Decreased expression of messenger RNAs encoding endothelin receptors and neutral endopeptidase 24.11 in endometrial cancer

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Summary In this study, we used reverse transcriptase–polymerase chain reaction (RT–PCR) to compare the expression of mRNAs encoding endothelin-1 (ET-1), endothelin receptors type A (ET$_A$-R) and type B (ET$_B$-R) and ET-1-degrading enzyme neutral endopeptidase 24.11 (NEP) in 15 endometrial cancer tissues and 13 normal endometrial tissues. The relative levels of ET-1 mRNA in endometrial cancer tissues did not differ from those in normal endometrium. Both ET$_A$-R and ET$_B$-R mRNA levels were significantly lower in endometrial cancer tissue than in normal endometrium ($P<0.001$). The complete lack of NEP mRNA in endometrial cancer tissues was in marked contrast to results from normal endometrium ($P<0.001$). In conclusion, differential expression of mRNAs encoding ET-R and NEP in normal endometrium and endometrial cancer suggests that ET action is altered in endometrial cancer compared with normal endometrium.

Keywords: endothelin; receptor; endometrium; cancer; neutral endopeptidase

Endothelin (ET) is a 21 amino acid peptide which was first described in 1988 as a potent vasoconstrictor localised in vascular endothelium (Yanagisawa et al., 1988). Subsequently, three ET isopeptides, called ET-1, ET-2 and ET-3, with different biological activities, have been identified by screening the human genomic DNA library (Inoue et al., 1989). Although ET-1 was originally isolated from cultured endothelial cells, it has become evident that the ETs are widely distributed in different tissues and organs (Nunez et al., 1990). The different potencies of the three isoforms of the ET family opened up the possibility of the existence of multiple ET receptor (ET-R) subtypes as well. Two distinct ET receptors with different specificities have been cloned (Arai et al., 1990; Sakurai et al., 1990). The type A endothelin receptor (ET$_A$-R) has high affinity for ET-1 and ET-2 but little cross-reactivity with ET-3 and sarafotoxin S6c, whereas type B receptor (ET$_B$-R) is non-selective with similar affinities for the different ETs and sarafotoxin S6c (Sakurai et al., 1992). A third ET-R subtype with superhigh affinity has been described but not yet cloned (Sokolovsky et al., 1992). The ET-Rs are widely distributed in cell lines and organs, but their relative abundance in different tissues varies. ET$_B$-R dominates in hippocampus, whereas ET$_A$-R dominates in the uterus (Williams et al., 1991). Neutral endopeptidase 24.11 (NEP), also called enkephalinase, is a zinc-containing plasma membrane enzyme that efficiently degrades a number of small bioactive peptides, including the ETs (Sokolovsky et al., 1990; Vijayaraghavan et al., 1990).

In the human endometrium, ET-1 has been shown to be produced by both stromal and epithelial cells, with increased concentrations observed before and during menstruation (Economos et al., 1992). The mRNAs for ET-1, ET-2 and ET-3 have been detected in human endometrium throughout the menstrual cycle (O'Reilly et al., 1992). Also, pregnancy endometrium (decidua) synthesises ET-1 (Kubota et al., 1992). The expression of ET$_A$-R and ET$_B$-R mRNA in the endometrium varies with the phase of the menstrual cycle, the ratio of ET$_A$-R to ET$_B$-R being lowest in late secretory phase (O'Reilly et al., 1992). NEP is present in the human endometrium throughout the menstrual cycle (Head et al., 1993). The specific activity of the enzyme is correlated with the plasma levels of progesterone and is highest in early and mid-secretory phase (Casey et al., 1991).

Little information on the role of the ET system in cancer is available. Several cancer cell lines, including endometrial cancer cell lines, have been shown to produce ET (Kusuhara et al., 1990; Pekonen et al., 1992), but information on ET-R mRNA expression in cancer tissue is scarce. In colon cancer, decreased ET receptor activity has been reported (Inagaki et al., 1992). Expression of NEP mRNA is typical for leukaemias, but has also been demonstrated in other malignancies such as melanomas, gliomas and mesenchymal tumours (Carrel et al., 1983; Mechtersheimer and Möller, 1989; Monod et al., 1992). Information on ET-1 mRNA expression in endometrial cancer tissue is lacking. Neither ET-R nor NEP mRNAs have been described in endometrial cancer tissue. In this study we compared the mRNA expression of ET-related substances in endometrial cancer and normal endometrium in an attempt to elucidate their potential role in the genesis of endometrial cancer.

