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ABERRANT OESTROGEN RECEPTOR SPECIES IN HUMAN MENINGIOMA TISSUE

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Summary—Meningiomas are very rich in progestin receptors (PR) whereas oestrogen receptors (ER) are seldomly found and only at low concentrations. These tumours might possess an ER which is defective in oestrogen binding but still functional in stimulating oestrogen-responsive genes such as PR. In human meningiomas a polymerase chain reaction fragment including the DNA binding domain, the hinge region and the ligand binding domain of ER was amplified. The size of the fragment obtained was as expected from wild type mRNA sequences. Moreover, a variant, which was overexpressed in meningiomas, with a major deletion in exons 2–6 was detected.

INTRODUCTION

Meningiomas are benign tumours arising from the leptomenginges. They are the only intracranial tumours, which occur more often in women than in men [1] and their symptoms may aggravate reversibly during pregnancy and in the second half of the menstrual cycle [2]. The occurrence of meningioma is associated with that of breast cancer [3] and intracranial metastases of breast cancer show a preference for meningiomas [4]. These findings suggest that female sex hormones are involved in the etiology of human meningiomas.

In the "classical" target tissues, i.e. the uterus and the mammary gland, steroids exert their effects through receptor proteins which are located in the nuclear compartment of the cells [5]. Moreover, in these tissues, the synthesis of progestin receptors (PR) is stimulated by oestrogens, through the available oestrogen receptors (ER).

The presence of high concentrations of PR in most human meningiomas has been well established by many groups. In contrast considerable controversy concerning the presence of ER exists [6–8]. This is based mainly on technical differences, e.g. the use of single saturating dose assays vs full range Scatchard plot analysis.

Because no correlation was observed between ER and PR content in meningioma [9], the PR content is not regulated and so does not conform with the "classical" model. Deletion mutants have been constructed from ER cDNA which constitutively activate transcription in the absence of oestradiol [10, 11]. Such ERs can bind DNA, but lack all or part of the hormone binding domain rendering them incapable of binding oestradiol. By consequence, they would also escape detection by ligand binding analysis or immunoassays based on antibodies directed against the hormone binding domain.

Fuqua *et al.* [12] detected mRNA coding for constitutively active receptor in ER negative/PR positive breast tumours. Apart from the wildtype (wt) ER in these breast tumours, an alternatively spliced product missing exon 5, which was transcriptionally active and hormone independent in *trans*-activation assays was detected [12].

To evaluate whether such a situation also prevails for meningioma, mRNA was prepared from meningioma tissues which were negative for ER by enzyme immunoassay as well as by ligand binding assay. Polymerase chain reaction (PCR) amplification of cDNA from these meningiomas was used to detect low amounts of ER transcript. Primers spanning the DNA binding domain (DBD), the hinge region and

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Abbreviations: cDNA, complementary DNA; DBD, DNA binding domain; ER, oestrogen receptor; LBD, ligand binding domain; mRNA, messenger RNA; PCR, polymerase chain reaction; PR, progestin receptor; wt, wild type.

the ligand binding domain (LBD) were used for PCR.

MATERIALS AND METHODS

Tissue collection

Human meningiomas were placed on ice immediately after removal from the patient. Representative specimens were frozen at −80°C until they were used for RNA extractions or receptor assay.

RNA extraction

Total RNA was extracted from 250 mg tissue, with an acid guanidinium thiocyanate–phenol–chloroform mixture as described by Chomczynski *et al.* [13].

Oligonucleotide primers and probes

The following oligonucleotides corresponding to the human ER cDNA were used in this study: (a) amplification exon 2–6, primer 1; sense 5′-GGAAGTATGGCTATGGAATCT-3′ (amino acid 171–178); primer 2; anti-sense 5′-GATCTTCGAACATGCTGCTGG-3′ (amino acid 423–430); (b) hybridization PCR products exon 2–6, probe 5; sense 5′-CATAACGACTATATGTGTCCAGCCACC-3′ (amino acid 216–227); (c) amplification exon 4–8, primer 3; sense 5′-GGAGACATGAGAGCTGCCAAC-3′ (amino acid 283–290); primer 4; anti-sense 5′-GAGACGGACCAAAGCCACTTG-3′ (amino acid 562–568); and (d) hybridization PCR products exon 4–8, probe 6; sense 5′-ACCAACCTGGCAGACAGGGAGCTG-3′ (amino acid 347–354).

