Endothelin Receptors in Human Myometrium and Endometrium

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We investigated endothelin (ET) receptors in the endometrium and the myometrium obtained from pre-menopausal 39-, 40-, and 49-year-old women, using quantitative receptor and emulsion autoradiographic methods with ^{125}I-endothelin-1 (^{125}I-ET-1) [radioligand for the ET receptors], BQ-123 [specific antagonist of the ET_A receptor], and sarafotoxin S6c [agonist for the ET_B receptor]. The radioligand binding reached a maximum at 36 hr of incubation and a plateau was maintained for up to 72 hr. No degradation of ^{125}I-ET-1 during incubation was observed at 72 hr. In cold-ligand saturation binding studies ^{125}I-ET-1 bound with a single affinity to the endometrium and the myometrium. In the myometrium the ET_A receptor was found to be predominantly present, and also a considerable amount of the ET_B receptor was quantified. There was a significant amount of the ET_A and the ET_B receptor in the endometrium, and the emulsion autroradiographic technique we used revealed the ET_A and the ET_B receptor to be on stroma cells and glandular epithelial cells, respectively. The existence of the the ET_A and the ET_B receptor in the endometrium and the myometrium supports the physiological significance of ET family of peptodes in the human uterus.

Key words : quantitative receptor autoradiography, emulsion autoradiography, endothelin, ET_A receptor, ET_B receptor, human uterus, endometrium, myometrium

Introduction

The endothelin family of peptides (ETs) initially purified from the conditioned medium of porcine aortic endothelial cells is a potent vasoconstrictor[1] and proliferative agent[2]. The functions of ETs are mediated through specific receptors, as two of which have been cloned. The ET_A receptor preferentially binds ET-1, while the ET_B receptor recognizes ET-1, ET-2 and ET-3 with a similar affinity[3,4].

There have been reports on the existence of a complete set of system composed of ETs production and ET receptors in the uterus[5-12]. Studies on the human myometrium have shown that the ET_A may be important in uterine contractility[5,13]. Other studies showed that the density of ET receptors increases in a dose dependent manner in the presence of 17 β -oestradiol and progesterone[14]. Clinical studies revealed a close relationship between the blood level of ovarian steroids and the density of ET receptors in the myometrium[10]. Thus, ETs seem to function in the uterus, presumably interacting with the ET_A and the ET_B receptor[3,4]. Possible paracrinal or autocrinal functions of ETs in the uterus led to use quantitative receptor and light-microscopical emulsion autoradiographic methods to investigate ET receptors in the human uterus.

Materials and Methods

Patients

Samples of endometrium and myometrium were obtained from three pre-menopausal women, aged between 39-(case No. 1), 40-(case No. 2), and 49-year-old women (case No. 3), undergoing abdominal hysterectomy because of uterine leiomyoma. After removal, all tissues were placed on ice and small pieces of approximately $5 \times 5 \times 5$ mm uterine tissues containing myometrium and endometrium were dissected, respectively. All these patients had not been treated with hormones in 3 months previous to the operation. Informed consent was obtained from each patient and the study was approved by the local ethical committee of the Nagasaki University School of Medicine.

Tissue preparation

Surgically excised tissues of human endometrium and myometrium were immediately washed with ice-cold saline and immersed in isopentane at $-30°C$. These samples were stored at $-80°C$ and used for the study within 5 weeks. Consecutive, 20-μ m-thick sections were cut in a cryostat at $-20°C$, thaw-mounted on gelatin coated glass slides, and dried overnight under vacuum at $4°C$.