Materials and methods

Tissues

Endometrial cancer tissue was obtained from 15 women who underwent hysterectomy for endometrial adenocarcinoma at the Department of Obstetric and Gynecology, Helsinki University Central Hospital. The mean age of the patients was 62.6 years (range 47–80 years). Samples of normal endometrium were obtained from 13 women who underwent laparoscopic tubal ligation at the Hyvinkää district Hospital, Finland. The mean age of these women was 42.2 years (range 35–47). The samples were collected with the approval of the Local Ethical Committees. The tissue samples were snap frozen in liquid nitrogen immediately after removal and stored at −80°C until processed. Routine haematoxylin and eosin-stained paraffin sections were prepared for histological evaluation. The dating of the endometrial samples was based on the first day of the last menstrual period and histological examination according to the method of Noyes et al. (1950).

RNA isolation

All reagents used for RNA isolation were molecular biology reagents from Sigma (St Louis, MO, USA). The guanidium thiocyanate method (Chomczynski and Sacchi, 1987) was used to isolate total RNA.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

A random-primed cDNA library was prepared from 1 μg of RNA with the Moloney murine leukaemia virus reverse transcriptase according to the manufacturer's recommendations (Gibco-BRL. NY. USA). The reaction was stopped by incubating at 95°C for 5 min and was then quick chilled on ice. The cDNA was amplified according to Takeda *et al.* (1992) using amplification primers based on previously reported sequences (Table I). The concentration of primers was 0.5 μM. of magnesium chloride. 1.5 mM and of *Taq* polymerase 1.25 U (Promega) in 50 μl buffer (Promega). In brief, 3 μl of cDNA reaction mixture was used for amplification in the presence of 1 mM each of dATP. dGTP and dTTP, 0.8 mM dCTP and 0.1 μl of [^{32}P]dCTP (3000 mCi mmol^{-1}. Amersham. Bucks. UK). Thirty cycle products, which were within the linear logarithmic phase of the amplification curve, were analysed (Figure 1). Actin (23 cycles, chosen to
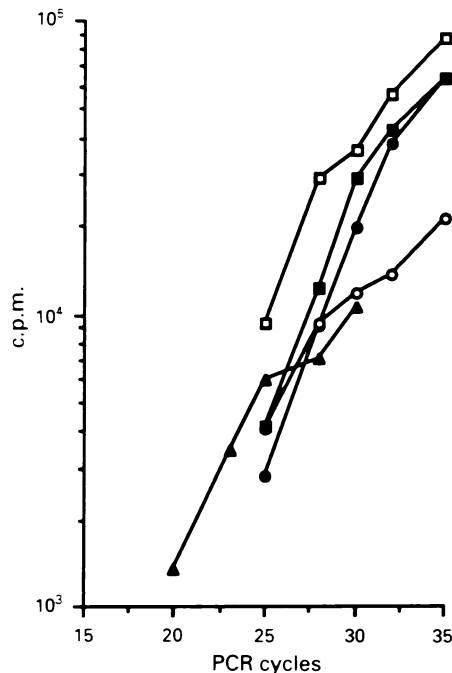

Figure 1 Amplification of ET-1 (●). NEP (O). ET$_A$-R (■). ET$_B$-R (□) and actin (▲) mRNA from human endometrium using RT-PCR. Quantification of RT-PCR product was based on incorporation of [^{32}P]dCTP into the specific amplification products at 24–36 cycles. The amplification products were separated by agarose gel electrophoresis and visualised by ethidium bromide. and radioactivities in specific bands were counted.

be within the logarithmic linear phase of actin amplification) was used as an internal control. and the reaction was performed in the same tube as the specific ET. ET-R and NEP reactions. The radioactivity in the specific PCR bands in low-melting Nu Sieve GTG agarose (FMC Bio Products. Rockland. ME. USA) gel was counted. Results were expressed as relative levels of specific mRNAs normalised to actin. In all experiments. two control reactions. one containing no mRNA and another containing mRNA but without reverse transcriptase. were included. The radioactivity in the control sample without mRNA was in each instance subtracted from the radioactivity in specific PCR bands.

