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Haplotype-dependent Differential Activation of the Human IL-10 Gene Promoter in Macrophages and Trophoblasts: Implications for Placental IL-10 Deficiency and Pregnancy Complications

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Abstract

Problem—Polymorphic changes in the IL-10 gene promoter have been identified that lead to altered IL-10 production. We hypothesized that because of these genotypic changes, the IL-10 promoter might be expressed in a cell type-specific manner and may respond differentially to inflammatory triggers.

Method of study—We created reporter gene promoter constructs containing GCC, ACC, and ATA haplotypes using DNA from patients harboring polymorphic changes at –1082 (G → A), –819 (C → T), and –592 (C → A) sites in the IL-10 promoter. These individual luciferase reporter constructs were transiently transfected into either primary term trophoblasts or THP1 monocytic cells. DNA-binding studies were performed to implicate the role of the Sp1 transcription factor in response to differential promoter activity.

Results—Our results suggest that the GCC promoter construct was activated in trophoblast cells in response to lipopolysaccharide (LPS), as demonstrated by reporter gene expression, but not in monocytic cells. The ACC construct showed weaker activation in both cell types. Importantly, while the ATA promoter was constitutively activated in both cell types, its expression was selectively repressed in response to LPS, but only in trophoblasts. DNA-nuclear protein binding assays with nuclear extracts from LPS treated or untreated cells suggested a functional relevance for Sp1 binding differences at the –592 position.

Conclusions—These results demonstrate cell type-specific effects of the genotypic changes in the IL-10 gene promoter. These responses may be further modulated by bacterial infections or other inflammatory conditions to suppress IL-10 production in human trophoblasts.

Keywords

Human trophoblasts; IL-10 gene promoter; polymorphism; promoter dysregulation

Introduction

IL-10 was originally identified in mice as a product of Th2 T cells with potent suppressive effects on inflammatory Th1 responses.¹ Recent observations have led to the identification of a family of IL-10-related cytokines that include IL-19, IL-20, IL-22, IL-24, and IL-26.² Importantly, IL-10 elicits pleiotropic immune responses and is produced not only by immune cells but also a variety of non-immune cells including trophoblasts.^{3,4} IL-10 is the most potent immunosuppressive cytokine, and its deficiency is associated with autoimmune diseases and heightened susceptibility to inflammation in both humans and mice.^{5–7} On the other hand, its localized production supports neoplastic growth by suppressing tumor-ablating immune responses.^{8,9} Thus, dysregulation of IL-10 expression is a key pathogenic event in a wide spectrum of human diseases. Despite therapeutic and disease-associated functions, the mechanisms regulating IL-10 expression are incompletely understood, even in immune cells that produce large amounts of this cytokine.

There is increased IL-10 production at the maternal–fetal interface during normal gestation as part of the polarized, intrauterine, anti-inflammatory milieu. This local increase in IL-10 has been shown to be produced by placental trophoblast cells and decidual innate immune cells and is implicated in controlling pro-inflammatory activities of these cells and their cytokine products.^{4,10–13} This increased intrauterine IL-10 production is not accompanied by a similar increase in systemic production by peripheral blood mononuclear cells (PBMCs). We have previously shown that this immunoregulatory cytokine is temporally regulated in the human placenta with significant attenuation at term.⁴ Importantly, we and others have demonstrated poor placental IL-10 production in decidua and placental tissue from a variety of pregnancy complications including unexplained spontaneous abortion, preterm birth, and preeclampsia.^{12,14,15} On the other hand, human trophoblasts expressing toll-like receptors (TLRs) produce IL-10 when exposed to lipopolysaccharide (LPS), a ligand for TLR4, suggesting a fail-safe mechanism for heightened inflammation.¹⁶ Nonetheless, it is not clear whether pregnancy complications are associated with defective systemic IL-10 production or whether local attenuation of IL-10 production intrinsically or in response to infection/inflammation contributes to their pathogenesis. It is thus plausible that IL-10 expression may be differentially regulated in the placenta and in circulating immune cells.

Recent reports provide evidence for genetically mediated regulation of IL-10 production. Although several polymorphic changes have been identified in the IL-10 gene promoter, the three sites at the –1082, –819, and –592 positions have been best characterized for their regulatory influence.^{6,17–21} At the –1082 position, the GG allele is associated with significantly increased production of IL-10 compared to the AA or AG alleles. On the other hand, the CC allele at the –592 position is less active compared to AA allele. The IL-10 promoter ATA haplotype constituted of polymorphic changes at the –1082, –819, and –592 positions has been shown to be associated with lower IL-10 production in several studies.²² In this regard, although several common haplotypes of the IL-10 promoter have been associated with a spectrum of pathologic conditions, expression studies are still inconclusive and there are only limited studies in pregnant patients.