Quantitative Receptor Autoradiography

Related tissue sections for the binding study were labeled in vitro with ^{125}I-ET-1 (specific activity, -81.4 TBq/mmol, New England Nuclear, U. S. A.) in 2.0 ml of incubation buffer. Briefly, after preincubation in the incubation buffer 50 mM Tris-HCl buffer (pH 7.4),

containing 100 mM NaCl, 5 mM MgCl₂, 10 mM EDTA-2Na, 10 μ M phosphoramidon, 1 mg/ml bacitracin 4 μ g/ml leupeptin, 2 μ g/ml chymostain, and 0.3% bovine serum albumin (proteinase-free), for 10 min at room temperature (23℃), consecutive tissue sections were incubated with ¹²⁵I-ET-1 in the absence (total binding) or presence of unlabeled 1.0 μ M ET-1 (non-specific binding). To characterize specific ¹²⁵I-ET-1 binding, we incubated consecutive sections with 0.83 nM of ¹²⁵I-ET-1, an excess amount of the radioligand, in the absence or presence of increasing concentrations of unlabeled ET-1, sarafotoxin S6c, selective agonist for the ETв receptor[15] and BQ-123, a highly selective antagonist for the ETₐ receptor[16,17], ranging from 10 pM to 10 μ M. After incubation, the slides were washed three times (1 min each) at 4℃ in 50 mM Tris-HCl buffer (pH 7.4), and rinsed quickly in ice-cold distilled water.

In the inital experiment, we investigated the appropriate incubation time, under the conditions described above, Incubations were done at 4℃ for 30 min, 1 hr, 2 hr, 6 hr, 10 hr, 24 hr, 36 hr, 48 hr and 72 hr. After these incubations, degradation of ¹²⁵I-ET-1 in the incubation buffer used was checked using high-performance liquid chromatographic (HPLC) analysis. One hundred ml sample of the incubation buffer with the radioligand was injected onto the HPLC system composed of a 6000A pump and reverse-phase μ Bondasphere C₁₈ column (5 μ m C₁₈, 3.9×150 mm, Waters, U. S. A.). ¹²⁵I-ET-1 was separated using a linear gradient of 12% to 45% acetonitrile in 0.1% (v/v) trifluroacetic acid, at a rate of 1.0 ml/min. Fractions of 2.0 ml were collected and the radioactivity was detected by γ - counting. A quantitation of ¹²⁵I-ET-1 binding sites in the related tissue sections was made with the computerized radioluminographic imaging-plate system[18,19]. The dry-labeled sections were exposed to a radioluminographic imaging plate coated with fine photostimulable phosphor crystals (BaFBr : Eu²⁺, Type BAS Ⅲ, Fuji Photo Film Co., Japan) with calibrated ¹²⁵I-standards ([¹²⁵I], micro-scales, Amersham, U. K.). The autoradiograms obtained were analyzed with the radioluminographic imaging-plate system (Bio-imaging analyzer BAS 2000, Fuji Photo Film Co.). The values for photostimulated luminescence obtained directly from the imaging plates by the computerized scanning system were converted to the bound radioactivity of the section, based on a comparison with standard curves for sets of standards run each autoradiogram. To obtain autoradiograms of a higher resolution, the dry-labeled sections were apposed against Hyperfilm-³H (Amersham) and the films were developed with a D19 developer (Eastman Kodak, U. S. A.) for 7 min at 4℃[20,21].

Emulsion autoradiography

To observe the cellular localization of ¹²⁵I-ET-1 binding sites, emulsion autoradiography was done. Radiolabeled sections were coated with NTB-2 nuclear emulsion (Eastman Kodak) and processed for light microscopic autoradiography. After exposure to Hyperfilm-³H, related tissue sections were defatted in xylene, rinsed in 100% ethanol, air dried, and then coated with NTB-2 nuclear emulsion at 42℃ for 5 sec. Coated slides were stored in dark at 4℃ for 4 days. After exposure to the nuclear emulsion, the slides were developed using a Kodak D19 developer at 16℃ for 5 min, rinsed briefly in distilled water and fixed. After washing in distilled water for 1 hr, slides were then counterstained with hematoxylim followed by coverslips.

Materials and data analysis

The data obtained by quantitative receptor autoradiographic studies were analyzed using the LIGAND computer program[22,23] to calculate binding parameters, dissociation constant (Kd), maximum binding capacity (Bmax) and inhibition constant (Ki). BQ-123 [cyclo (-D-Asp-Pro-D-Val-Leu-D-Trp)] was a gift from the Banyu Pharmaceutical Co., Japan. Peptides and drugs used were purchased from the Peninsula Lab., U. S. A., and the Peptide Institute Inc., Japan.

