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CONSTITUTIVE CO-EXPRESSION OF ESTROGEN AND PROGESTERONE RECEPTOR mRNA IN HUMAN MENINGIOMAS BY RT-PCR AND RESPONSE OF *IN VITRO* CELL CULTURES TO STEROID HORMONES

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Although it is well recognised that human meningiomas are rich in progesterone receptor (PgR), controversy has existed about the presence of the estrogen receptor (ER) in these tumours. We have investigated the presence of both ER and PgR in a series of 20 human meningiomas, spanning the main histological groups, using reverse transcription linked PCR (RT-PCR). Total RNA was extracted from whole tissues and reverse transcribed to yield cDNA. This was amplified using primers specifically designed to detect ER and PgR. All samples co-expressed ER and PgR mRNA, irrespective of tumour classification, patient age or sex. In general, transcripts for PgR appeared considerably stronger than those for ER, and although this was a purely qualitative study, it suggests increased expression of PgR. Addition of exogenous 17β-estradiol or progesterone to meningioma cell cultures showed that 2/4 cultures responded to these steroids. Our results confirm that human meningiomas do express gene transcripts for ER, and that previous failures to detect ER in these tumours may be due to the lack of sensitivity of the techniques employed. However, these receptors may not be functional in all tumours. ***Int. J. Cancer*** **72:714–719, 1997.**
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Meningiomas are mostly benign primary neoplasms derived from the leptomeninges of the brain and spinal cord. They account for approximately 18% of intracranial tumours and are distributed unevenly over the sexes, with roughly two-thirds of all patients being middle-aged women (Kepes, 1982; Mirimanoff *et al.*, 1985). The positive association of some meningiomas with breast cancer (Schoenberg *et al.,* 1975) and the observed acceleration of meningioma cell growth during pregnancy have led to investigations into the presence of receptors for the female sex hormones estrogen and progesterone in meningioma tissues.

Steroid hormones, including estrogens and progestogens, play a key regulatory role in controlling growth and/or differentiation of several target tissues, as well as in the development and growth of hormone-sensitive tissues (Vihko and Apter, 1989). Studies looking at the direct effects of estrogens and progestogens on growth of human meningiomas *in vitro* have been carried out, and it has been shown that although progestogens have minimal effects on growth, some reports suggest that estrogen may have proliferative effects (Jay *et al.*, 1985; Blankenstein *et al.*, 1989; Koper *et al.*, 1990).

Whilst it is well recognised that human meningiomas possess progesterone receptors (PgR; Blankenstein *et al.,* 1983; Schrell *et al.,* 1990), considerable controversy exists over the presence, or otherwise, of estrogen receptor (ER) in these tumours. The first suggestion of ER in meningiomas was described over 15 years ago (Donnell *et al.*, 1979). This was the starting point for a number of studies investigating steroid hormone receptor status in meningiomas and has provoked some controversy over the years. However, using either immunohistochemistry or ligand binding assays with Scatchard analysis, the general consensus is that the majority of meningiomas are devoid of ER but positive for PgR (Blankenstein *et al.,* 1983; Markwalder *et al.,* 1983; Courriére *et al.,* 1985; reviewed in Table I). In this study, we have re-investigated the PgR and ER status of a series of human meningiomas using the highly sensitive technique of reverse transcriptase-linked polymerase chain reaction (RT-PCR), and we provide conclusive evidence of constitutive co-expression of transcripts for both ER and PgR. Further, we have investigated the proliferative effects of steroid hormones on a number of meningioma cell cultures.

MATERIAL AND METHODS

Tissue samples

Tissue obtained from 20 surgically removed human meningiomas was used, comprising 7 males (mean age, 53.6; range, 24–75 years) and 13 females (mean age, 54.8; range, 36–71 years) who presented sequentially. Samples were received without prior knowledge of the clinical or biochemical presentation of the patients. The age, sex and pathological details of each sample are given in Table II. Upon receipt, all samples were dissected and a portion of tissue from the centre of each meningioma was snap-frozen in liquid nitrogen upon receipt and stored at −80°C until required. This ensured that only tumour tissue was used and not tissue from normal meninges.