Southern blot hybridisation

The digoxigenin-11-dUTP (DIG)-based labelling and detection system from Boehringer Mannheim. with 3'-tailing of oligonucleotides. was used for Southern hybridisation. The PCR products were blotted by capillary transfer onto nylon membrane (Zeta-Probe. Bio-Rad). The blotted membrane was probed according to the manufacturer's instructions in 5 ml of hybridisation mixture containing 5 × SSC. 0.1% *N*-laurosylsarcosine. 1% blocking reagent. 0.02% sodium dodecyl sulphate (SDS). and DIG-labelled oligonucleotides which occurred between the two primers used in the specific PCR reactions (Table I).

Statistics

Student's *t*-test for non-paired samples was used when mRNA expression in cancer tissues and normal tissues was compared.

Results

The 30 cycle PCR products were within the linear logarithmic phase of the amplification curve for ET-1. NEP. ET$_A$-R and ET$_B$-R (Figure 1). The sizes of the RT–PCR products were as predicted from the genomic maps (Table I and Figure 2). By Southern hybridisation single bands corresponding to the RT–PCR bands in agarose gels were seen in each case (results not shown). The ET-1 mRNA levels in human endometrial cancer tissues and samples of normal endometrium did not differ ($P = 0.959$) (Figure 3). In contrast. both ET$_A$-R and ET$_B$-R mRNA levels were significantly lower ($P < 0.001$) in endometrial cancer tissues than in normal endometrial tissues (Figure 4). and NEP mRNA could not be detected in any of endometrial cancer tissues (Figure 3). This was in striking contrast to the results from normal endometrial tissues. in which NEP mRNA was detectable in each tissue studied (Figure 3. $P < 0.001$).