Results

In the initial experiments done at 4℃ with 0.83 nM of ¹²⁵I-ET-1, we found that specific ¹²⁵I-ET-1 binding to the uterine sections reached a maximum at 36 hr, and a plateau was maintained for up to 72 hr (Fig. 1A). The radioligand was stable during the 72 hr incubation time, as the HPLC analysis revealed no degradation of ¹²⁵I-ET-1 in the incubation buffer (Fig. 1B). Therefore, the following binding experiments were carried out at 4℃ for 48 hr.

Under the binding condition, we observed considerable amounts of specific ¹²⁵I-ET-1 binding in the myometrium of three women studied here (Fig. 2). Unlabeled ET-1 significantly diminished ¹²⁵I-ET-1 binding densities from all sections, at a concentration of 1.0 μ M (Fig. 2D, E, and F). These non-specific binding amounts were less than 5% of the total binding. The section A obtained from 39-years-old-woman had the endometrium with a considerable amount of specific ¹²⁵I-ET-1 binding sites (e in Fig. 2A), and these binding sites were of higher density than that in the myometrium (m in Fig. 2A).

¹²⁵I-ET-1 binding sites in the human myometrium were characterized, in consecutive and related tissue sections, in the presence of increasing concentrations of unlabeled ET-1, BQ-123, a selective antagonist for the ETₐ receptor[16,17], and sarafotoxin S6c, an agonist for the ETв receptor[17]. Figure 3 shows typical receptor autoradiograms of binding sites in related tissue sections obtained from 39-years-old woman by incubating consecutive tissue
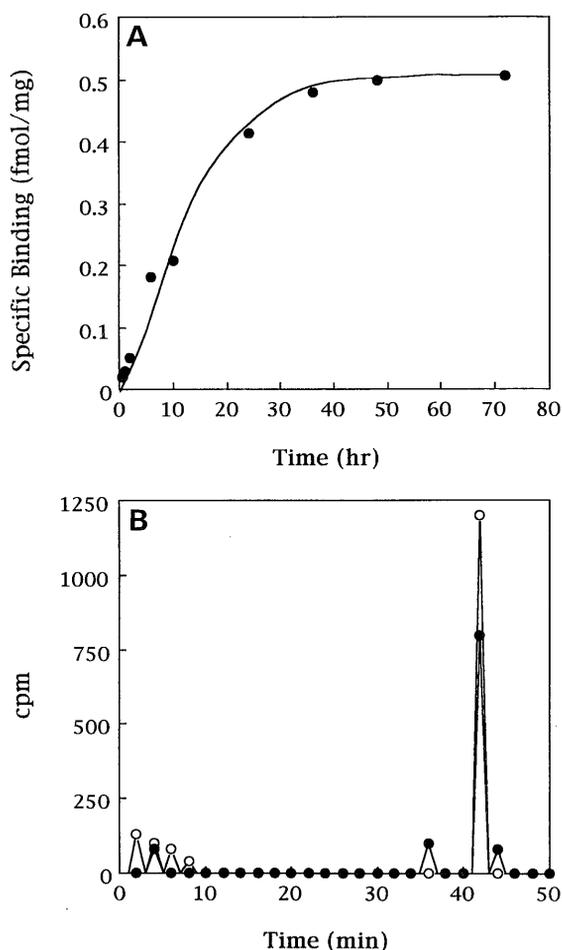
Fig. 2. Receptor autoradiographic localization of ^{125}I-endothelin-1 (^{125}I-ET-1) binding sites in the human uterus. Consecutive, 20-μ m-thick tissue sections obtained from 39-(A, D), 40-(B, E), and 49-years-old woman (C, F) were labeled with 0.83 nM of ^{125}I-ET-1 in the absence (total binding, A, B, C) or presence of 1.0 μ M ET-1 in the absence (total binding, A, B, C) or presence of 1.0 μ M ET-1 (non-specific binding, D, E, F), in vitro. After incubation, dried sections were exposed to Hyperfilm-^{3}H for 2 days. A significant amount of specific ^{125}I-ET-1 binding sites is detected in the endometrium (e) and the myometrium (m). Scale bar = 3 mm.