RNA extraction and cDNA synthesis

Tissue was pulverised using a mortar and pestle and total RNA extracted according to Chomczynski and Sacchi (1987). RNA (1 µg) was reverse transcribed to yield cDNA in a 50 µl volume which contained: 0.5 mM each of dATP, dCTP, dTTP and dGTP (Boehringer Mannheim, Lewes, UK), 2 µg oligo dT, 20 U RNA guard (both Pharmacia, St. Albans, UK), 10 mM dithiothreitol, 10× reverse transcriptase buffer and 20 U/µg RNA of MMLV reverse transcriptase (Life Technologies, Paisley, UK). The reaction was incubated on ice for 5 min, followed by 60 min at 37°C and concluded by freezing at −20°C. Substitution of reverse transcriptase with distilled water served as a control to ensure that RNA rather than genomic DNA was being extracted and subsequently amplified, and was consistently negative.

PCR reaction

PCR was performed to yield fragments of amplified cDNA corresponding to unique sequences contained within the ER and PgR. To check the integrity of the cDNA, fragments of β-actin, a standard housekeeping gene, were amplified in parallel. ER (Wu *et al.,* 1993) and β-actin primers (Birch *et al.*, 1993) were obtained from Cruachem (Glasgow, UK). PgR primers were a generous gift from Dr. E.F. Adams (Erlangen-Nürnberg, Germany). Sequences, product sizes and restriction mapping details for PgR and ER primer pairs are illustrated in Table III. Primers to detect ER spanned the DNA binding domain of the human ER, details of which have been published previously (Wu *et al.,* 1993); PgR primers spanned exons 4–7 of the human PgR. The PCR reaction contained: 2 units of BIOTAQ, 10× PCR buffer (containing 1.5 mM $MgCl_2$; both Bioline, London, UK), 0.5 µg of each oligonucleotide primer, 200 µM each of dATP, dCTP, dGTP and dTTP, 1 µl

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TABLE I – EXPRESSION OF ER AND PgR IN HUMAN MENINGIOMAS: A REVIEW (1986–1996)

Author	ER (% expression)	PR (% expression)	Sample size	Type of analysis[1]
Blankenstein *et al.* (1986)	0	NA	21	IHC
Ironside *et al.* (1986)	0	53	45	IEF/LBA
Lesch and Gross (1987)	11	76	70	LBA
Whittle *et al.* (1987)	0	55	29	LBA
Kornblum *et al.* (1988)	3	28	29	LBA
Halper *et al.* (1989)	33	69	52	NBA
	2	76		LBA
	0	89		IP
Horsfall *et al.* (1989)	20	74	57	LBA
	0	NA		IHC
Schrell *et al.* (1990)[2]	0	98	50	IHC
Stojkovic *et al.* (1990)	0	66	6	LBA
Piquer *et al.* (1991)	36	92	39	LBA
Huisman *et al.* (1991)	19	76	24	LBA
Perrot-Applanat *et al.* (1992)	0	72	36	IHC
Meixenberger *et al.* (1992)	11	64	28	LBA
Brandis *et al.* (1993)	0	61	61	IHC
Koehorst *et al.* (1993*a*)	100	NA	8	PCR/FH
Magrassi *et al.* (1993)	100	50	12	PCR/FH
Maxwell *et al.* (1993)	0	100	9	Northern blot/ISH
Khalid (1994)	0	100	34	IHC
Rubenstein *et al.* (1994)	33	84	51	LBA
Bouillot *et al.* (1994)	0	53	52	IHC
Bozzetti *et al.* (1995)	18	89	46	LBA
	0	70		IHC
Zorludemir *et al.* (1995)	0	77	14	IHC

[1]NA, not analysed; IHC, immunohistochemistry; IEF, isoelectric focusing; LBA, ligand binding assay; NBA, nuclear binding assay; ISH, *in situ* hybridisation; IP, immunoperoxidase; PCR, polymerase chain reaction; FH, filter hybridisation.–[2]10/50 samples expressed ER at <4 fmol/mg protein. For the purpose of this review, only those samples with ER >10 fmol/mg protein were considered positive.

