Expression of a functional endothelin (ET$_A$) receptor in human meningiomas

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Endothelin (ET) receptor subtypes (ET$_A$ and ET$_B$) in human meningiomas were characterized using quantitative receptor autoradiography. A single class of high-affinity $^{125}$I-ET-1 binding sites was localized in all meningioma tissue studied (dissociation constant: 2.4 ± 0.3 nM, maximum binding capacity: 3.19 ± 66 fmol/mg (mean ± standard error of the mean for 13 tumors)). Unlabeled ET-1 showed a strong affinity for $^{125}$I-ET-1 binding to tissue sections of the tumors with a 50% inhibiting concentration (IC$_{50}$) of 2.9 ± 0.7 × 10$^{-9}$ M, whereas ET-3 showed a much lower affinity (IC$_{50}$: 8.4 ± 2.5 × 10$^{-6}$ M). Sarafotoxin S6c, a selective agonist for the ET$_A$ receptor, could not compete for $^{125}$I-ET-1 binding to meningiomas. Endothelin-1 significantly stimulated deoxyribonucleic acid (DNA) synthesis in a dose-dependent manner in cultured human meningioma cells. In contrast, no significant stimulation of DNA synthesis occurred with an S6c concentration up to 10$^{-7}$ M. Pretreatment of the meningioma cells with pertussis toxin, a bacterial toxin that adds adenosine 5'-diphosphate-ribose to the α subunit of guanine nucleotide binding (G) proteins such as G$_i$ or G$_o$, induced a concentration-dependent reduction in ET-stimulated DNA synthesis in meningioma cells, but did not affect the epidermal growth factor-induced DNA synthesis. These observations suggest that the ET$_A$ receptor is predominantly expressed in human meningioma tissue and that ET may act as a growth factor on the meningioma cells by interacting with the ET$_A$ receptor and by pertussis toxin-sensitive mechanisms.

KEY WORDS · endothelin receptor subtype · endothelin (ET$_A$) receptor · sarafotoxin S6c · pertussis toxin · meningioma · DNA synthesis

ENDOTHELIN (ET), a potent vasoconstrictor peptide originally isolated from a conditioned medium of cultured porcine aortic endothelial cells, also acts as a growth-promoting factor in vascular smooth-muscle cells and in cultured glial cells. Specific high-affinity binding sites for ET have been demonstrated in the central nervous system (CNS) and in the cardiovascular system. Subsequent studies revealed the existence of peptides ET-1 and ET-3 in the CNS and their neurophysiological roles. Messenger ribonucleic acids encoding for ET-1 and ET-3 have been found in human CNS. Specific ET receptors linked to mitogenesis were found in rat glioma cell lines; hence, this peptide may have a role in the cell growth of certain brain tumors. Of particular interest is the finding that specific ET binding sites, candidates for biologically active ET receptors, have been found in human glioma tissues using quantitative receptor autoradiography. Additional work revealed that A-172, a human glioma cell line, expresses functional ET receptors that induce intracellular Ca$^{2+}$ accumulation and inositol 1,4,5-triphosphate formation in vitro.

Two ET receptor subtypes (ET$_A$ and ET$_B$) have been cloned from the complementary deoxyribonucleic acid (DNA) library of bovine and rat lungs, respectively. In the order of affinity for ET$_A$, ET-1 is similar to ET-2 which is greater than ET-3, whereas ET$_B$ shows an equipotent affinity for all three ET's. In addition, sarafotoxin S6c, a snake venom toxin, acts as a highly selective ET$_B$ receptor agonist. Both subtypes belong to the superfamily of guanine nucleotide binding protein-coupled receptors. Differential expression and the distinct function of each receptor subtype have been
found in tissues or cell culture systems. Endothelin receptor subtypes in human brain tumors were given less attention. Specific ET receptor subtypes in human meningiomas were characterized using quantitative receptor autoradiography. The biological significance of these subtypes in cell growth became evident in 3H-thymidine incorporation studies.