Fig. 1. (A) Effect of incubation time on specific binding of ^{125}I-endothelin-1 (^{125}I-ET-1) to human uterus sections. Each point represents the mean of four determinations. Related tissue sections obtained from 39-years-old woman were incubated at 4 ℃ with 0.83 nM ^{125}I-ET-1 in the absence or presence of unlabeled 1.0 μ M ^{125}I-ET-1.

(B) High-performance liquid chromatographic analysis of ^{125}I-ET-1 before (open circle) and after (closed circle) incubation of 72 hr at 4℃. The sample was loaded on a reverse-phase μ Bondasphere C$_{18}$ column (3.9×150 mm) and eluted at 1.0 ml /min with a linear gradient of 12% to 45% acetonitrile in 0.1% (v/v) trifluroacetic acid. Fractions of 2.0 ml were collected and the radioactivity was detected by γ -counting.

sections with ^{125}I-ET-1 in the absence (total binding, Fig. 3A), in the presence of 10 μ M BQ-123 (Fig. 3B), 1.0 μ M ET-1 (non-specific binding, Fig. 3C), and 10 μ M sarafotoxin S6c (Fig. 3D). BQ-123 significantly inhibited ^{125}I-ET-1 binding to the myometrium, however, dot-shaped binding sites were cleary appeared in the autoradiograms (Fig. 3B). There was a considerable binding remained in the endometrium in the presence of BQ-123. In contrast to the ET$_A$ drug, the compound acting for the ET$_B$ receptor, sarafotoxin S6c also reduced ^{125}I-ET-1 binding density in the
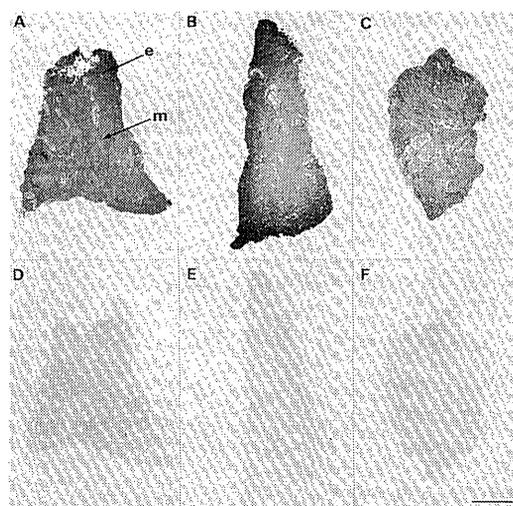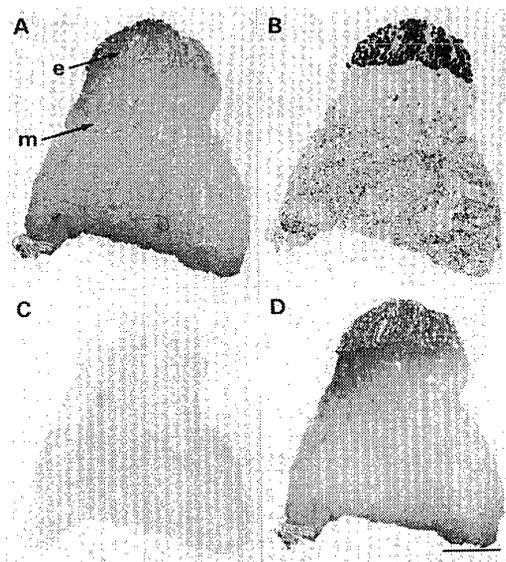
Fig. 3. Receptor autoradiographic characterization of specific ^{125}I-endothelin-1 (^{125}I-ET-1) binding sites in the human uterus. Consecutive, 20- μ m-thick tissue sections obtained from 39-years-old woman were incubated with 0.83 nM ^{125}I-ET-1 in the absence (total binding, A) or presence of 10 μ M BQ-123 (B), 1.0 μ M ET-1(C) and μ M sarafotoxin S6c (D) and exposed to Hyperfilm-^{3}H for 2 days. Scale bar = 3 mm.

myometrium. A significant binding was observed in the endometrium in case of the ETʙ compound. Interestingly, we noted a difference between the binding images in the endometrium remained in the presence of ETᴀ and ETʙ compounds. This means a different localization of the ETᴀ and the ETʙ receptor within the area of endometrium.

