ORIGINAL ARTICLE

The allelic modulation of apolipoprotein E expression by oestrogen: potential relevance for Alzheimer's disease

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Background: The ε4 allele of the apolipoprotein E (*APOE*) gene is a major genetic risk factor for Alzheimer's disease but appears to be associated with greater risk in women than in men. Some studies suggest that the level of *APOE* may of its own modulate the risk for Alzheimer's disease. Sex differences and an apparent benefit of oestrogen therapy suggest a role for oestrogen. *APOE* expression is influenced by oestrogen and oestrogen therapy may not benefit women bearing an *APOE* ε4 allele. These findings suggest an interaction between oestrogen and *APOE* in the Alzheimer's disease process.
Aim: To explore the hypothesis that *APOE* expression is regulated by a genomic mechanism and is modified by the polymorphisms in *APOE* associated with risk for Alzheimer's disease.
Methods: In vitro binding studies were undertaken between oestrogen receptors and fragments of the human *APOE* gene. *APOE* gene expression was studied to investigate a possible functional interaction.
Results: *APOE* ε2/ε3/ε4 coding and –219 G/T promoter polymorphisms influenced binding to the oestrogen receptor and altered transcriptional activity in response to oestrogen.
Conclusions: An allele dependent modulation of oestrogen induced regulation of *APOE* might be involved in the increased risk for Alzheimer's disease in women bearing an ε4 allele.

In the main, Alzheimer's disease is inherited in a complex pattern that probably involves multiple genes and environmental factors. Many genes have been investigated for their potential to modulate the risk for this disease, but so far only variations in the apolipoprotein E gene (*APOE*) have consistently been found to be associated with the complex inherited forms. The ε2, ε3, and ε4 alleles are encoded by polymorphisms in exon 4 of the *APOE* gene. The ε4 allele occurs two to three times more often in Alzheimer's disease than in non-demented controls, whereas the ε2 allele may be protective.[1] The relation is less clear in familial forms. A lower age of onset is reported in epsilon 4 bearers with mutations in the amyloid precursor protein (APP),[2] but not with mutations in presenilin-1.[3] The *APOE* ε4 allele is estimated to only account for increased risk in around 50% of complex forms of Alzheimer's disease. Furthermore, individuals bearing an ε4 allele do not necessarily develop Alzheimer's disease. Thus other determinants are involved independently or by modifying the risk associated with ε4. Polymorphisms influencing the risk for Alzheimer's disease may lie within the promoter of the *APOE* gene itself.

There is also growing evidence that the level of expression of *APOE* may of its own play a role in Alzheimer's disease, and that polymorphisms influencing the risk for the disease might lie within the promoter of the *APOE* gene. We and others have found the –491 A/T and –219 G/T polymorphisms modify risk for Alzheimer's disease, whereas a role for +113 G/C and –427 T/C has received less attention or support.[4–10] We undertook a meta-analysis in over 2000 cases and 2000 controls and showed that despite linkage disequilibrium with ε4, these polymorphisms are independent risk factors for Alzheimer's disease.[10] Both –491 A/T and –219 G/T promoter polymorphisms were found to modify *APOE* expression in vitro and so could potentially do so in vivo.[11 12] Some but not all studies report evidence of differential expression of *APOE* alleles in human brains.[13 14] Laws *et al* found that the –491 A/T polymorphism was associated with altered levels of ApoE in human brains.[15]

Several studies provide support for different functional roles of the ApoE promoter polymorphisms. First, altered *APOE* expression may in turn modify the A load, as suggested in mice bearing the human APP transgene. Greater amyloid deposition was found with increasing copy number of the murine *APOE* gene.[16 17] Indeed, the promoter polymorphisms have been associated with increasing amyloid deposition in the brain of controls and cases of Alzheimer's disease.[18 19] Second, another potentially enlightening functional association derives from our report of the –219 G/T promoter polymorphism and its possible influence on poor recovery after traumatic brain injury (TBI).[20] Intraneuronal ApoE is markedly increased after acute TBI. The ε4 allele has also been associated with poor outcome after TBI and there is evidence that TBI can itself predispose to Alzheimer's disease.[21–27]

An understanding of the mechanisms of control of *APOE* expression may identify other risk factors and even alternative approaches to treatment. One such mechanism might involve an oestrogen signalling pathway whereby oestrogen, acting as a nuclear transcription factor, may influence *APOE* expression. Many of the effects of oestrogen arise at the genomic level by the classical molecular mechanism of oestrogen binding to nuclear oestrogen receptors, ESR1 and ESR2. This is followed by binding of oestrogen receptors to consensus oestrogen response elements (ERE) in the target gene and leads to altered transactivation of gene expression.[28] It is not yet clear whether a potential interaction between *APOE* and sex may result from oestrogen receptor dependent mechanisms; however, this is possible because ESR1 and ESR2 are found in the regions of the brain that include the areas affected by Alzheimer's disease.[29] Recently, the *ESR1*

Abbreviations: ApoE, apolipoprotein E; APP, amyloid precursor protein; EMSA, electrophoretic mobility shift assay; ERE, oestrogen response element; RLA, relative luciferase activity; TBI, traumatic brain injury

gene itself was reported to be a possible risk factor for Alzheimer's disease.[30 31]