Table I Primers used in PCR amplifications

| Gene	Oligonucleotide sequence	Nucleotide number	Product size (bp)	Reference
ET-1	5' primer: TGCTCCTGCTCGTCCCTGATGGATAAAGAG	157–186	462	Itoh *et al.*. 1988
	3' primer: GGTCACATAACGCTCTCTGGAGGGCTT	592–618		
	internal oligonucleotide: CCAAATGATGTCCAGGTGGCAGAAGTAGTA	193–219		
ETA-R	5' primer: CACTGGTTGGATGTGTAATC	38–57	367	Adachi *et al.*. 1991
	3' primer: GGAGATCAATGACCACATAG	386–405		
	internal oligonucleotide: GCAAGACTGGCTATCAGCGCGTTG	345–368		
ETB-R	5' primer: TCAACACGGTTGTGTCCTGC	308–368	529	Sakamoto *et al.*. 1991
	3' primer: ACTGAATAGCCACCAATCTT	818–837		
	internal oligonucleotide: GGATGAAGCAAGCAGATTCGCAG	754–776		
NEP	5' primer: GGTCATAGGACACGAAATCAC	1736–1757	520	Malfroy *et al.*. 1988
	3' primer: TGAAGATCACCAAACCCGGCACTT	2232–2256		
	internal oligonucleotide: CACCAGTCAACGAGGTCTCCATC	1794–1816		
b-actin	5' primer: CCCAGGCACCAGGGCGTGAT	154–173	260	Ponte *et al.*. 1984
	3' primer: TCAAACATGATCTGGGTCAT	396–415		
	internal oligonucleotide: TACAATGAGCTGCGTGTGGCTCCCGAG	312–338		
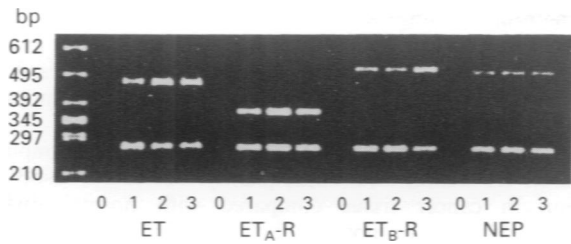
Figure 2 Amplification products of actin, ET-1, ET_A-R, ET_B-R and NEP mRNAs in three representative endometrial samples. The mRNA was reverse transcribed and amplified by PCR (30 cycles). The RT–PCR products were separated by agarose gel electrophoresis and visualised by ethidium bromide. The base pair markers are indicated on the left. Lane 0; control samples without mRNA; lanes 1–3; three different endometria (cycle days 8, 19, 27). The predicted sizes for the amplification products were: ET, 462 bp; ET_A-R, 367 bp; ET_B-R, 529 bp; NEP, 520 bp; actin 260 bp.
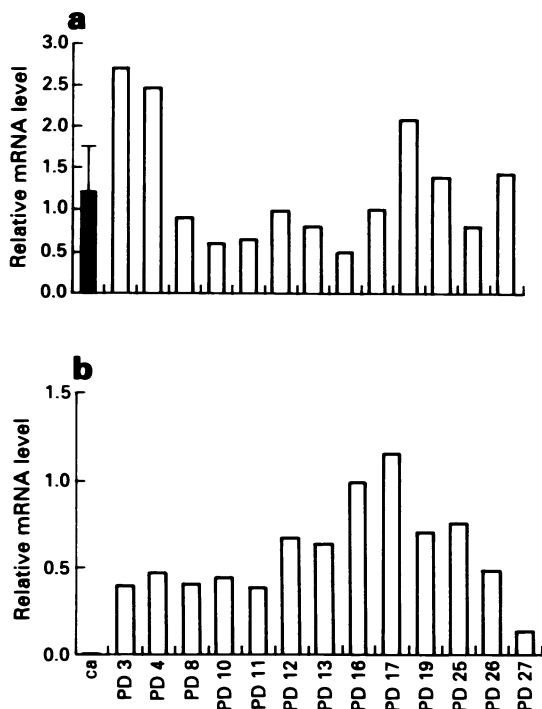

Figure 3 The relative levels (mean + s.d.) of mRNAs for (a) ET-1 and (b) NEP in endometrial cancer tissues (ca, solid column) and mean of triplicate measurements of normal endometrial tissue specimen. The day of the cycle is indicated below each column. Quantification of RT–PCR product (30 cycles) was based on incorporation of [^{32}P]dCTP in the specific amplification product. Actin mRNA was amplified in the same RT–PCR reaction (23 cycles). The amplification products were separated by agarose gel electrophoresis, visualised by ethidium bromide and radioactivities in the specific bands were counted. Data are expressed as relative level of specific mRNA (= c.p.m. of specific bands divided by c.p.m. of actin bands). Radioactivity in control samples without mRNA was subtracted from all values.

Figure 4 The mean (+s.d.) relative levels of mRNA for (a) ET_A-R and (b) ET_B-R in 15 endometrial cancer tissues (solid column) and in 13 normal endometrial tissues (the day of the cycle is indicated below each column). Quantification of RT–PCR product was performed as described in legend to Figure 3. The samples are the same as in Figure 3.