TABLE II – PATHOLOGICAL DETAILS OF THE TUMOURS USED IN THE PRESENT STUDY

Sample	Age (yr)	Sex	Pathology[1]
M1	47	F	Fibroblastic
M2	61	F	Fibroblastic
M3	56	M	Atypical
M4	70	M	Psammomatous
M5	71	F	Fibroblastic
M6	53	F	Transitional
M7	59	F	Transitional
M8	31	M	Meningothelial
M9	24	M	Transitional
M10	66	F	Fibroblastic
M11	64	M	Infiltrating atypical
M12	45	F	Meningothelial
M13	37	M	Meningothelial
M14	64	F	Psammomatous
M15	52	F	n.a.
M16	75	M	Fibroblastic
M17	66	F	Meningothelial
M18	56	F	Meningothelial
M19	36	F	Transitional
M20	37	F	Meningothelial

[1]Samples were collected between 1993 and 1994 and classified by a consultant neuropathologist at the hospital from which the tissue was obtained. The older WHO method of classification was used (Zulch, 1979) since the majority of samples were collected at or around the time the new WHO classification was being implemented (Burger and Scheithauer, 1993).

n.a.: pathology report not available.

nascent cDNA and sterile distilled water to bring the volume to 50 µl. As a positive control, cDNA from the ER/PgR-positive human breast cancer cell line T47D was used. Negative controls included substitution of RNA or cDNA with distilled water, or substitution of cDNA with an irrelevant cDNA; cDNA synthesised from 4 day cultures of pituitary fibroblasts served as an ER-negative control and cDNA synthesised from human tibialis anterior muscle served as a PgR-negative control. ER/PgR transcripts were analysed in parallel in a thermal cycler (Hybaid OmniGene, Teddington, UK) with the following cycle: a denaturation step at 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec and a final primer extension step of 72°C for 5 min. PCR products were analysed by electrophoresis through a 1.2% agarose gel and visualised by ethidium bromide staining under UV light.

Restriction enzyme digests

To confirm the identity of the PCR products, restriction digests were performed on representative PCR products. Amplified product (5 µl) was digested with the appropriate restriction enzyme (Table III) for 2 hr at 37°C. The digested products were then electrophoresed through a 2% agarose gel and visualised as above.

Immunohistochemical detection of ER

Formalin-fixed paraffin-embedded sections from 4 meningiomas were dewaxed and rehydrated. Prior to immunostaining, sections were subjected to microwave enhancement as previously recommended for detection of ER (Sannino and Shousa, 1994). Sections were immunostained with the ER antibody 1D5 (Dako, High Wycombe, UK) purchased as an optimally prediluted solution, according to the manufacturer's instructions. Methanol-fixed cultures of the ER-positive breast carcinoma cell line MCF-7 served as a positive control, and omission of the primary antibody served as negative control. Positive immunostaining was detected using the ABC method (Hsu *et al.*, 1981) with diaminobenzidene as a substrate.

Effects of estrogen and progesterone on meningioma cell proliferation

Cell cultures were prepared from selected meningiomas (n = 4) as previously described (Boyle-Walsh *et al.*, 1994), except that phenol red-free culture medium (phenol red has weak estrogenic activity) and charcoal-stripped FCS (to remove remove endogeneous steroidogenic activity which is associated with FCS) were used and seeded into 24-well plates. Log phase cultures were incubated with either 17β-estradiol (E2; 45 and 90 nM) or progesterone (PG; 0.35 and 0.7 µM) for 4 days at 37°C in a humidifed atmosphere containing 5% CO_2. On the 4th day, cells were lysed for nuclei counting by adding 500 µl of 10 mM Hepes/1.5 mM $MgCl_2$ and 50 µl of Zaponin (Coulter, Luton, UK). Released nuclei were counted in a Coulter Counter model ZM.