Although we and others found that polymorphisms in the oestrogen receptor genes are poor genetic determinants of Alzheimer's disease,[32 33] it is possible that *APOE* expression may be modulated directly by oestrogen. Most oestrogen responsive genes possess one or more EREs that act alone or synergistically with each other and with other sites, such as SP1 and AP1, to modulate gene expression. An ERE was determined to have the consensus sequence 5′-GGTCAnnnTGACC-3′. However, few oestrogen responsive genes contain perfect consensus EREs because ERE-like sites and half sites can modulate transcription. Furthermore, a contribution from the flanking sequences may allow EREs to retain function despite core sequence variations.[34] Nevertheless, the flanking sequences that are needed to define these functional non-consensus EREs are unknown. Thus the current method of assessment of ERE in addition to sequence inspection is a functional approach, first to determine if oestrogen receptors are able to bind to the putative ERE, and second to determine if oestrogen can transactivate expression through an oestrogen receptor dependent pathway. We have used this approach to address our hypothesis that the polymorphisms –491 A/T, −219 G/T, and ε2/ε3/ε4 modulate oestrogen receptor binding to ERE-like sequences in the *APOE* gene, and thus potentially modify gene transactivation. We used MCF-7 breast cancer cells which allowed a pilot study using suitably designed expression vectors in a cell line known to contain functional oestrogen receptors and known to respond to oestrogen. We then carried out the same experiments in a human glioma cell line that is more relevant to brain, glia being a major source of ApoE in the human brain.

METHODS

Screening for ERE consensus sequences

Sequences within the *APOE* gene were searched for the number of matches to the ERE consensus sequence, GGTCAnnnTGACC, at every nucleotide, using a program designed in-house. The program predicts the same putative EREs as other software packages such as TRANSFAC and MATINSPECTOR, except that it only looks for sequence similarities for a specific consensus sequence of interest, not every factor's sequence, and produces a graphic output.

Electrophoretic mobility shift assays

The specificity and relative affinity of binding of putative ERE sequences to oestrogen receptors were determined by electrophoretic mobility shift assays (EMSA). Labelled DNA was bound to oestrogen receptor and assessed for specificity using specific and non-specific sequences. Relative affinity was determined by increasing concentrations of specific competition with unlabelled sequences.

In the following oligonucleotide sequences, polymorphisms are shown in parentheses and the putative ERE underlined. For the promoter polymorphisms, the following single stranded oligonucleotides were annealed to their respective complementary oligomer to form oligonucleotide probes. The –491 AT polymorphism probe was: 5′-end DIG labelled-GGGGTTTCACCATGTTGGCCAGGCTG-GTCTCAA(A/T)CTCCT GACCTT-3′ and 5′-AAGGTCAGGAG(T/A)TTGAGACCAGCCT GGCCAAC-ATGGTGAAACCCC-3′. The same unlabelled sequences were used to generate probes in competition assays. The –219 GT polymorphism probe was: 5′-AGGG TGTCTG(G/T)ATTACTGGGCGAGGTGTCCT-CCCTTCCTGGG-GAC-3′ and 5′-end DIG labelled-GTCCCCAGGAAGGGAG GACACCTCGCCCA-GTAAT(C/A)CAGACACCCT-3′. The same unlabelled sequences were used to generate probes in competition assays. Tests of binding specificity were done by specific and non-specific competition with unlabelled probes and random scrambled non-ERE-containing probes designed according to Jeltsch *et al*[35]: 5′-GCGAGATATGCGA GATATGAGATTCCTTAGCGAG-3′ annealed to 5′-CGCTCTAT ACGCTCT-ATACTCTAAGGAATCGCTCTA T-3′.

For the ε2/ε3/ε4 polymorphism probes, an exon 4 polymerase chain reaction (PCR) fragment was amplified containing both polymorphic sites and putative ERE (see fig 1B), using the following primers: forward oligonucleotide, 5′-GGCACGGCTGTCCAAGGAG-3′; reverse oligonucleotide, 5′-end DIG-labelled GCCCCGGCCTGGTACACTGCCA-3′.

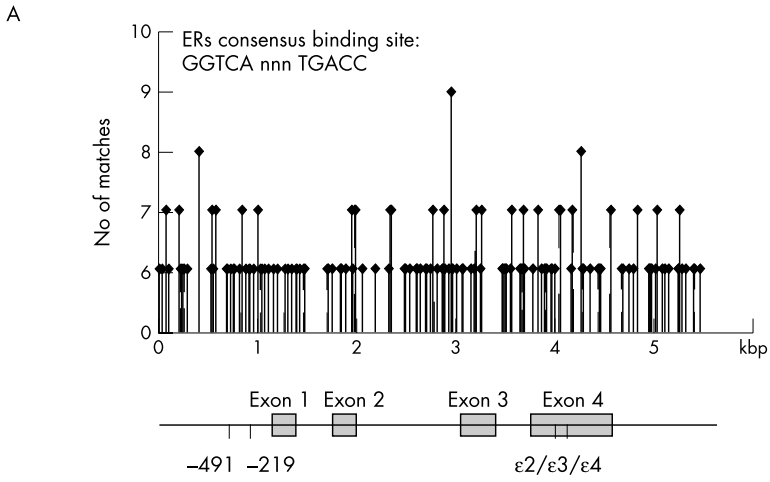
PCR product probes were purified by gel extraction and the concentration estimated spectrophoretically. Tests of specificity were done using the following unlabelled probes: (1) an oligonucleotide probe containing the putative ERE sequence of interest (5′-TCCTCCGCGATGCCGATGACCTGCAGAAG(C/T)GCCTGGCAGT-3′ annealed to 5′-ACTGCCAGGC(G/A)CTTCTGCAGGTCATCGGCATCGCGGAGGA-3′); (2) the random scrambled non-ERE oligonucleotide probe described above; and (3) a probe corresponding to a sequence located at the 5′ end of the PCR product but which did not include a putative ERE (forward, 5′-GGCCCGGCTGGGCGCGGAC-ATGGA-3′; reverse, 5′- CTCCATGTCCGCGCCCAGCCGGGCC-3′). All 5′-end DIG labelled oligonucleotides were purified by high performance liquid chromatography (HPLC; Oswell).