Given the importance and temporal production of IL-10 at the maternal–fetal interface and its variance with PBMCs, we undertook this study to compare the common IL-10 promoter haplotypes for their transcriptional activity in trophoblasts and monocytic cells. Our results suggest that the ATA haplotype leads to differential repression of IL-10 production in human trophoblasts, particularly under LPS-inducible conditions. These observations provide a mechanistic basis for the link between microbial infections, inflammation, reduced IL-10 production, and adverse pregnancy outcomes.

Materials and Methods

Genomic DNA and Reporter Constructs

After approval by the Institutional Review Boards of Linköping University Hospital, Linköping, Sweden and Women & Infants Hospital of Rhode Island, USA, genomic DNA was isolated from blood mononuclear cells from normal pregnancy patients and patients with recurrent spontaneous abortion (RSA). Three well characterized single nucleotide polymorphic variants are located at positions -1082, -819, and -592, where the numbering starts from the transcription start site. The homozygous -1082 (G/G), -819 (C/C), and -592 (C/C) genotypes can be further characterized by Mnl I, MaeIII, and Rsa I restriction, respectively (Fig. 1a). An example of the Mae III restriction polymorphism at -819 is depicted in Fig. 1b. PCR amplified fragment of DNA from different patients with a history of RSA encompassing the -819 site was digested with Mae III. Restriction patterns included heterozygous (C/T) genotype (samples 1, 2, and 7), homozygous (G/G) genotype (samples 3, 5, 6, 8, 9, and 10), or homozygous (T/T) genotype (sample 4). The polymorphisms at the -1082 site were characterized by Mnl I restriction. The polymorphism at the -592 site has been shown to be in linkage disequilibrium with the -819 site and this was confirmed accordingly. Promoter DNA was subsequently amplified from homozygous patients harboring either GCC, ACC, or ATA haplotypes encompassing the three polymorphic sites using PCR with forward primers (5'-NheI GCTAGCAAAGTGAATGCAGGCAA-3') (encompassing sequences from -1332 to -1313) and containing an NheI restriction site and with reverse primer (5'-CAAGACAGACTTGCAAAAGAAGGC-CTCGAGXhoI-3') containing a XhoI site (encompassing sequences from +6 to +33) (Operon, Huntsville, AL, USA) (Fig 1a). The fragments containing the three distinct haplotypes were then digested with NheI and XhoI restriction enzymes and ligated into the pGL3-basic luciferase expression vector (Promega, Madison, WI), which had been digested with the same enzymes. All constructs were confirmed by bidirectional DNA sequencing. Resulting plasmids were propagated in *E. coli* and purified.

Placental Tissue and Trophoblast Isolation

Primary cytotrophoblasts were isolated from term placental tissue ($n = 9$) as previously described.^{4,23} Placental tissue was digested four times with decreasing concentrations of trypsin-DNase 1 (starting concentrations: trypsin, 1 mg/mL and DNase, 1.5 mg/mL) at 37 °C for 20 min each. The cell mass obtained was treated with a red blood cell lysis buffer (0.15 M NH₄Cl, 1 mM KHCO₃, and 0.1 mM EDTA (pH 7.3) for 5 min at room temperature with constant shaking. Following a Percoll density gradient (Sigma, St Louis, MO, USA), the layer enriched in primary trophoblast population was further purified by negative selection of CD45⁺ cells using CD45⁺ human micro-beads and magnetic antibody cell sorting large cell separation columns (Miltenyi Biotec Inc. Auburn, CA, USA). The cells collected in the flow through were then cultured in D-MEM (20% FBS) and allowed to adhere overnight. The cells were subsequently analyzed for purity by fluorescence-activated cell sorter analysis for cytokeratin 7 and CD45 (BD Biosciences, San Jose, CA, USA). Cytotrophoblasts isolated in this manner were >98% positive for cytokeratin 7.