^{125}I-ET-1 binding characteristics were examined in cold-ligand saturation and displacement studies using related consecutive tissue sections in the presence of a fixed amount of ^{125}I-ET-1 (0.83 nM) and increasing concentrations of unlabeled ET-1, BQ-123 and sarafotoxin S6c. Typical cold-ligand saturation and displacement curves obtained from binding to the myometrium done on the related tissue sections of 39-years-old-woman are shown in Fig. 4A. Unlabeled ET-1 monophasically inhibited ^{125}I-ET-1 binding to the myometrium area. The computer program LIGAND fitted the data obtained from the cold-ligand saturation study to a one-site model, as evidenced by the straight line of the Scatchard plots (Fig. 4B). The Kd,and the Bmax of the myometrium obtained from three cases of women, case No. 1 of 39-years-old-woman, case No. 2 of 40-years-old-woman, and case No. 3 of 49-years-old-woman are listed in Table 1.

As shown in Fig. 4A and Table 2, BQ-123, a selective antagonist of the ETᴀ receptor, and sarafotoxin S6c, an agonist for the ETʙ receptor, significantly inhibited ^{125}I-ET-1 binding to the myometrium. With the maximum dose of 10 μ M, BQ-123 inhibited 58% of specific binding and 42% of specific binding remained in the case No. 1. Sarafotoxin S6c was also a potent inhibitor in specific binding of ^{125}I-ET-1 to the myometrium. Forty seven% of specific binding was inhibited by 10 μ M of sarafotoxin S6c. As listed in Table 2, we obtained the Ki values of three cases examined in this study to be 5 to 8 nM for BQ-123 and 1.5 to 2.5 nM for sarafotoxin S6c. The LIGAND program also calculated the receptor occupation values of each inhibitor. In the case No. 1, BQ-123 and sarafotoxin S6c occupied the amount of 0.35 and 0.26 fmol/mg, respectively, among the total ET receptor density of 0.67 fmol/mg, a Bmax value obtained from the cold-ligand saturation sutdy. This indicates that BQ-123-sensitive ETᴀ receptor and sarafotoxin S6c-sensitive ETʙ receptor
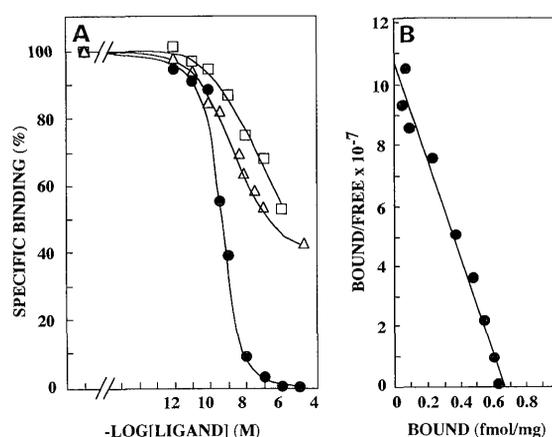

Fig. 4. Typical cold-ligand saturation and displacement studies of ^{125}I-endothelin-1 (^{125}I-ET-1) binding to the myometrium done on use of related tissue sections obtained from 39-years-old woman (A). Consecutive tissue sections were incubated with 0.83 nM ^{125}I-ET-1 in the absence or presence of increasing concentrations of unlabeled ET-1 (closed circle), BQ-123 (open triangle), or saraforoxin S6c (open square). A scatchard plor (B) was obtained by displacing the binding of 0.83 μ M ^{125}I-ET-1 by unlabeled ET-1 (closed circle in A), by using the LIGANDcomputer program.