Recombinant ESR1 (Cambridge Biosciences) (100 fmol) was added to a final volume of 20 μl of a mixture containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol (DTT), 15% glycerol, 2.5 mg/ml bovine serum albumin (BSA), 2.5 mg/ml poly(dIdC), and 250 fmol of 5′-end labelled PCR product or 1 pmol of probe (annealed DIG labelled oligonucleotides). The reaction was incubated for 25 minutes in ice before gel analysis. Increasing amounts of unlabelled oligonucleotide probes or PCR product probes (from 0.5- to 20-fold molar excess) were used in competition experiments. The complexes were separated on a 5% non-denaturating polyacrylamide gel followed by semi-dry electrophoretic transfer to nitrocellulose membranes. Detection was as described by the supplier (Roche Diagnostics).

The results from at least three EMSAs were analysed by densitometric analysis (Alpha Imager TM 2000) and used to generate graphs of relative binding (that is, density of probe-ESR1 complex relative to zero competition) at increasing levels of competition. The mean (SEM) values were plotted at each competition level for each allele. Data in the linear region of the competition curves were used in the analyses described below.

Construction of reporter plasmids

We cloned 140 bp and 240 bp fragments containing the –491 A/T and –219 G/T polymorphisms, respectively, into a luciferase reporter vector pGL3 promoter, upstream of a SV40 promoter (to ensure a detectable basal level of transcription and the detection of an increase or decrease in transcription). The *APOE* exon 4 PCR fragment previously used for our EMSA experiments was cloned into the pGL3 promoter vector downstream of the firefly luciferase gene enhancer cloning site (this position was preferred as the exon 4 is downstream of the *APOE* promoter and consistent with the location of a downstream enhancer/repressor). The fragments containing the polymorphisms of interest were amplified by PCR from genomic DNA of homozygous individuals using the following forward and reverse oligonucleotides: exon 4 ε2/ε3/ε4 polymorphism, 5′-GCGG-ATCCGGCACGGCTGTCCA-AGGAC-3′ and 5′-GCGGATCC-GCCCCGGCCTGGTACACTGCCA-3′; −491 AT polymorphism, 5′-GGTACCCTCAGCCTCCCAAGTAGG-3′ and 5′-AGATC-TCAGTGGGCGAATCACTTA-3′; −219 GT polymorphism,



B

–491 AT:
caccatgttGGCCAggcTGGTCtcaa (a→t) ctcctg

–219 GT:
tcg (g → t) attactGGGCGaggTGTCCtcccttcctg

ε2, ε3 and ε4:
tggaggacgtg(c → t)gcggCcgcctggtgcagtaccgcggcgaggtgcaggccatgctcggccagagcaccgaggagctgcg
ggtgcgcctcgcctcccacctgcgcaagctgcgtaagcggctcctccgcGATGCcgaTGACCtgcagaag (c → t) gc

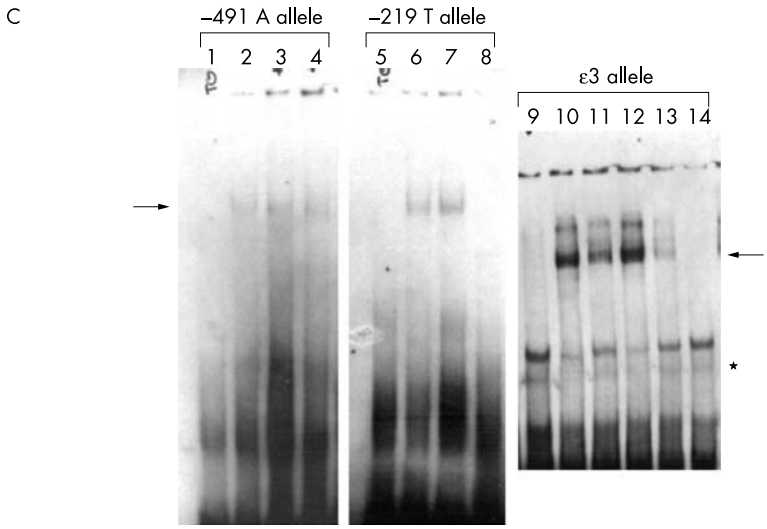


Figure 1 Screening of putative oestrogen response elements (ERE) in the *APOE* gene. (A) The number of matches to consensus ERE for six or more matches out of 10; *APOE* sequence nucleotides number is represented along the y axis. An *APOE* gene schema is shown illustrating the positions of promoter, intron, exon structure, and polymorphisms relative to putative ERE matches. (B) Sequence and localisation of putative EREs near the −491 A/T, −219 G/T, and ε2/ε3/ε4 polymorphisms. (C) Electrophoretic mobility shift assay (EMSA): the labelled −491 A, −219 G, and exon 4 (ε3 allele). Polymerase chain reaction (PCR) probes were incubated as follows: without ESR1 (lanes 1, 5, 9); with ESR1 (lanes 2, 6, 10); with ESR1 and 30-fold excess of random scrambled unlabelled probe (lanes 3, 7, 11); with ESR1 and 30-fold excess of specific unlabelled probe (lanes 4, 8, 13) or unlabelled exon 4 PCR product probe (lane 14); with ESR1 and 30-fold excess of a probe sequence located outside of the ESR1 binding site in exon 4 of the *APOE* gene (lane 12). Arrows indicate specific band showing the binding complex (ESR1 protein–*APOE* labelled exon 4 PCR product); asterisk, non-specific band.

5′-AGATCTACTCAAGGATCCCAGACTTG-3′ and 5′-GGTACC TACTTTCTTTCT-GGGATCCAG-3′. Fragments were cloned using the T-easy plasmid kit (Promega). After excision of the fragments of interest by enzyme restriction and gel purification, the APOE exon 4 fragment was cloned into the *Bam*H I site, downstream of the Firefly luciferase gene in the pGL3-promoter vector (Promega). The *APOE* promoter fragments were cloned into the *Kpn* I and *Bgl* II sites upstream of the firefly luciferase gene of the pGL3 promoter vector. The integrity of every insert was confirmed by sequence analysis using the Taq Big Dye Terminator sequencing kit on an ABI 377 sequencer (Perkin-Elmer Applied Biosystems).