Materials and Methods

Tumor Specimens

Fresh surgically excised human meningioma specimens obtained from 13 patients were immediately placed in liquid nitrogen and stored at −80°C for less than 1 month. The histological subtypes of the meningiomas are listed in Table 1. Sections 20 μm thick were cut in a cryostat at −20°C, thaw-mounted on gelatin-coated glass slides, and dried overnight in a desiccator at 4°C while under vacuum.

Quantitative Receptor Autoradiography

Sections were labeled in vitro with 125I-ET-1 (specific activity: 2200 Ci/mmol) using experimentally determined incubation parameters. Each section was pre-incubated for 10 minutes at 22°C in 50 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl, 10 mM Na2 ethylenediamine tetra-aceitic acid (EDTA), bacitracin (1 mg/ml), leupeptin (4 μg/ml), chymostatin (2 μg/ml), 10 μM phosphoramidon, and 0.3% protease-free bovine serum albumin (BSA). Sections were then incubated for 48 hours at 4°C (various periods of incubation were used for time-course experiments) in fresh buffer containing the appropriate ligand (we had earlier con-

<table>
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<th>Case No.</th>
<th>Age (yrs)</th>
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<th>Histological Type</th>
<th>Kd (nM)</th>
<th>Bmax (fmol/mg)</th>
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* Consecutive tissue sections were incubated with 0.1 nM 125I-ET-1 in the presence of increasing concentrations of unlabeled ET-1. To determine Kd and Bmax, binding data were analyzed using the LIGAND computer program.

Culture of Human Meningioma Cells

Immediately after the removal of tumor from three patients (Cases 6, 9, and 12), the specimens were minced into 1-sq mm pieces and incubated in 0.125% trypsin and 0.02% EDTA solution for 15 minutes at 37°C. Trypsin activity was inhibited by adding Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). After the cell pellet was centrifuged at 1500 G, it was resuspended in complete medium (DMEM supplemented with 10% FBS) and the cells were mechanically dispersed. The cells were seeded in 75-sq cm tissue culture flasks and cultured at 37°C in 5% CO2/95% air with 100% humidity until subconfluence occurred at 1 week.

The morphological appearance of the cultured meningioma cells was similar to that described in previous studies. The cells are polygonal in shape, have ovoid or round nuclei with one to four nucleoli, and manifest formations of whorls.

3H-Thymidine Incorporation Studies

To test the effect of ET analogs (ET-1 and S6c) on stimulation of DNA synthesis in cultured human meningioma cells, 3H-thymidine incorporation studies...
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were performed according to a modification of the method of Russell, et al., as described previously by us. The cultured meningioma cells, after a single passage, were treated with 0.1% trypsin and 0.02% EDTA solution, suspended in DMEM/Ham's F-12 medium (1:1) containing 0.1% FBS and plated onto 24-well tissue culture plates at a density of 3 x 10^4 cells/well. The cells were cultured at 37°C for 48 hours; next, the medium was changed to serum-free DMEM/F-12 (1:1) and the preparation was incubated for another 48 hours. The cultures were then incubated with increasing concentrations (10^{-12} to 10^{-7} M) of ET-1, S6c, and 5% FBS, after being washed with fresh DMEM/F-12 (1:1). The control cultures were incubated with DMEM/F-12 (1:1) containing 0.1% BSA. After incubation for 24 hours, 3H-methyl thymidine (0.5 μCi/well) was added to the cultures and incubation was continued for another 4 hours at 37°C. The reaction was halted by aspiration of the incubating solution and washing the cells three times with ice-cold phosphate-buffered saline. The cultures were then incubated with 5% trichloroacetic acid (TCA) at 4°C for 20 minutes. The cultures were washed with fresh 5% TCA, after which they were dissolved in 1% sodium dodecyl sulfate. The resultant solution was added to 10 ml of a scintillation cocktail and counts were made using a scintillation counter. Results were analyzed by unpaired t-test; a p value of less than 0.05 was considered statistically significant.

† Scintisol EX-H scintillation cocktail manufactured by Wako Pure Chemical Industries, Ltd., Osaka, Japan.