Cell Culture and Transient Transfection Assays

The evening before transfection, cells were washed and cultured in Opti-MEM Reduced Serum media (GIBCO/Invitrogen, Carlsbad, CA, USA). Fugene-6 transfection reagent (Roche, Indianapolis, Ind) was equilibrated in a microfuge tube with Opti-MEM media, and plasmids were added using a ratio of 3:1 Fugene to DNA. This mixture was incubated at room temperature for 30 min prior to its addition to cells for overnight incubation. Post overnight incubation, cells were stimulated with 5 µg/mL LPS (0111:B4) (Sigma) for 8 hr and lysed in luciferase cell lysis buffer (BD Biosciences). Luciferase activity was quantified

by addition of BD Monolight Luciferase Reagents A and B (BD Biosciences) and read on Perkin Elmer TopCount NXT Luminometer (Downs Grove, IL, USA).

Real-Time RT-PCR

Total RNA was isolated from cells with Tri-Reagent (Sigma) and transcribed to cDNA with SuperScript III first strand synthesis system for RT-PCR (Invitrogen) as per the vendors' instructions. Real-Time PCR was performed with Applied Biosystems TaqMan Universal Master Mix and inventoried Applied Biosystems TaqMan primers for IL-10 (Hs00961622_ml) and normalized to Beta-2 microglobulin (Hs0018743_ml) (Applied Biosystems, Foster City, CA, USA). The Real-Time PCRs were run on an ABI Prism 7000 (Applied Biosystems) using 50° 2 min., 95° 10 min, followed by 40 cycles of 95° 15 s, 60° 1 min.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclei were isolated from primary placental cells cultured overnight as previously described in Dulbecco's modified Eagle's medium (DMEM) with or without LPS (5 ug/mL).²⁴ Similarly, nuclei were isolated from THP-1 cells cultured overnight in RPMI with or without LPS (5 ug/mL). Cells were harvested in 1× PBS by scraping and spinning down cell pellet at 1000 rpm for 5 min at 4°C. Pellets were re-suspended in 1 mL of Buffer A (10 mM HEPES, pH 7.9, 15 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, and 0.2 mM PMSF), then spun for 5 s. The supernatant was removed and the pellets were re-suspended again in 1 mL of Buffer A and incubated on ice for 10 min. Samples were then vortexed for 30 s and spun for 5 s. The supernatant was again removed and pellets were re-suspended in an equal volume of Buffer C (10 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF) and incubated on ice for 15 min. Samples were spun for 5 min at 4°C, and the protein content in supernatant was quantified via bicinchoninic acid (BCA) protein assay (Pierce/Thermo Scientific, Rockford, IL, USA), aliquoted and frozen at -80°C until use.

Ten-microgram nuclear protein extract from either primary placental cells or THP-1 cells was incubated for 30 min at room temperature with ³²P-labeled probe (10,000 cpm), 200 ng poly(dIdc)-poly(dIdc), 4 uL 5× ABR Binding Buffer (20 mM HEPES, pH 7.9, 60 mM KCl, 5 mM MgCl₂, 2 mM DTT and 50% glycerol), and adjusted to 20 uL with dH₂O. Reactions were then run on an 8% polyacrylamide gel (PAGE). Free probe was run in parallel to distinguish slower migrating protein-DNA complexes from the free probe. Supershift reactions were done as above with the addition of 1 or 5 ug of anti-Sp1 rabbit polyclonal IgG or control IgG (Upstate). Nuclear protein extracts from primary placental cells and THP-1 cells were also tested with radiolabeled Sp1 consensus sequence (Promega, Madison, WI, USA). The specificity of DNA-protein binding was confirmed in competition electrophoretic mobility shift assay (EMSAs), which were carried out as described above with the addition of 50×, 100×, and 200× of the appropriate cold probe.

Radiolabeling of double-stranded nuclear probes was carried out as previously described.²³ Briefly, appropriate primer sets were annealed by taking 2 ug each primer and adding 1 uL 0.3 M NaCl and 15 uL dH₂O and boiling for 3 min. A volume of 4 uL of 0.5 M NaCl was then added, and the reaction was allowed to slowly cool to room temperature. Probes were then end labeled with ³²P using Klenow exo minus (Promega, Madison, WI). The primer sets containing -1082 G or A genotypes or -592 C or A genotypes and used in this study are listed in Table I.

Statistical Analysis

All data are shown as mean $\pm$ SEM. Transient transfection data were compared by analysis of variance followed by Student Neuman Keuls correction for multiple comparisons. In all analyses, a P value <0.05 was considered statistically significant.