Cell culture and transfection experiments

MCF7 (gift of Dr Moray Campbell, Birmingham, UK) and U138MG glioblastoma (ATCC) cells were maintained in Dulbecco's modified eagle's medium/F12 (1:1) (Gibco), supplemented with 10% fetal calf serum (FCS) (Gibco) and

1×antibiotic/antifungal solution (Gibco). For transfection experiments, the cells were seeded in hormone deficient growth medium (DMEM/F12 (1:1), lacking phenol red and with 10% dextran coated charcoal FCS (Perbio)), at 2.5×10^5 cells/well in a 12 well culture dish (Costar). After 48 hours, cells were transfected for five hours using fugene 6 (Roche) at a ratio of 1:3 DNA to fugene, plus cotransfection with renilla vector for normalisation of transfection efficiency. Triplicate wells were then treated either with vehicle only (β-cyclodextrin, DMSO 0.1%; Sigma); or with water soluble oestrogen 10^{-7} M (Sigma); or with water soluble 17-oestradiol (oestrogen) 10^{-7} M and ICI 182,780 10^{-5} M dissolved in DMSO (a gift of Dr Ian White, Leicester, UK).

Cells were harvested 24 hours later and lysed in reporter lysis buffer (Promega). Firefly luciferase activities (LAf) and renilla luciferase activities (LAr) were measured sequentially by luminometry, using a dual luciferase reporter assay system (Promega). To adjust for any variation in transfection efficiency and DNA uptake, the relative luciferase activity (RLA) was calculated as RLA = LAf/LAr. To compare the RLA of one construct with another, relative RLA was normalised to the relative luciferase activity of empty pGL3 promoter vector before and after treatments. At least three independent transfection experiments were done in triplicate using fresh construct preparations.

Statistical analysis

Differences in binding and transcriptional activity were determined by unpaired *t* test using SPSS for windows, version 9.0 (Chicago, Illinois, USA).

RESULTS

Screening of ERE in the APOE gene

Several ERE-like consensus sequences and half sites were found in the *APOE* gene sequence. Three regions (promoter, intron 2, and exon 4) showed high match to consensus (fig 1A). We detected putative EREs with 7/10 matches to consensus within a few bases of the promoter −491 A/T, −219 G/T, and exon 4 ε2/ε3/ε4polymorphisms (fig 1B). We next investigated whether the EREs in the locality of these polymorphisms were able to bind oestrogen receptor.

ERE near the −219 G/T and ε2/ε3/ε4 polymorphisms specifically bind recombinant ESR1

The −491 A and −219 G oligonucleotide probes were used in EMSA with recombinant oestrogen receptor and revealed weak protein–DNA complex formation (fig 1C, lanes 2 and 6) compared with free probe (fig 1C, lanes 1 and 5). The ESR1-219 G probe binding was competed by an unlabelled −219 G probe (lane 8), supporting the specificity of ESR1 binding. In contrast, the ESR1-491 A probe binding was not specifically competed by an unlabelled −491 A probe (lane 4), suggesting non-specific binding to ESR1. Binding in both cases was not competed by an unlabelled random scrambled oligonucleotide sequence (lanes 3 and 7).

A putative ERE lies between the two *APOE* coding polymorphisms in exon 4 (fig 1B). Thus EMSA experiments were done using DIG labelled exon 4 PCR fragment probes containing the polymorphisms. We detected a strong band—a complex of the labelled PCR fragment with recombinant ESR1 (fig 1C, lane 10). This was compared with free probe in the absence of oestrogen receptor (fig 1C, lane 9). Complex formation appeared stronger than observed with the –219 G probe (Fig. 1b). The shift from free to ESR1 bound exon 4 probe was specifically competed by incubation with an unlabelled probe only containing the putative ERE sequence (lane 13) or excess of unlabelled PCR product (lane 14). In contrast, competition with a scrambled probe (lane 11) or probe corresponding to a part of the *APOE* PCR fragment not containing this putative ERE (lane 12) showed little competition, supporting the specificity of binding of the putative exon 4 ERE to ESR1. Similar results were observed with recombinant ESR2 (data not shown).

In vitro binding of ESR1 is influenced by the −219 G/T and ε2/ε3/ε4 exon 4 polymorphism alleles

We next considered whether there was an allele dependent binding of ESR1 to the sequence containing the −219 G/T and ε2/ε3/ε4 polymorphisms (−491 A/T was not pursued because binding to oestrogen receptor was not specific). We compared the affinity of ESR1 for oligomers containing the −219 G and T alleles. We found the G allele displayed an average 1.7-fold lower affinity than the T allele under equilibrium conditions (fig 2). Similarly, we compared the affinity of ESR1 for exon 4 PCR fragments containing the ε2, ε3, and ε4 alleles. The ε2 and ε4 alleles showed an average twofold lower affinity than the ε3 allele under equilibrium conditions (fig 3). Similar results were observed using recombinant ESR1 (data not shown).