To investigate the possible involvement of guanine nucleotide binding proteins in the mitogenic action of ET, we compared the effects of pertussis toxin on ET-1 and epidermal growth factor (EGF)-induced 3H-thymidine incorporation on meningioma cells. Cultures of meningioma cells (Case 6) were incubated for 3 hours with increasing concentrations (0.1 to 100 ng/ml) of pertussis toxin prior to exposure to 10^{-7} M ET-1 or 10^{-5} M EGF. Radioactivity of 3H-thymidine incorporated into DNA was determined as described above.

Results

Binding Conditions

Figure 1 shows the time course of 125I-ET-1-specific binding in meningioma tissue sections at 4°C. The binding reached equilibrium after 24 hours and remained stable for at least 72 hours. Thus, we used 48 hours as an incubation time in the following experiments.

Localization of 125I-ET-1 Binding Sites

Specific ET binding sites were localized in the tissue sections of all human meningiomas tested. Conversely, normal dural membrane adjacent to the tumor tissue did not exhibit specific ET binding. Nonspecific binding was less than 5% of the total binding. Diffuse and dense ET binding was observed in most cases (Fig. 2). Identification of precise cell types exhibiting these binding sites in tissue sections of the tumors was not feasible with our quantitative macro-autoradiography system because of the inherent limitation in the resolution of the technique. However, when compared to adjacent sections stained with hematoxylin and eosin, ET binding sites showed a nonvascular pattern of distribution, as was noted in human glioma tissues.

Characterization of ET Receptor Subtypes in Meningiomas

The 125I-ET-1 binding sites in human meningiomas were characterized using the cold-ligand (unlabeled ET-1) saturation method. The LIGAND computer program fitted the data for the one-site model statistically better than did the two-site model, as evidenced by a straight line on the Scatchard plot (Fig. 3 inset). The binding parameters K_d and B_max are listed in Table 1. A single class of high-affinity sites was localized in all 13 meningiomas studied, regardless of histological subtypes of the tumor specimens (K_d: 2.4 ± 0.3 nM, B_max: 319 ± 66 fmol/mg).

Specific ET binding in the tissue sections of meningiomas was completely displaced by incubation in the presence of ET-1 with an IC_{50} of 2.9 ± 0.7 x 10^{-8} M, whereas the affinity of ET-3 in these tissues was three to four orders of magnitude lower than that of ET-1 (IC_{50}: 8.4 ± 2.5 x 10^{-6} M). In contrast, no significant displacement occurred with a concentration of S6c up to 10^{-6} M in meningioma tissue sections (Figs. 2 and 3 and Table 2).
The stimulation of $^3$H-thymidine incorporation into meningioma cells (Case 6) in the presence of $10^{-7}$ M ET-1 or $10^{-7}$ M EGF was about 200% or 650% above the basal level, respectively (apparent units of the data (cpm/3 x 10^5 cells): basal stimulation in serum-free condition = 3935 ± 336; $10^{-7}$ M ET-1 = 8269 ± 1061 (p < 0.01 vs. basal stimulation); $10^{-7}$ M EGF = 26,169 ± 4226 (p < 0.0001 vs. basal stimulation)). A 3-hour preincubation period of the cells with pertussis toxin resulted in a concentration-dependent reduction of ET-1-induced DNA synthesis, whereas EGF-induced DNA synthesis was not affected (Fig. 5). The highest concentration of pertussis toxin (100 ng/ml) reduced DNA synthesis by 40% (p < 0.01).

**Discussion**

Based on quantitative receptor autoradiography, the ETA receptor was predominantly expressed in human meningiomas. Our results show that only the ETA receptor is functionally associated with intracellular signaling mechanisms coupled to DNA synthesis in meningioma cells. This is the first report of evidence for the expression of the biologically active ETA receptor on human brain tumor cells.

Other workers have revealed that the ETA receptor is involved in the mitogenic action of ET in cultured smooth-muscle cells, using BQ-123, a selective ETA receptor antagonist. In Swiss 3T3 fibroblasts, ET stimulated phospholipase C with the production of inositol triphosphate and 1,2-diacylglycerol, thereby leading to an increase in intracellular free Ca^{2+} mobilization through ETA receptors present on the cell surface. Furthermore, the ETA receptor has been shown to mediate the stimulation of anchorage-independent cell growth by ET in NRK 49F fibroblasts, a cell line derived from peripheral mesenchymal tissue. Meningiomas are believed to originate from CNS mesenchymal tissues, including arachnoidal cap cells (cells forming the outer lining of the arachnoid membrane) and related cells such as arachnoidal fibroblasts.