Discussion

The diverse distribution of ET isoforms and subtypes of ET receptors suggest that ET has multiple functions in different tissues. This study shows that in human endometrial cancer ET-1 mRNA is expressed, whereas the relative levels of mRNAs encoding ETRs or NEP are low or undetectable. Although ET production has been demonstrated in many human cancer cell lines (Kusuhara et al., 1990), little information on the ET system in human cancer tissue is available. It
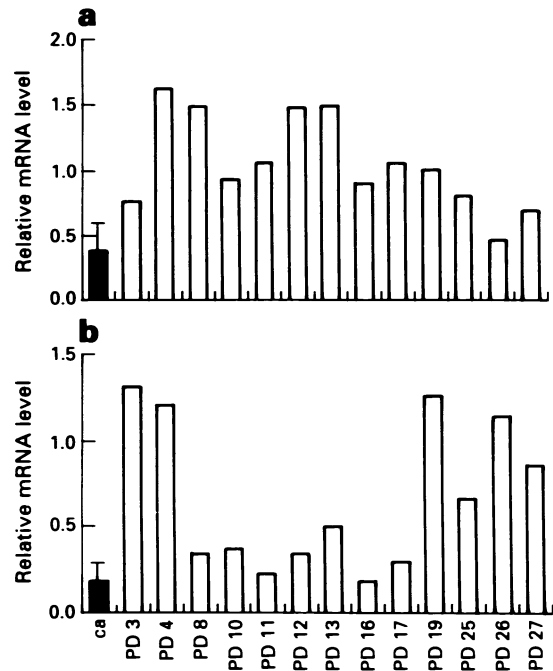
has been shown that some pulmonary tumours express ET-1 mRNA (Giaid et al., 1990), and that the epithelial cancer cells in human colon cancer tissue bind ET-1 minimally (Inagaki et al., 1992). Our results on low or lacking ET-R mRNA expression in endometrial cancer are thus in good agreement with those of Inagaki et al. (1992). The data are also in keeping with a recent observation that ET binding in endometrial cancer tissue is lower than that in normal endometrium from premenopausal women (Ben-Baruch et al., 1993). Endometrial adenocarcinoma tissue typically consists of epithelial cells with few stromal elements. The absence of a stromal compartment might explain decreased ET-R expression in endometrial cancer if stromal cells were the major site of ET-R expression in the endometrium as described in breast tissue (Baley et al., 1990). On the other hand, it has been shown that in normal endometrium the glandular epithelium and blood vessels have a high density of ET binding sites (Davenport et al., 1991). If this is the case, it suggests that endometrial adenocarcinoma cells differ from normal endometrial epithelial cells regarding ET binding. Contaminating endothelial cells (Davenport et al., 1991) might account for the low levels of ET-R mRNA in endometrial cancer tissues. Further studies are, however, needed to clarify the cellular localisation of ET binding in endometrial cancer. No information on ET-R expression in postmenopausal endometrium is available, but ET-1 binding to myometrial membranes is lower in tissue samples obtained from post-menopausal women than from premenopausal pregnant women (Schiff et al., 1993).

The relative levels of ET-1 mRNA in different endometrial cancer cell lines are reflected in their ET-1 secretion, as shown previously (Pekonen et al., 1992). The expression of ET-1 mRNA in endometrial cancer tissues is in agreement with an epithelial cell origin of ET-1. The finding that the relative levels of ET-1 mRNA in endometrial cancer tissue did not differ from those in normal endometrium during different phases of the menstrual cycle suggests that the ET-1 expression in endometrial epithelium remains unchanged despite malignant transformation.

Immunoreactive NEP has been localised exclusively to the

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stromal cells in the human endometrium with maximal activity in mid-secretory phase (Casey et al., 1991; Head et al., 1993). In agreement with this, the highest levels of NEP mRNA were detected in early secretory phase endometrium in this study. Stromal cell origin may account for the absence of NEP mRNA in endometrial cancer tissues. Another explanation may be the age of endometrial cancer patients. In this study, they were all post-menopausal. It is obvious that post-menopausal endometrium differs from premenopausal endometrium regarding autocrine and paracrine factors regulated by ovarian steroid hormones. It appears that the NEP mRNA expression in the endometrium is not regulated by oestrogens, since NEP mRNA levels in post-menopausal endometrium have been reported to be similar to those in proliferative phase endometrium (Head et al., 1993). Even progesterone's role in the regulation of NEP remains unclear. Immunoreactive NEP was weak in proliferative

endometrium, strong in mid-secretory endometrium and almost non-existent in predecidualised/decidualised endometrium in spite of high serum progesterone levels (Head et al., 1993). Thus, NEP expression in the endometrium appears to be more differentiation than hormone dependent.

The low expression of ET-R in endometrial cancer implies decreased ET action and, consequently, decreased vasoconstriction in cancer tissue compared with normal endometrium. Whether or not the lack of NEP has a role in endometrial carcinoma is more difficult to speculate, since NEP hydrolyses ET-1 as well as a number of other bioactive peptides, which may have effects on tumour growth.

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