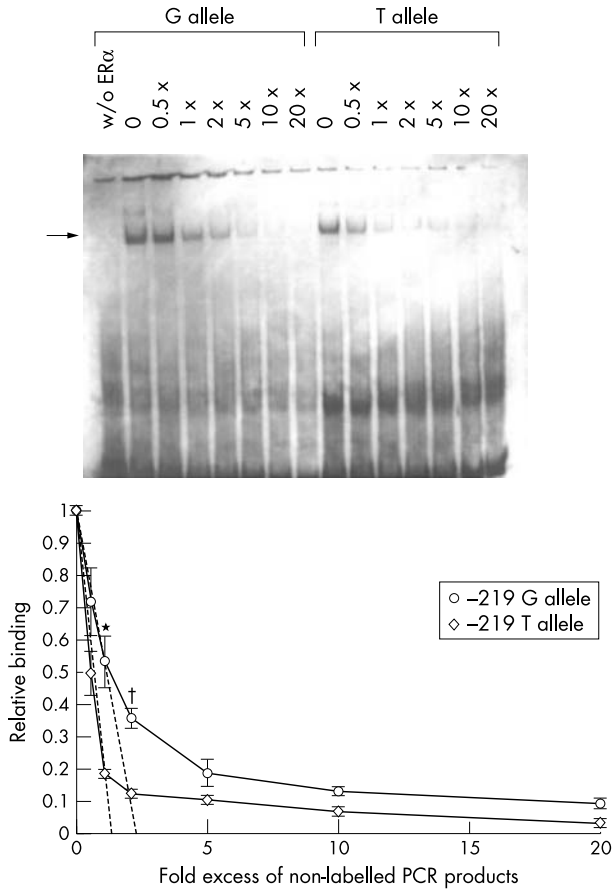


Figure 2 Modulation of the binding of ESR1 by the –219 G/T polymorphism. The binding complex (ESR1 protein–*APOE* labelled probe) is indicated by the arrow; lane 1 is labelled probe product without ESR1, subsequent lanes show decreasing complex formation owing to increasing competition for each allele. Top panel: Competition of binding to ESR1 with increasing concentration of excess of unlabelled probe under equilibrium conditions for alleles G and T. Bottom panel: Relative binding of allele G compared to allele T of the −219 G/T polymorphism with increasing competition by excess unlabelled probe under equilibrium conditions. The slopes of the dashed lines that represent the initial linear phases of competition are described by the equations $y=-0.232x+1.00$ for the T allele and $y=-0.407x+1.00$ for the G allele. Results are the average of at least four experiments; significant differences between alleles at each concentration are shown: *$p<0.05$, †$p<0.005$.

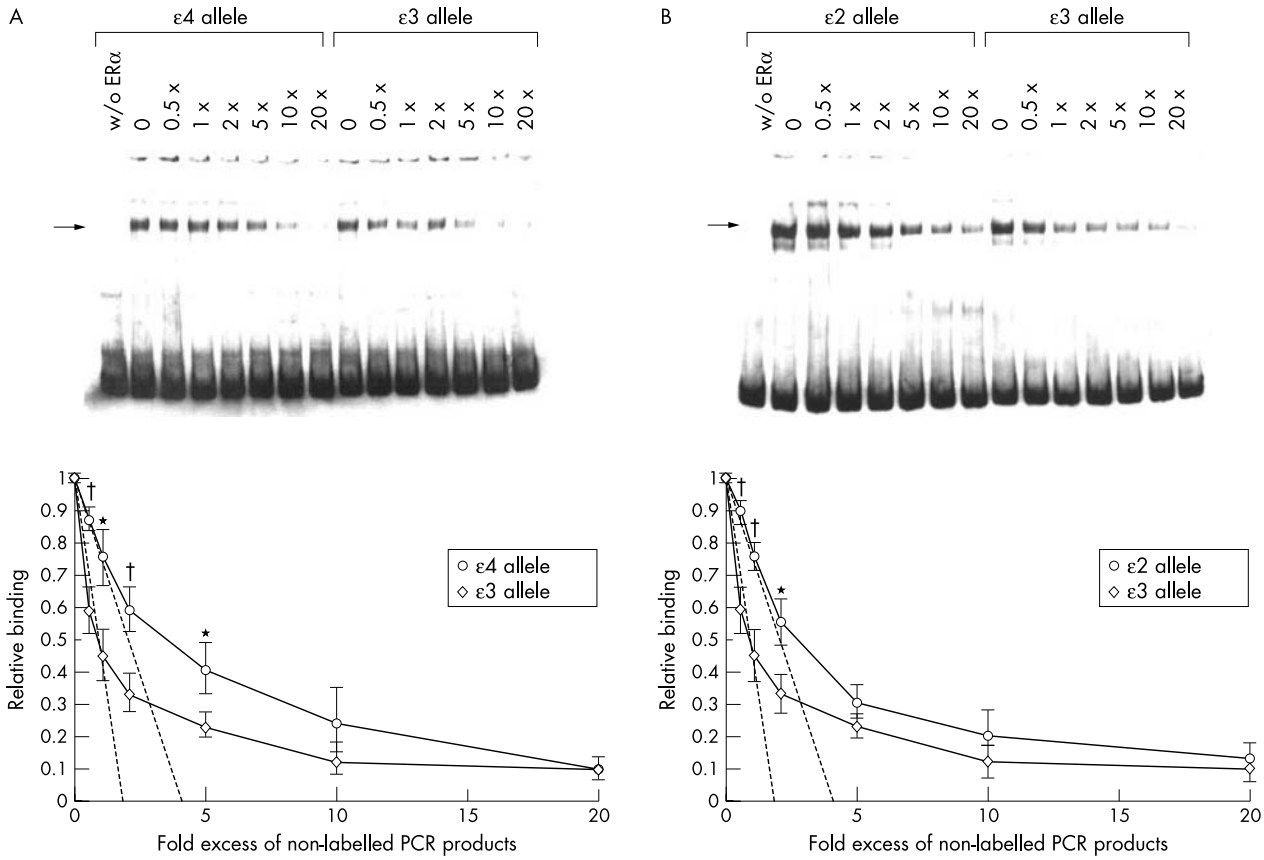


Figure 3 Modulation of the binding of ESR1 by the ε2/ε3/ε4 polymorphisms. The binding complex (ESR1 protein–*APOE* labelled exon 4 polymerase chain reaction (PCR) product) is indicated by the arrow; lane 1 is the labelled PCR product probe without ESR1, subsequent lanes show decreasing complex formation owing to increasing competition (unlabelled PCR product probe) under equilibrium conditions for each allele. Panel (A) compares alleles ε4 with ε3, panel (B) compares alleles ε2 and ε3. The slopes of the dashed lines that represent the initial linear phase of competition are described by the following equations: for the ε2 allele, $y = -0.25x + 1.00$; for the ε3 allele, $y = -0.56x + 1.00$; and for the ε4 allele, $y = -0.24x + 1.00$. Results are the average of at least four experiments. Significant differences between alleles at each concentration are shown: *$p<0.05$, †$p<0.005$.