In conjunction with the present observation of enhanced expression of the functional ETA receptor in meningiomas, we believe that the ETA receptor may also have an important biological role in the proliferation of neoplastic cells derived from CNS mesenchymal tissues such as the leptomeninges.

Although precise EC_{50} values were not determined, the affinity of ET-1 for stimulation of DNA synthesis in cultured meningioma cells seems higher than that obtained in a competitive study using meningioma tissue sections and autoradiography. This discrepancy may be partially explained by differences in the preparations used. To minimize the possibility that the biological properties of CNS tumor cells might be altered during cell culture, we used only cells after a single passage. However, cultured cells may not always exhibit the same receptor characteristics as those in original tissue *in situ*. Meningioma cells during the logarithmic growth phase (subconfluent cultures), which we used in our $^3$H-thymidine incorporation study, might exhibit a higher affinity for the ETA receptor.

**$^3$H-Thymidine Incorporation Studies**

Incubation of meningioma cells (from Cases 6, 9, and 12), which were made quiescent by serum deprivation with increasing concentrations (10^{-13} to 10^{-7} M) of ET-1 for 24 hours, induced a marked elevation of DNA synthesis in a dose-dependent manner. During this period, $^3$H-thymidine incorporation into cells increased about 200% to 300% above basal levels (Fig. 4). The stimulation activity of ET-1 was 35% to 40% of that induced in the presence of 5% FBS. To determine ET receptor subtypes mediating the mitogenic action of ET, $^3$H-thymidine incorporation into meningioma cells was examined in the presence of increasing concentrations (10^{-13} to 10^{-7} M) of S6c, a selective agonist for ET_B receptors; S6c failed to stimulate $^3$H-thymidine incorporation in concentrations up to 10^{-7} M (Fig. 4). Although precise 50% effective concentration (EC_{50}) values were not determined, ET-1 induced a significant DNA synthesis, even in a concentration as low as 10^{-12} M (Cases 9 and 12) or 10^{-11} M (Case 6) (Fig. 4).
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when compared with receptors in tissue sections of meningiomas. The apparent binding affinities determined by the $K_a$ of ET-1 binding to meningiomas ($K_a$: $10^{-10}$ to $10^{-9}$ M) were comparable to reported values for the binding to other nonvascular tissues.\textsuperscript{7,14,26,33,54,56} It has not yet been determined whether the binding parameters ($K_a$ and $B_{max}$) differ between tissue sections and cultured cells of meningiomas. Alternatively, the half-maximum concentration of ET-1 for stimulation of DNA synthesis ($EC_{50}$) in cultured meningioma cells could be lower than that for inhibition of $^{125}\text{I}$-ET-1 binding ($IC_{50}$) to the cell-surface receptors, as previously explained by the conception of a "spare receptor" (receptor reserve).\textsuperscript{12,26} A similar difference between $EC_{50}$ and $IC_{50}$ has been reported in the $ET_A$ receptor on human neuroblastoma cells in the stimulation of phosphoinositide hydrolysis.\textsuperscript{55}

It is noteworthy that stimulation of DNA synthesis in cultured cells from an anaplastic meningioma (Case 12, Fig. 4A) was higher than in a transitional meningioma (Case 9, Fig. 4B) or a fibroblastic meningioma (Case 6, Fig. 4C). Since no significant difference in ET binding characteristics ($B_{max}$ and $K_a$) among histological subtypes of meningiomas was found in this study,
Capillary endothelial cells can produce and secrete ET, and meningioma is a hypervascular CNS tumor. Thus in a paracrine fashion, the endothelium of the tumor vessels might be a source of ET for meningioma cells. Plasma-derived ET may be another source of peptide for meningioma cells, since the blood-tumor barrier in meningiomas is incomplete and reported ET concentrations in human plasma (1 to 20 pg/ml), which approximately correspond to $10^{-13}$ to $10^{-12}$ M, are sufficient to stimulate DNA synthesis in meningioma cells, as demonstrated in this study.