Probes were synthesized using an Applied Biosystems (San Jose, CA, U.S.A.) DNA synthesizer.

cDNA synthesis

Following the establishment of the integrity by gel electrophoresis, 1 μg of total RNA was used, for the preparation of cDNA. Reactions were carried out in 20 μl 10 mM Tris–HCl (pH 8.3) containing 50 mM KCl, 5 mM $MgCl_2$, 10 ng random hexamer primer, 0.5 mM dNTP, 20 U RNAse inhibitor (Boehringer, Mannheim, Germany) and 40 U of M-MLV H RT superscript (BRL, Bethesda, MD, U.S.A.). Synthesis of cDNA was performed in a PCR apparatus (Perkin Elmer, Norwak, CT, U.S.A.). The synthesis consisted of one cycle: 30 min incubation (42°C), 5 min enzyme inactivation (95°C), and 5 min at 5°C. 10 μl was used for the PCR.

PCR assay

PCR reactions were carried out in 50 μl 10 mM Tris–HCl (pH 8.3) containing: 50 mM KCl, 1.5 mM $MgCl_2$, 100 ng primers and 1 U ampli Taq (Perkin Elmer). To amplify exons 2–6 primers 1 and 2 were used and for exons 4–8 primers 3 and 4 were used. Each cycle of amplification consisted of 30 s denaturation (95°C), followed by 1 min annealing (53°C), followed by 90 s extension (72°C). The ramp-time between annealing and extension was 90 s. Each PCR consisted of 35 cycles.

Hybridization in agarose gels

Half of the DNA of the PCRs was separated on 1% agarose gels. Gels were denatured for 30 min in 0.5 M NaOH solution containing 0.15 M NaCl and subsequently neutralized for 30 min in 0.5 M Tris–HCl buffer (pH 7.0) containing 0.15 M NaCl followed by a 10 min wash in water. Gels were dried under vacuum for 30 min at room temperature followed by 30 min at 60°C. The gels were prehybridized for 2 h in 5 × SSPE (1 × SSPE: 0.18 M NaCl, 10 mM sodium phosphate, pH 7.7, 1 mM Na_2 EDTA), 5 × Denhardts (1 × Denhardts: 0.02% w/v bovine serum albumin, 0.02% w/v Ficoll, 0.02% w/v polyvinyl pyrollidone), 0.1% SDS and 100μg/ml herring sperm (Boehringer Mannheim, Germany) at 42°C. Gels were hybridized overnight at 42°C with end-labelled oligonucleotides. After hybridization the gels were washed twice with 5 × SSC (1 × SCC: 0.15 M NaCl, 0.015 M Na_3 citrate), 0.1% SDS at 42°C for 15 min and exposed to X-ray films for 12 h. For the detection of exons 2–6 and 4–8 of ER, probes 5 and 6 were used, respectively as end-labelled oligonucleotides.

RESULTS

ER−/PR+ meningioma were examined. The ER content in these meningiomas was determined by the ligand binding assay as described by the EORTC [14]. The ER and PR content is given in Table 1. The MCF-7 breast cancer cell line and a solid breast tumour specimen were used as positive controls, and an ER/PR-negative solid breast tumour was used as a negative control. The controls in which no cDNA was used were all negative and are therefore not shown. The DBD, the hinge re-

Table 1 ER and PR content in human breast cancer cell-line MCF-7, human breast cancers and meningiomas measured by ligand binding assay [14]

Sample	Tissue/ cell-line	Receptor level (fmol/mg protein) ER	PR
1	MCF-7 cell-line	150	220
2	Breast cancer	188	874
3	Meningioma	7	92
4	Meningioma	<3	309
5	Meningioma	<3	267
6	Meningioma	<3	179
7	Meningioma	<3	135
8	Meningioma	<3	<3
9	Breast cancer	<3	<3

gion and the LBD were examined. mRNA from six meningiomas was reverse transcribed, and the cDNA was amplified by PCR. The amplified PCR products were then analysed by gel electrophoresis and hybridization with an internal ER oligonucleotide probe (see Figs 1 and 2).