Comparison of the basal level of transcriptional activity in human MCF-7 breast cancer and human brain derived U138MG glioblastoma cell lines

We next investigated whether the putative EREs were able to mediate oestrogen transcriptional activity and whether this was related to the ESR1 allele dependent binding found alone. Constructs containing the fragments of interest (see Methods) were transfected into breast and glial cancer cells (MCF7 and U138MG, respectively), both previously described as expressing oestrogen receptors.[36 37] The −491 A allele in MCF-7 and U138MG was associated with a higher basal level (control—that is, construct plus vehicle) of transcriptional activity compared with the T allele ($p<0.001$ and $p<0.01$, respectively; fig 4A, 4D). No significant difference in the basal transcriptional activity was observed with the −219 G or T alleles in either cell line (fig 4B, 4E). We could not detect a difference in the basal level of transcription in the MCF-7 breast cancer cells between those transfected with the ε2, ε3, or ε4 constructs (fig 4C). However, in U138MG glioblastoma the ε4 allele displayed a lower basal level of transcription compared with the ε3 and ε2 allele ($p<0.03$ and $p<0.008$, respectively; fig 4F).

Transcriptional activity is influenced by the −219 G/T and ε2/ε3/ε4 alleles in response to oestrogen in MCF-7 human breast cancer cells

The −491-pGL3 construct was not responsive to oestrogen, irrespective of the allele present in MCF-7 human breast cancer cells (fig 4A). In contrast, oestrogen decreased transactivation with the –219 and exon 4 constructs in MCF-7 cells. An allele specific difference in the magnitude of the increase in transcriptional response to oestrogen was found (fig 4B, 4C). For the −219 G allele, oestrogen decreased transcriptional activity by (mean (SEM)) 35.7 (4.0)% ($p<0.001$), and for the T allele, by 21.4 (2.4)% ($p<0.007$). Comparison between the −219 alleles showed that the G allele was more efficient at reducing transcription than the −219 T allele ($p<0.04$). Similarly, for the ε2 allele, oestrogen decreased transcriptional activity by 29.4 (5.3)% ($p<0.05$), and for the ε4 allele, by 39.1 (2.5)% ($p<0.001$), whereas the ε3 allele showed only a non-significant decrease of 7.9 (1.7)%. No significant difference was observed between the relative reduction in transcription in the presence of the ε2 and ε4 alleles.

Transcriptional activity is influenced by the −219 G/T and ε2/ε3/ε4 alleles in response to oestrogen in U138MG glioblastoma cells

The direction of the response to oestrogen was cell line specific. In contrast to MCF-7 cells, oestrogen increased transcription in the presence of the −219 G/T and ε2/ε3/ε4 constructs in glioblastoma cells (fig 4E, 4F). Oestrogen treatment resulted in an increase in transcription with the G allele of the −219 construct by 27.0 (6.2)% ($p<0.01$). The oestrogen induced increase produced by the −219 T allele was not significant compared with untreated cells, at 16.0 (3.5)%. Comparison between the −219 alleles showed that the G allele was more efficient at increasing transcription