The transmembrane signals mediating the biological action of ET in the meningioma cells were not fully investigated in this report; however, pretreatment of meningioma cells with pertussis toxin induced a concentration-dependent reduction in ET-stimulated DNA synthesis of up to 60% of the control level; these findings are consistent with data on cultured aortic smooth-muscle cells. This effect was not due to any nonspecific toxicity, since EGF-induced DNA synthesis was unaffected. Insensitivity to pertussis toxin is a common feature of growth factors acting via tyrosine kinase-dependent mechanisms. Our data suggest that ET may stimulate mitogenesis in meningioma cells partially through a pertussis toxin-sensitive pathway.

Endothelin receptors are functionally coupled to effectors by guanine nucleotide binding proteins. Pertussis toxin, a bacterial toxin secreted by Bordetella pertussis (the "whooping cough" bacterium), catalyzes transfer of the adenosine 5'-diphosphate-ribose from the oxidized form of nicotinamide adenine dinucleo-

**FIG. 4.** Graphs showing the effects of endothelin (ET) analogs on $^3$H-thymidine incorporation into DNA of cultured meningioma cells derived from an anaplastic type (Case 12, A), a transitional type (Case 9, B), and a fibroblastic type (Case 6, C). The cells, preincubated in serum-free Dulbecco's modified Eagle's medium/Ham's F-12 medium (1:1) for 48 hours, were incubated with increasing concentrations of ET-1 (circles) or sarafotoxin S6c (squares) for 24 hours, then pulsed with 0.5 μCi/well of $^3$H-thymidine for 4 hours. The control values were defined as the incorporation in the presence of 0.1% bovine serum albumin and were 1700 (A), 1900 (B), and 1570 (C) cpm/3 × 10⁴ cells. Values are means ± standard error of the means (vertical bars) for triplicate determinations.
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The cultured cells derived from a fibroblastic meningioma (Case 6) were preincubated for 3 hours with increasing concentrations of pertussis toxin before exposure to $10^{-7}$ M ET-1 (closed circles) or $10^{-7}$ M epidermal growth factor (open circles) dissolved in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (1:1) containing 0.5 μCi/well of $^3$H-thymidine. Radioactivity incorporated into DNA was determined as described in the Materials and Methods section. Data are means ± standard error of the means (vertical bars) for triplicate determinations. The control values were 3940 cpm/3 × 10⁴ cells and 26,170 cpm/3 × 10⁴ cells for ET-1 and EGF, respectively. The baseline incorporation of $^3$H-thymidine into the cells in the presence of serum-free medium was 3940 cpm/3 × 10⁴ cells. Statistical differences between ET-1 and EGF were analyzed using the unpaired t-test. * = p < 0.05; ** = p < 0.01.

Conclusions

The results suggest that the specific ET₄ receptor may play an important role during the proliferation of meningioma cells. The ET₄ receptor, expressed in human meningiomas in vivo, is probably associated with intracellular signal transduction mechanisms coupled to DNA synthesis via pertussis toxin-sensitive mechanisms. In this regard, newly developed specific antagonists for the ET₄ receptor, such as BO-123,BO-123 or BO-153,BO-153 might be useful as antitumor compounds for patients with meningioma, especially in inoperable, invasive, or malignant cases. The investigation of the effect of selective ET₄ receptor antagonists on proliferation of meningioma cells in vitro is under way in our laboratory.

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References


Lin WW, Lee CY, Chuang DM: Comparative studies of phosphoinositide hydrolysis induced by endothelin-related peptides in cultured cerebellar astrocytes, C3 glioma, and cerebellar granule cells. *Biochem Biophys Res Commun* **168**:512–519, 1990


Russell WE, Van Wyk JJ, Pledger WJ: Inhibition of the mitogenic effects of plasma by a monoclonal antibody to somatomedin C. *Proc Natl Acad Sci USA* **81**:2389–2392, 1984


Wilkes LC, Border MR: Characterization of endothelin re-

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