Table 1 Binding parameters of specific ^{125}I-endothelin-1 binding sites in human myometrium tissues

Case No	Age (years)	Histological Tissue Type	ET-1	
			Kd	Bmax
1	39	myometrium	0.41	0.67
2	40	myometrium	0.18	0.26
3	49	myometrium	0.22	0.49

*The experiments were done using tissue sections labeled with 0.83 nM of ^{125}I-endothelin-1 (^{125}I-ET-1) in the presence of increasing concentrations (10 pM-10 μ M) of unlabeled ET-1. To determine binding parameters, data were analyzed using the LIGAND computer program. Bmax, maximun binding capacity (fmol/mg); Kd, dissociation constant (nM).

Table 2 Binding parameters of specific ^{125}I-endothelin-1 binding sites in human myometrium tissues*

Case No	Age (years)	Histological Tissue Type	BQ-123		S6c		ETᴀ : ETʙ
			Ki	Receptor occupation	Ki	Receptor occupation	
1	39	myometrium	6.91	0.35	2.42	0.26	52 : 39
2	40	myometrium	7.85	0.12	1.49	0.13	46 : 50
3	49	myometrium	4.98	0.17	2.38	0.13	35 : 27

*The experiments were done using tissue sections labeled with 0.83 nM of ^{125}I-endothelin-1 in the presence of increasing concentrations (10 pM-10 μ M) of unlabeled BQ-123 and sarafotoxin (S6c). To determine binding parameters, data were analyzed using the LIGAND computer program. Receptor occupation (fmol/mg); Ki, inhibition constant (nM).
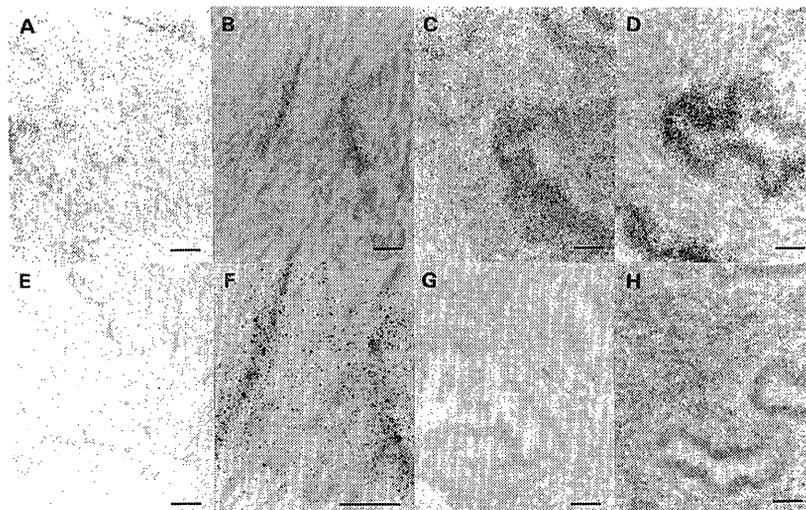
Fig. 5. Emulsion autoradiographic localization of specific ^{125}I-endothelin-1 (^{125}I-ET-1) binding sites in the human myometrium (A, B, E, F)and the endometrium (C, D, G, H). Consecutive, 20-μ m-thick tissue sections obtained from 39-years-old woman were labeled wiht 0.83 nM ^{125}I-ET-1 in the absence (total binding, A, C) or presence of 10 μ M BQ-123 (B, D, F), 1.0 μ M ET-1 (non-specific binding, E, G), 1.0 μ M sarafotoxin S6c (H) in vitro. Labeled sections were coated with NTB-2 liquid autoradiographic emulsion at 4℃. The ET$_B$ receptor is present in smooth muscle cells in the myometrium (B, F). In the endometrium, the ET$_A$ receptor was localized in stromal cells (H), and the ET$_B$ receptor was detected in glandular epithelial cells (D). The panel F is a high-magnification photograph from B. Scale bar = 3 mm.

co-exist in the myometrium of this case in the ratio of approximate 52 : 39. Similarily, the proportion of ET$_A$ and ET$_B$ receptor was calculated to be approximately 46 : 50 in the case No. 2, and 35 : 27 in the case No. 3, respectively (Table 2).