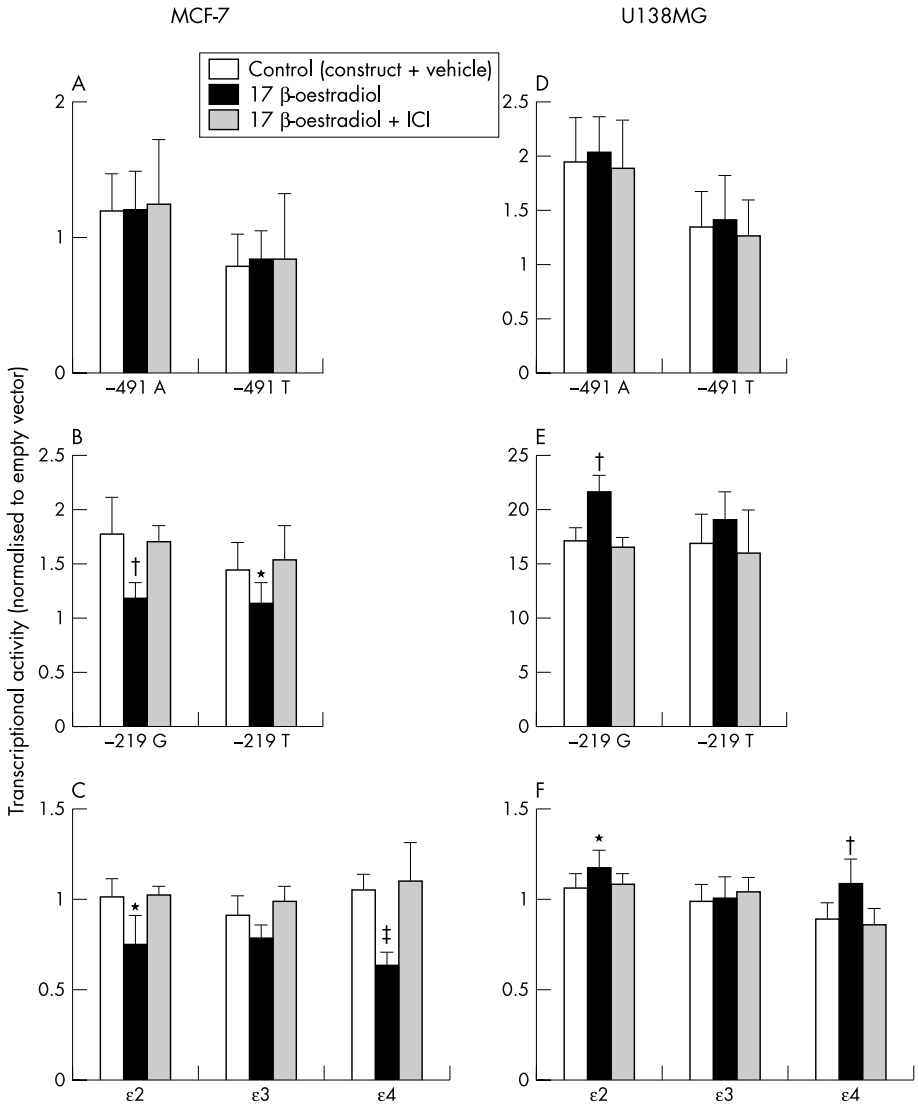


Figure 4 Transcriptional activity associated with the alleles of the −491 A/T, −219 G/T, and ε2/ε3/ε4 polymorphisms: comparison of the effect of vehicle (unfilled bars), oestrogen (black bars), and oestrogen plus oestrogen receptor antagonist (stippled bars). Transfection in MCF-7 cells is shown in panels A, B, and C. Transfection in glioblastoma U138MG cells is shown in panels D, E and F. Statistical comparison of the effect of oestrogen *v* control is shown: *p<0.05, †p<0.01; ‡p<0.001; error bars = SEM. Results are the average of at least three experiments.

than the −219 T allele (p<0.02). We detected significant increases in the transcriptional level in the presence of the ε2 and ε4 alleles (16.2 (2.9)%, p<0.05, and 26.5 (2.3)%, p<0.01, respectively) but not in the presence of the ε3 allele (2.0 (2.3)%). The increase associated with the ε4 allele was greater than the increase in transcriptional level in the presence of either the ε2 or the ε3 alleles (p<0.02 and p<0.001, respectively). The increase in transcription was greater for the ε2 allele than for the ε3 allele (p<0.005).

Modulation of transcriptional activity in response to oestrogen appears to be oestrogen receptor dependent

The oestrogen receptor antagonist ICI 182,780[38] abolished the oestrogen mediated alteration in the transcriptional activity for all responsive constructs in both cell lines (fig 4).

DISCUSSION

Outside of a role in the reproductive system, oestrogen has many effects on the brain: modulation of higher cognitive function, pain mechanisms, fine motor skills, altered mood, susceptibility to seizures, and neuroprotective actions in relation to stroke damage and Alzheimer's disease.[29] Some of the beneficial effects of oestrogens in Alzheimer's disease may include antiamyloidogenic properties, antioxidant effects, modulation of the cholinergic system, actions on neurotrophic factors, and neuroprotection.[39 40]

ApoE is also implicated in several of these processes[41 42] and an ε4 allele–environment interaction has been suspected. Several studies report that women bearing at least one ε4 allele are at increased risk of developing Alzheimer's disease compared with men of the same *APOE* genotype.[1 43–45] Oestrogen has been implicated in Alzheimer's disease because women have a greater risk of dementia than men, particularly in their later years.[42] Various retrospective studies have found a protective effect of exogenous oestrogen on the risk for dementia and impaired cognitive function.[46–55] Not all studies of oestrogen therapy—particularly the prospective studies—showed beneficial effects,[56–61] and it has even been reported that oestrogen/progestin treatment may increase the risk for probable dementia in postmenopausal women aged 65 years or older.[62]

How oestrogen acts at a molecular level has received much attention.[29] Genomically, oestrogen is a nuclear transcription factor that binds nuclear oestrogen receptors, ESR1 and ESR2. This is followed by binding of oestrogen receptors to consensus ERE, ERE-like sites, and ERE half sites in DNA, and leads to altered transactivation of gene expression.[28] This mechanism could result in the induction of neurotrophic factors and ApoE. Support for the influence of oestrogen on *APOE* expression is given, first, by an oestrogen induced increase of *APOE* mRNA expression in murine astrocytes and microglia[63]; second, by the finding that oestrogen promotes synaptic sprouting in response to injury in mice through an ApoE dependent mechanism[64]; and third, by the fact that oestrogen influences murine *APOE* expression through an ESR1 pathway.[65]

Here we report the identification of putative EREs in the human *APOE* gene and show that those in the vicinity of the −219 promoter and ε2/ε3/ε4 exon 4 coding polymorphisms can specifically bind to oestrogen receptors. We piloted the study of our expression vectors using MCF-7 human breast cancer cells because they are known to contain functional oestrogen receptors. The human glioma U138MG cells we used are more relevant to our investigations of ApoE in Alzheimer's disease because glia area major source of *APOE* in the human brain. Our in vitro expression studies in MCF7 cells and U138MG glioblastoma show an allelic dependent modulation of *APOE* transactivation in response to oestrogen by the −219 G/T promoter and exon 4 ε2/ε3/ε4 polymorphisms. Antagonism of these effects by the ICI 182,780 oestrogen receptor antagonist is consistent with a genomic mechanism of action through oestrogen receptors.