PCR amplification of exons 2–6

Exons 2–6 include the DBD, the hinge region and the first 102 amino acids of the LBD. PCR amplification of cDNA of these exons produced a transcript of the expected size of 770 bp as seen in the positive controls. In addition to the expected product, two products of smaller size were seen in the controls. The product with a size of approx. 450 bp could hardly be seen in the control but clearly seen in the meningiomas (see Fig. 1).

PCR amplification of exons 4–8

Exons 4–8 cover the entire LBD of the ER. The positive control as well as the meningiomas expressed, an approx. 500 bp product in addition to the expected 850 bp PCR product (see Fig. 2).

DISCUSSION

Since PR is normally induced by oestrogens it was a paradox that in most meningiomas no ER was detectable by ligand binding assay [6], or with a monoclonal antibody against the ER [15].

Possible ER mutants, which do not bind their ligands but have retained the DBD and hence the potential to induce transcription, cannot be detected by ligand binding or immunoassay. Because no hormone binding domain is present in the protein and the anti-ER antibody has its epitope in the hormone binding domain [16], the variant would thus be undetected and the tissue would be classified as oestrogen-negative. Other methods must be used to detect ER mutants.

In this study PCR was used to detect ER transcripts in meningiomas. Amplification of cDNA coding for exons 2–6, which include the DBD, the hinge region and the first 102 amino acids of the LBD produced a transcript of the expected size of 770 bp. In addition to the expected 770 bp product, a 450 bp variant was detected, which was overexpressed compared to

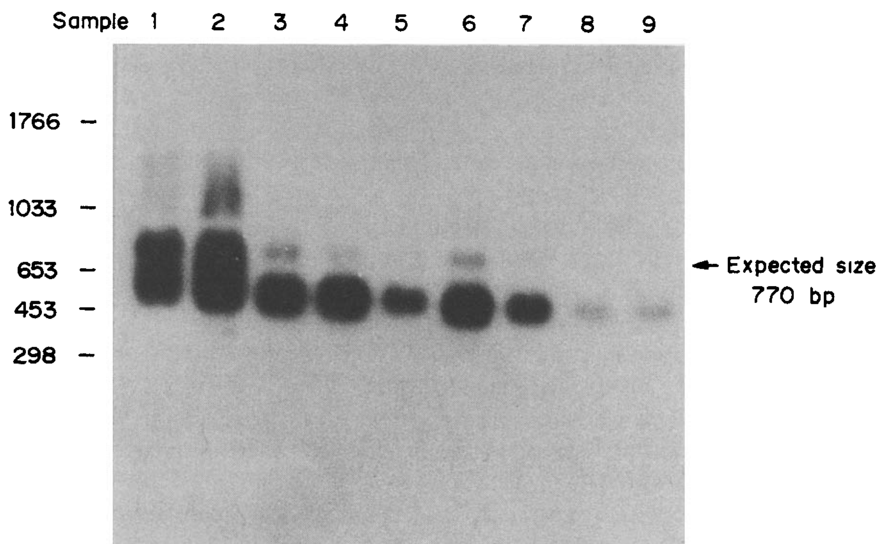

Fig. 1. PCR amplification of cDNA from meningiomas coding for exons 2–6 of the human ER. cDNA was prepared from: ER+/PR+ MCF-7 cells and solid breast tumour (samples 1 and 2), six meningiomas (samples 3–8), and one ER-/PR-solid breast tumour (sample 9). The receptor content of the samples is given in Table 1. PCR products were separated on a 1% agarose gel. The gel was dried and hybridized with the ER-specific end-labelled probe 5.

