endothelium of feto-placental vessels, in the extravillous cytotrophoblast of the basal and chorionic plates and in trophoblasts in culture (37–39). ET-1 secreted by trophoblasts could reach other nearby placental structures. Because the trophoblast and fetal vessels lie close together, ET-1 may influence regulation of the feto-placental circulation. ETs could also directly enter the maternal intervillous blood space and contribute, along with the ET produced by decidual cells (40), to the large amount of irETs found at term in this circulation, which is specific to the human feto-maternal interface (16). Consequently, these circulating ETs could act in a paracrine way to regulate myometrial contractility, especially at parturition. The high affinity ET-1 binding sites on the microvillous membrane may indicate an autocrine feedback pathway, whereby ET-1 regulates its own synthesis or release and may help specific trophoblast functions. ET-1 and ET-3 both increase the release of progesterone by syncytiotrophoblasts in culture (41), suggesting an autocrine role of ETs in the control of placental steroidogenesis. The fact that ET-3 is more effective than ET-1 in stimulating progesterone production is in agreement with the presence of predominantly ET_B-R subtypes on the microvillous membrane. The mitogenic properties of ETs might also indicate that these peptides are involved in placental growth, as ET-1 stimulates mitogenesis in first-trimester human placental fibroblasts (36) and the proliferation and invasion of first-trimester cytotrophoblastic cells *in vitro* (42). The *in vitro* ET-1 effect on proliferation is mediated by both ET_A-R and ET_B-R subtypes, while the effect of ET-1 on the invasion of trophoblastic cells is mediated by the ET_B-R subtype only (43). We found that the number of ET_A-R on early gestation microvillous membrane accounts for 28% of total receptors. The physiological function of these ET_A-R in placental development remains to be clarified.

The fate of the ETs released from the placenta at the feto-maternal interface is not well understood. Functional ET_B-Rs are clearance receptors in the rat lung and

human astrocytoma U373 MG cells (44, 45). The presence of mainly ET_B-R on the placental microvillous membrane suggested that this membrane may be an important site of ET clearance. ET_B-R could play a major protective role by modulating the concentration of ETs in the maternal intervillous blood space. These receptors could thus be important in disorders of gestation such as pre-eclampsia and premature labor. The etiology of pre-eclampsia is unknown, but the placenta appears to play a major role, since the trophoblasts do not invade the maternal uterine tissues correctly, leading to placental ischemia and altered endothelial cell function, which in turn increases ET production (42, 46, 47). Thus, altered ET_B-R production and/or function in the placental microvillous membrane may contribute to pathogenesis by reducing ET clearance, leading to higher concentrations of circulating ET at the feto-maternal interface.

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