In agreement with previous studies of the functionality of other putative EREs,[34] we found that we could not have predicted from sequence alone that the ERE in the vicinity of the −491 A/T polymorphism would bind only weakly and non-specifically to ESR1. Thus this finding was consistent with the apparent lack of functionality in our in vitro expression paradigm using the same promoter fragment.

It is clear that other factors influence oestrogen receptor binding and the functionality of putative ERE and half sites, such as the surrounding sequence.[34] We have only examined single EREs and ERE half sites within 33 mer oligonucleotides or ~150 bp PCR constructs. It is known that several factors such as DNA bending can influence ERE mediated gene expression—for example, the cooperation of other EREs at a distance as far upstream or downstream as 2000 nucleotides. EREs can cooperate with other binding sites and factors such as Sp1.[66 67] Sequences both proximal and distal to the *APOE* gene contain a variety of regulatory regions.[8 9] Of particular relevance to the ERE located near the −219 G/T polymorphism are several potential SP1 and AP1 binding sites. Thus these data do not preclude oestrogen action in the vicinity of −491 A/T or modulation by its alleles.

At the level of cells and tissues, differences in the genomic responses to oestrogen occur because of the different intracellular concentrations of oestrogen receptors, the relative proportions of ESR1 to ESR2,[68 69] and the variation in cell specific factors that contribute to selective modulation of oestrogen receptors, cofactor binding, and transactivation.[28 69] The resultant cell specific variation could explain the directional difference in transactivation by oestrogen that we observed between MCF-7 breast cancer and glioblastoma cells. For both MCF-7 and U138MG glioblastoma cells, we observed differential binding to ESR1 and greater modulation of transcriptional activity of −219 G/T polymorphism in the promoter construct with the G than with the T allele. The greatest modulation of *APOE* construct expression occurred in the presence of the ε4 allele. It is plausible that the combination of nucleotides in the presence of ε4 allows stronger binding to the oestrogen receptor, or perhaps to the secondary structure arising from DNA bending when the ε3 allele is present and limits oestrogen receptor access.

Several mechanisms whereby the ε4 allele increases the risk of Alzheimer's disease have been suggested. These include enhancing Aβ aggregation,[40 41] Aβ toxicity,[40 41] reduced Aβ clearance,[70] impaired dendritic outgrowth and arborisation,[71] a reduced neuroprotective effect,[72] enhanced oxidative neuronal damage,[73] impaired neuronal plasticity, actions involving immunomodulation, glial activation, and CNS inflammatory response,[74 75] and ApoE fragment toxicity.[76] An overstimulation of the ApoE response may well be damaging. If enhanced production of the ε4 allele is detrimental then it is logical to suppose that increased production of the ε4 allele may be deleterious and further exacerbate the Alzheimer's disease process. In vitro studies of ApoE isoforms led to the proposal that increased levels of ApoE may be beneficial up to a point, but a further increase may then lead to cytotoxicity.[77] Previously we reported a 1.5-fold greater relative level of expression of the ε4 allele compared with the ε3 allele in human Alzheimer's disease brains bearing the ε3/ε4 heterozygote than were found in age and genotype matched brains from non-demented individuals.[78]

Experimental and epidemiological findings, although unable to establish a causative effect, are consistent with an accentuated detrimental effect of the ε4 allele in the presence of oestrogen. From experiments in mice bearing the human ε3 or ε4 *APOE* transgenes and driven by the neurone specific enolase promoter, it was reported that the human ApoE isoforms had differential effects on brain function in vivo (specifically in spatial learning tasks) and that the susceptibility to ApoE4 induced deficits was critically influenced by age and sex, these deficits being primarily seen in females.[79]

Our in vitro data show that the expression of the ε2 allele is greater than of the ε3 allele. We might invoke a similar explanation for oestrogen induced increased expression of the ε2 allele. Oestrogen induced differential expression of the ε2 allele could accentuate any beneficial effects of this allele. However, the ε2 allele is rare and there is no epidemiological evidence yet to suggest that the impact of the ε2 allele is sex dependent.[1] In our experiments the changes in expression levels induced by oestrogen were modest. We cannot predict from these experiments what local levels or effects within specific brain cells might occur. Interestingly an increased risk for dementia is found in women bearing the ε4 allele who have a longer reproductive period (that is, a longer lifetime exposure to oestrogen).[80]

If oestrogen does modify *APOE* expression in vivo, perhaps a low but sustained increase over many years might be necessary or sufficient to modify the risk for disease. Mutagenesis in and around the putative ERE sequences reported here could be used to test predicted outcome in response to oestrogen, using paradigms designed to explore the many mechanisms of action of the ε4 allele implicated in Alzheimer's disease, such as neurite outgrowth. Furthermore, it would be interesting to explore our findings by comparing the effects of oestrogen on the relative levels of ε4 and ε3 allele mRNA and E4 and E3 isoforms and function in transgenic mice, particularly where the human transgene is controlled by different promoters—the human *APOE* promoter, neurone specific enolase, and glial fibrillary acidic protein promoters.[80–82]

Given the caveats and limitations of extrapolation from in vitro studies, the oestrogen accentuated expression of the ε4 allele we report here might explain why the beneficial effects of hormone replacement therapy (HRT) are less effective in women bearing an ε4 allele.[55] They may also contribute to

some of the inconsistencies found in epidemiological studies and clinical trials. To further explore this potential pharmacogenetic relation, *APOE* genotyping should be carried out on the studies relating HRT with cognitive abilities and decline in Alzheimer's disease and in non-demented individuals.

Conclusions

Polymorphisms may alter an oestrogen modulated control of *APOE* expression and the levels of the deleterious ε4 allele. Such factors may be involved in influencing the risk for complex inherited forms of Alzheimer's disease. They may also contribute to some of the inconsistencies found in epidemiological studies and clinical trials. The great relevance of gene regulation in disease is emerging and it is important to learn more of the intricacies of the regulation of *APOE*.

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