Cellular distribution of ^{125}I-ET-1 binding sites in the human myometrium and the endometrium was investigated on the same section used for quantitative receptor autoradiography, using an emulsion autoradiographic techique. Light microscopic autoradiograms revealed that most of ^{125}I-ET-1 binding grains seemed to be over smooth muscle cells (Fig. 5A). As we noted dot-shaped ^{125}I-ET-1 binding with a much lower affinity for BQ-123, as shown in the receptor autoradiogram of Fig. 3, we performed the emulsion autoradiographic study to see the cellular localization of BQ-123-insensitive ^{125}I-ET-1 binding sites, candidates for the ET$_B$ receptor. In emulsion autoradiogram obtained at incubation with the radioligand in the presence of 10 μ M BQ-123, ^{125}I-ET-1 binding grains were also concentrated over smooth muscle cells (Fig. 5B and F). Thus, although we could not discriminate types of smooth muscle cells, we obtained evidence on the existence of smooth muscle cells with the ET$_B$ receptor in the human myometrium.

In the endometrium, ^{125}I-ET-1 binding grains were seen in glandular epithelial cells, when related tissue sections were incubated with the radioligand in the presence of 10 μ M BQ-123 (Fig. 5D). Conversely, silver grains remaining in the presence of 10 μ M sarafotoxin S6c were concentrated
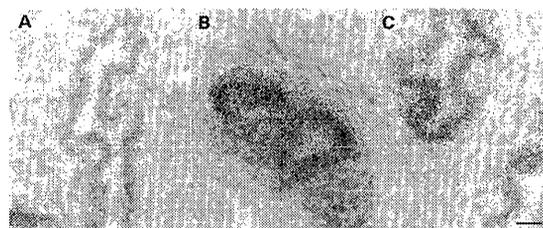

Fig. 6. Emulsion autoradiographic localization of specific ^{125}I-endothelin-1 (^{125}I-ET-1) binding sites in the human endometrium. Related tissue sections obtained from 39-years-old woman were labeled wiht 0.83 nM ^{125}I-ET-1 in the presence of 10 μ M BQ-123. Labeled sections were coated with NTB-2 liquid autoradiographic emulsion at 4 ℃. A difference in the ET$_B$ receptor density among glandular epithelial cells is noted in the endometrial part of A, B, and C. Scale bar = 3 mm.

over stromal cells (Fig. 5H). The light microscopic autoradiography we used revealed that the ET$_A$ and the E T$_B$ receptor are present on different components of the human endometrium ; the ET$_A$ receptor on stromal cells and the ET$_B$ receptor on glandular epithelial cells.

Interestingly, we observed a difference in the ET$_B$ receptor density among glandular epithelial cells in the endometrium of the same sections obtained from 39-years-old woman (Fig. 6). Fig. 6B shows the highest density of ^{125}I-ET-1 binding grains remaining in the presence of 10 μ M

BQ-123 in glandular epithelial cells in a part of the endometrium, whereas in another part a scanty density of ^{125}I-ET-1 grains was detected on glandular epithelial cells (Fig. 6A). A difference in the density of binding grains within glandular epithelial cells forming an epithelial canal is also presented in Fig. 6C. This observation may mean that the ET$_B$ receptor is differentially expressed in glandular epithelial cells in response to the cell circle.

Discussion

Our quantitative receptor and emulsion autoradiographic studies revealed heterogeneous expressions of the ET$_A$ and the ET$_B$ receptor among smooth muscle cells in the human myometrium, and stromal cells and glandular epithelial cells in the endometrium of the human uterus. We extended the previous finding that ETs act as paracrinal and/or autocrinal factors within the uterus[7,8] by studying the ET receptor binding characteristics and the precise localization of ET$_A$ and ET$_B$ receptor in the human uterus. Taken together with the previous finding of the expression of ET$_A$ and ET$_B$ receptor mRNA in the human uterus[24], we confirmed the existence of functional ET receptors in the human myometrium and the endometrium, based on the binding characteristic obtained here.