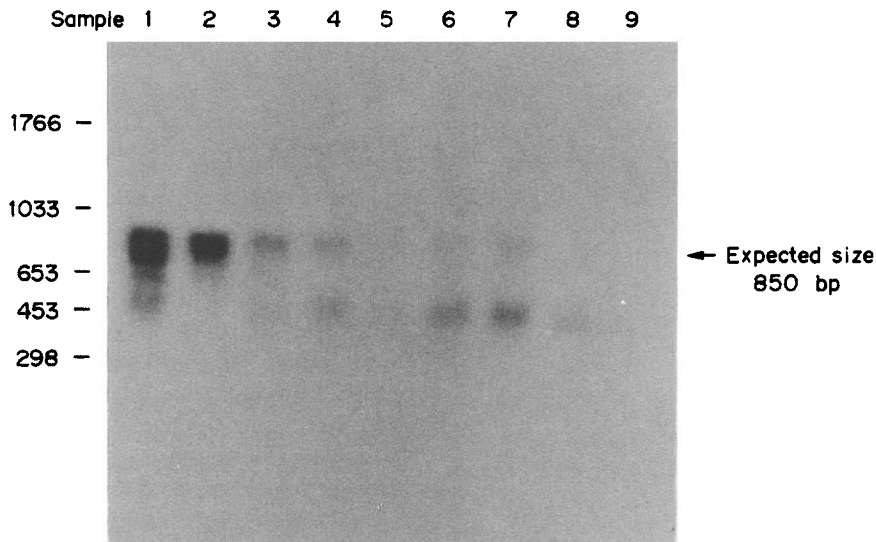

Fig. 2. PCR amplification of cDNA from meningiomas coding for exons 4–8 of the human ER. cDNA was prepared from ER+/PR+ MCF-7 cells and solid breast tumour (samples 1 and 2), six meningiomas (samples 3–8), and one ER−/PR− solid breast tumour (sample 9). The receptor content of the samples is given in Table 1. PCR products were separated on a 1% agarose gel. The gel was dried and hybridized with the ER-specific end-labelled probe 6.

the wt product in meningiomas. Amplification of exons 4–8 gave a product, corresponding in size to the wt product in the controls and in the meningiomas. In the meningiomas, variants were detectable of approx. 500 bp which were also present in the controls. Compared to the wt receptor this variant is expressed with similar intensity in meningiomas.

ER variants were described earlier in human breast cancer or human breast cancer cell lines. By using Northern blots, smaller molecular weight ER-like molecules were detected in some human breast cancer biopsy samples. These variants were only found in the presence of normal transcripts. In some tumours, however, the abundance of these smaller transcripts was the same or greater than the normal sized transcripts [17].

In meningiomas ER mRNA was not detectable by Northern blot analysis compared to the positive control (data not shown). This analysis is probably not sensitive enough to detect low amounts of ER mRNA.

Graham *et al.* [18] described in the ER-positive, but estrogen-resistant, $T47D_{co}$ cell line in addition to the wt ER, three mutant ERs, which were not detectable by Northern blot analysis, but only by screening a cDNA bank derived from this cell line.

Recently PCR was used for the detection of ER variants in breast tumours. Exons 4–6, sequences encoding part of the LBD of the ER, were amplified and the presence of a variant ER mRNA which is deleted entirely of exon 5 was detected. This transcript was a minor component of ER/PR-positive tumours, but was the predominant species in the ER-negative/PR-positive tumours. The variant ER constitutively activates transcription of a normally oestrogen-dependent gene construct in yeast cells [12].

The variant found in meningiomas, by amplification of exons 2–6, with a deletion of approx. 300 bp compared to the wt receptor, was overexpressed in the PR-positive meningiomas. In the PR-negative meningioma this product was poorly expressed. No conclusions can be drawn from one PR-negative meningioma yet, but it is tempting to speculate that this variant might play a role in the regulation of the PR transcription.

Cloning and subsequent sequencing of the ER PCR products are performed to establish that no minor deletions or point mutations are present, which can give rise to a receptor which is transcriptionally active and hormone-independent. The presence of a variant, which is overexpressed and has a major deletion in the hinge region and/or LBD, is a first indication for the regulation of the PR transcription managed by a truncated ER receptor in human meningioma.

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