RESULTS

Detection of ER and PgR transcripts in human meningiomas

Steroid receptor profiles of all 20 meningiomas were determined in parallel on at least 2 separate occasions. As illustrated in Figure 1, all samples co-expressed message for both PgR and ER by RT-PCR, giving predicted product sizes of 389 and 263 bp, respectively. Although the PCR was entirely qualitative, message for PgR appeared considerably stronger than that obtained for ER.

Confirmation of product identity

The identity of the PCR products was further confirmed using restriction enzymes selected to cleave the PCR product into definitive sizes. Using specific restriction enzymes, the 389 bp PgR product was cleaved into fragments corresponding to 243 and 146 bp, while the 263 bp ER product was cleaved into fragments of 197 and 66 bp. Results of restriction mapping experiments from 3 representative amplifications for PgR and ER are illustrated in Figure 2.

Immunostaining

Meningioma sections were immunostained with a primary antibody directed against the N-terminal region of the ER. There was no evidence of ER positivity in any of the sections, although

TABLE III – PCR PRIMERS, PREDICTED PRODUCT SIZES AND RESTRICTION MAP DETAILS

Primer sequence	cDNA product (bp)	Restriction enzyme	Cleaved products (bp)
ER			
5′-TGCCAAGGAGACTCGCTA-3′			
5′-TCAACATTCTCCCTCCTC-3′	263	Ava II	197, 66
PgR			
5′-ACCTCCAGTTCTTTGCTGACAAGTC-3′			
5′-GTTTGACTTCGTAGCCCTTCCAAAG-3′	389	Hinf I	243, 146

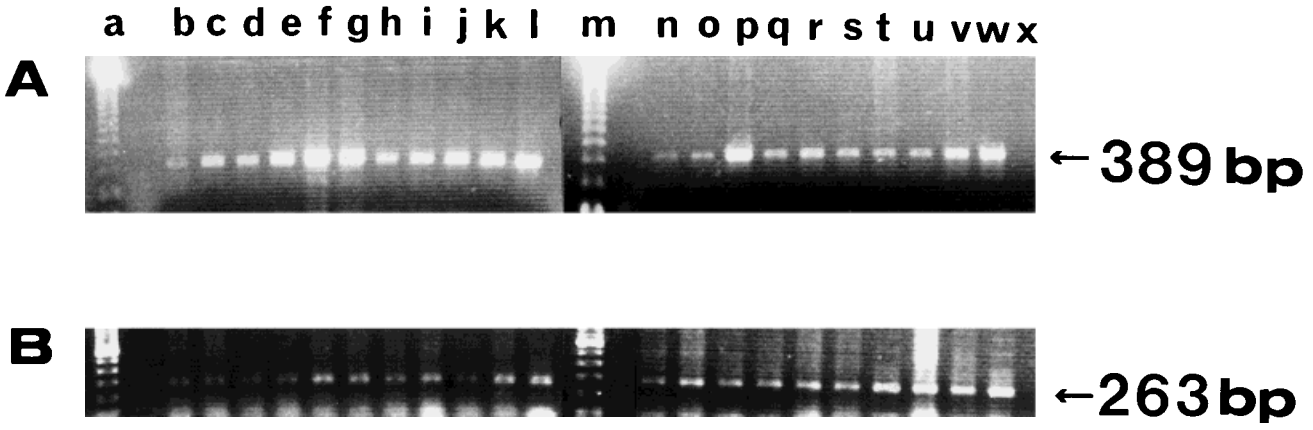

FIGURE 1 – Steroid receptor expression assessed by RT-PCR in 20 human meningiomas. *(a)* Results for PgR. *(b)* Results for ER. Lanes a and m, 123 bp ladder (Life Technologies); lanes b–l, M1–M11 inclusive; lane n–v, M12–M20 inclusive; lane w, T47D; lane x, negative control. The intense bands at the bottom of *b* represent excess primers and dNTPs which have not been utilised in the PCR reaction.