We report here what seems to be the first evidence that there are two types of smooth muscle cells in the myometrium ; ones with the ET$_A$ receptor and others which have the ET$_B$ receptor. As the ET-1 functions as a potent vasoconstrictor, by interacting with the ET$_A$ receptor on vascular smooth muscles[3], the present observation on the existence of numerous smooth muscle cells equipped with the ET$_A$ receptor in the myometrium seems pertinent to the previous evidence that ETs act as an endogenous uterine contractor[5,13]. Functions performed by uterine smooth muscle cells with the ET$_B$ receptor need to be elucidated. Accumulating evidence revealed that cultured rat astrocytes proliferate and differentiate by interacting with their own ET$_B$ receptor[25,26], presumably linked to activation of the mitogen-activated-protein kinase cascade[27,28]. Hence, the possibility that the ET$_B$ receptor expressed in uterine smooth muscle cells is functionally related to uterine growth in cases of organic disease, such as uterine leiomyoma or malignancy have to be considered. Furthermore, we obtained data on the different proportion in the number of myometrial ET$_A$ and ET$_B$ receptor in three cases of women examined in this study. Since Schiff et al.[10] observed phenomenal changes in the density of ET receptors in the myometrium of postmenopausal, pre-menopausal, and pregnant women, a finding suggesting that ovarian steroids regulate the expression of myometrial ET receptors, the different proportion of ET receptors sheds some light on the regulatory mechanism of functional ET$_A$ and ET$_B$ receptor in the myometrium.

The emulsion autoradiography method we used provided evidence on the cellular localization of the ET$_A$ receptor on stromal cells and the ET$_B$ receptor on glandular epithelial cells in the endometrium. The function of ET$_B$ receptor in the endometrium remains to be elucidated, but they may have an autocrinal or paracrinal role of releasing ETs or other substances such as prostaglandin F2 α[29,30]. In fact, Baley et al.[31] reported that hormonal stimulation of prolactin enhanced the expression of ET mRNA in breast epithelial cells, a finding suggesting a paracrinal role of ET in the human breast. There have been reports on the hormonal regulation of ET system, and the change of ET-1 production and ET$_A$ and ET$_B$ receptor expression throughout the menstrual cycle in the human endometrium[8]. Also, the role of ovarian steroids in the functional layer of endometrium during cyclical changes is well known. Taken together, our findings on the cellular localization of ET$_A$ and ET$_B$ receptor in the endometrium support the idea that ETs play a function regulating menstrual bleeding in the cyclic endometrium, in collaboration with ovarian hormones.

The incubation time of specific binding ^{125}I-ET-1 to human uterus sections was tested in kinetic experiments, reaching a maximum after 36 hr of incubation and maintaining a plateau for up to 72 hr at 4℃. This slow association supports previous reports[32,33] in which a incubation time of 48 hr and 24 hr were found respectively. We added phosphoramidon[34], an inhibitor of endothelin neutral endopeptidase (EC 3. 4. 24. 11) to the incubation buffer to prevent the radioligand from degradation during a long incubation time.

In summary, we have obtained evidence that there are two types of ET receptors, ET$_A$ and ET$_B$ receptor, in the pre-menopausal human uterus. In the myometrium smooth muscle cells equipped with the ET$_A$ receptor and cells with the ET$_B$ receptor co-exist. On the other hand, the endometrial ET$_A$ and ET$_B$ receptor are detected in stromal cells and glandular epithelial cells, respectively. The ET$_B$ receptor carried by myometrial smooth muscle cells may be operative as an etiological factor of uterine leiomyoma. Studies to elucidate the possible role of ET$_B$ receptor carried by uterine smooth muscle cells are under way in our laboratory.

Acknowledgements

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