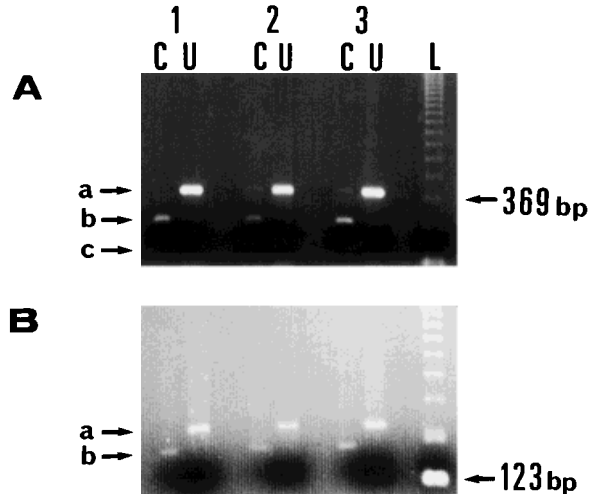

FIGURE 2 – *(a)* Restriction maps of 3 representative RT-PCR reactions amplified with PgR primers. Lane 1, M12; lane 2, M8; lane 3, M2. C, cut product; U, uncut product; a, expected product size of 389 bp; b and c, restriction mapped products of 243 and 146 bp, respectively. *(b)* Restriction maps of 3 representative RT-PCR reactions amplified with ER primers. Lane 1, M16; lane 2, M12; lane 3, M8. C, cut product; U, uncut product; a, expected product size of 263 bp; b, restriction mapped product of 197 bp. The 66 bp restriction fragment migrated ahead of the dye front and is not visible on the photograph.

strong nuclear ER staining was observed using the MCF-7 breast cancer cell line as a positive control (Fig. 3).

Effect of steroids on meningioma proliferation in vitro

Cell cultures were prepared from selected meningiomas and incubated with either E2 or PG for 4 days in phenol red-free medium. The results are illustrated in Figure 4. Two of the 4 cultures responded similarly to both steroids, giving an approximately 2-fold increase in terminal cell density compared with steroid-free controls.

DISCUSSION

In this study, we have presented conclusive evidence of co-expression of both ER and PgR gene transcripts in human meningiomas, irrespective of tumour type or patient age or sex. This is in contrast to most previous reports on ER/PgR status in these tumours but may be explained by the extreme sensitivity of the method we used, RT-PCR.

Routinely, biochemical assays of ER levels involve measurement of high-affinity and saturable ligand binding or employment of monoclonal antibodies directed against the hormone-binding domain of the ER. Consequently, the majority of previous studies on ER expression in meningiomas (reviewed in Table I) have concentrated primarily on immunohistochemistry and ligand binding assays, of which the former depends on the presence of immunoreactive protein and has relatively low sensitivity, while the latter, although highly specific, usually has a cut-off value of 10–20 fmol receptor/mg protein (Leake and Habib, 1987), above which levels samples are considered to be ER positive. This, coupled to the well-recognised thermolability of the ER (Crawford *et al.*, 1984; Bojar, 1986), which necessitates proper sample collection and storage, may have accounted for the negative results previously reported by others. We too were unable to obtain positive staining for ER in meningioma sections using immunohistochemistry, even though ER transcripts were detected by PCR in the same samples.

Although we were able to detect both ER and PgR transcripts in all the tumours, this does not give any indication of the functionality of either receptor, as detection of a PCR product does not necessarily imply translation into active protein. In the case of PgR, there has been a long-standing debate as to whether or not this is functional in meningiomas, with earlier studies indicating a non-functional PgR *in vitro* (Adams *et al.*, 1990). However, studies

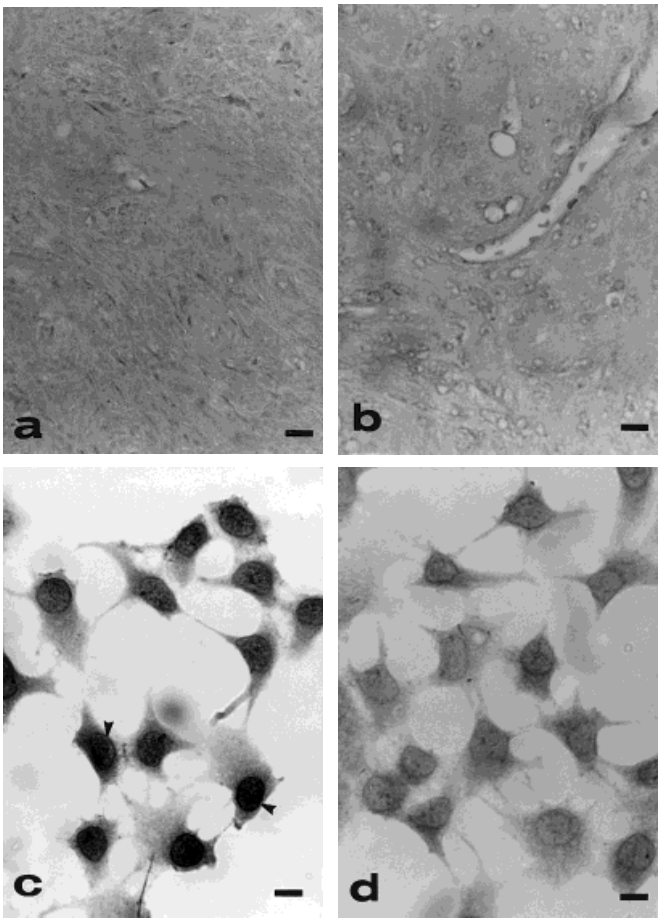

FIGURE 3 – Immunostaining for ER in meningioma sections. Sections/cell cultures were incubated with a monoclonal antibody to ER which was visualised by diaminobenzidene. *(a)* Fibroblastic meningioma. *(b)* Transitional meningioma. No staining is evident in either section. *(c)* MCF-7 breast cancer cell line positive control showing strong nuclear staining for ER (arrowheads). *(d)* MCF-7 negative control. No staining is evident. Scale bars = 15 μm (a and b), 20 μm (c and d).

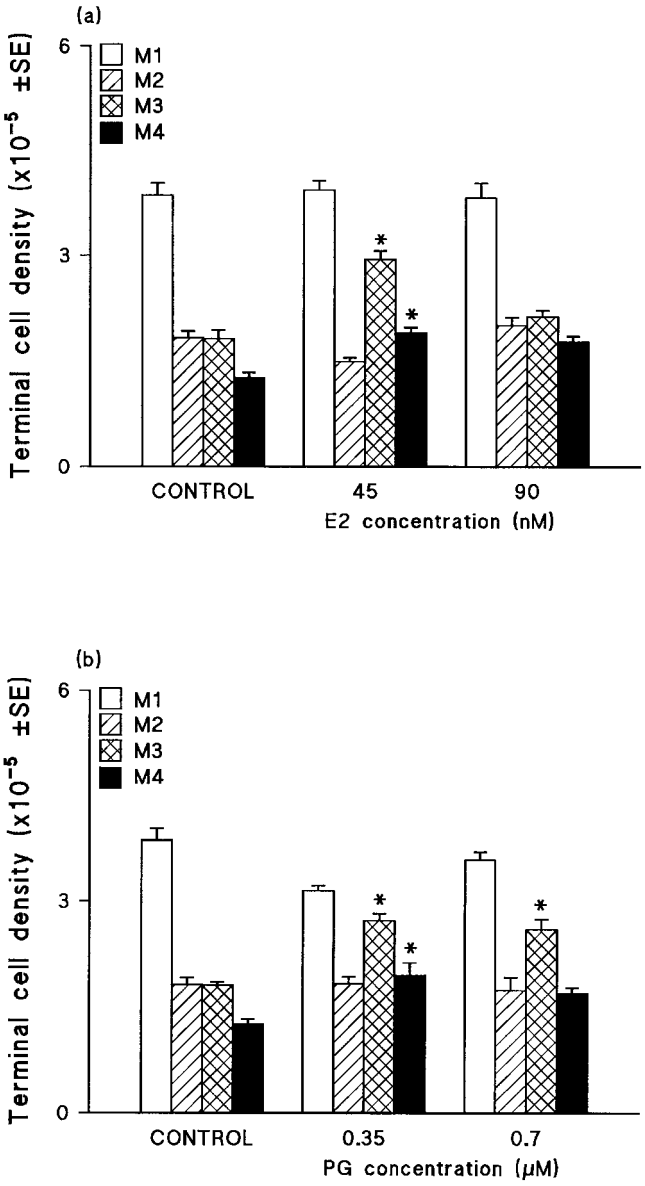

FIGURE 4 – Effects of 17β-estradiol *(a)* and progesterone *(b)* on terminal cell density of cultured meningiomas. Cells were incubated with the above for 4 days and then lysed for nuclei counting as described in the text. $*p < 0.05$ *vs.* appropriate untreated control, by unpaired Student's *t*-test.

have indicated nuclear localisation of PgR in meningioma cell cultures, and transient transfection of these cultures with a progesterone-responsive element indicate that the receptor is indeed functional in these tumours (Carroll *et al.*, 1995). The functionality of the ER is less clear, and it has been suggested that ER within meningiomas may not meet the specific criteria for true steroid receptor proteins (Schwartz *et al.*, 1984). It is possible that the ER within meningiomas may exist as a truncated or mutated receptor, which is unable to bind ligand but still able to induce transcription, perhaps acting as an oncogene. Both wild-type and mutated ER have been detected in a small number of meningiomas (n = 5) using the technique described herein (Koehorst *et al.*, 1994), and ER variants, lacking exons 4 and 7, have been described in 8/8 PgR-positive meningiomas using PCR (Koehorst *et al.*, 1993*a*). However, there have been results showing that physiologically relevant concentrations of estrogens induced growth stimulation of meningiomas *in vitro*, providing evidence for the biological activity of estrogen in meningioma cell growth (Jay *et al.*, 1985; Blankenstein *et al.*, 1989; Koper *et al.*, 1990).

In the present study, under phenol red-free and reduced serum conditions, 2/4 cell cultures prepared from meningiomas gave a modest rise in cell number in response to exogenous E2 and PG, with an E2 response corresponding to a parallel response to PG. This would be as predicted in hormone-dependent tumours such as breast, in which expression of PgR is directly regulated by ER (Horwitz and McGuire, 1978); however, this is not the case in meningiomas in which expression of PgR is independent of estrogen (Blankenstein *et al.*, 1995). The lack of effect of steroids in the remaining samples suggests that although gene transcripts for their receptors are expressed, they are probably non-functional, at least through the conventional ligand-receptor pathway, although activation of other signal transduction pathways by these steroids remains a possibility. The identification of a soluble ER-like protein in meningioma tissues of both ER^+ or ER^- phenotype further supports this hypothesis (Koehorst *et al.*, 1993*b*). It is also worth noting that the enzyme 17β-hydroxysteroid dehydrogenase, which catalyses the reversible conversion of estrone to the biologically more potent E2 has been identified in meningiomas (Carsol *et*

al., 1995), lending further support for a possible role of estrogens in these tumours.

In summary, using an RT-PCR technique, we have detected co-expression of ER and PgR transcripts in a series of human meningiomas. Further, we provide evidence for a proliferative effect of E2 and PG in some meningioma cell cultures. The fact that not all cultures responded to these steroids suggests that the receptors may not always be functional and may thus lack transcriptional activity. However, the function(s) of these receptors and their ligands may have relevance with regard to the biological behaviour of these tumours and deserves further investigation.

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