Results

Haplotype-based Promoter Activity in Term Trophoblasts and THP1 Cells and Its Modulation by LPS

To determine whether the polymorphic changes at the -1082 , -819 , and -592 positions resulting in GCC, ACC, and ATA haplotypes affect the IL-10 promoter activity and its regulation by inflammatory stimuli such as LPS, we assayed promoter activity in primary human trophoblasts and monocytic THP1 cells. Reporter constructs harboring GCC, ACC, and ATA haplotypes were constructed as described in Fig. 1. These constructs were used in transient transfection assays as described in Materials and Methods. It is possible that inflammatory events and/or infectious agents lead to poor IL-10 production as seen in abnormal pregnancy outcomes, and this scenario may be modified by genetic determinants. To address this issue, a comparative analysis was carried out using primary trophoblasts from term normal pregnancy and monocytic THP1 cells. THP1 cells have been widely used as a model system of circulating macrophages to study the regulatory mechanisms for the IL-10 promoter. Transcription activity in transient transfection assays was normalized to that of parallel experiments employing transfection of the empty pGL3 basic vector, which was assigned a transcription index of zero. In primary trophoblast cells, the GCC construct showed LPS-mediated induction of transcription activity (Fig. 2a). This augmentation of expression ($\sim$ two fold) was statistically significant, $P < 0.05$. In contrast, this increase in expression was not observed with the ACC construct (Fig. 2a). The ATA construct, which includes a genotypic change (C to A) at the -592 position, showed enhanced basal activity that was in contrast inhibited by LPS treatment, $P < 0.05$ (Fig. 2a). This is in agreement with published observations that the A allele at -592 increases promoter activity compared with that of a promoter containing a C allele at this position.¹⁹ These data are intriguing and suggest that the constitutive activation of ATA haplotype in trophoblast cells can be reversed by microbial products. As shown in Fig. 2b, THP1 cells showed robust IL-10 promoter activity (~ 30 fold) for all haplotypes. However, in contrast to the results in primary placental trophoblasts, LPS treatment had a minimum additional effect on IL-10 expression. RT-PCR and IL-10 production as evaluated by cytokine-specific ELISA (data not shown) confirmed the promoter activity observations.

DNA-Protein Binding at -1082 and -592 Sites

It has been shown that a repressor element controlled by the Sp1 transcription factor binds in the vicinity of the -592 site.²⁰ To explain our findings of constitutive activation of the ATA promoter in trophoblast cells and its repression by LPS, the DNA sequences surrounding the -1082 and -592 sites (see Materials and Methods) were examined for nuclear protein binding using EMSA. Nuclear extracts were prepared from LPS untreated and treated trophoblasts and THP1 cells. Extracts were subjected to EMSA with radiolabeled, double-stranded oligonucleotides harboring either of the -1082 sites (G or A allele) and the -592 (C or A allele) sequences. For comparison, we included Sp1 consensus sequences in DNA-protein binding assays. The specificity of all DNA-protein complexes was confirmed by competition with cold DNA probes (data not shown). As shown in Fig. 3, Sp1 consensus sequences exhibited differential binding patterns with nuclear extracts from trophoblasts and THP1 cells, respectively. THP1 cell nuclear extract gave rise to three major bands indicated by arrows. The IL-10 promoter -592 sequences with C or A alleles showed only the middle, albeit a stronger binding affinity with A allele sequence. Curiously, the -1082 sequences

also showed a binding complex at the same position. In the case of trophoblast cells, the lower complex with –592 sequences was apparent irrespective of G or A allele. The complex formation with the –1082 sequence was weak at best. These data warranted an analysis of Sp1 complex specificity for both the –592 and –1082 sequences.

The DNA–Protein Complex with the –1082 Sequence Lacks LPS or Sp1 Specificity

To assess whether DNA–protein complex detected with the –1082 sequence using trophoblast nuclear extract was induced by LPS or involved Sp1 binding activity, we used nuclear extracts from LPS untreated and treated trophoblast cells and performed binding assays in the presence of a Sp1 antibody. As shown in Fig. 4, no major changes were observed for either complex mobility or its disposition by Sp1 antibody, suggesting that the –1082 DNA–protein complex represents a constitutive transcription factor not related to Sp1 or representing a non-consensus Sp1 site.

The –592 A Allele Forms an Sp1 Specific and LPS-inducible Complex in Trophoblast Cells

To explain LPS-mediated repression of the IL-10 promoter containing the ATA haplotype in human term trophoblasts, we hypothesized that LPS induced a repressor activity for the –592 site that was controlled by Sp1 transcription factor. It has been previously shown that Sp1 complementation in an Sp1-deficient cell line decreased human IL-10 promoter function.²⁰ We performed EMSA with –592 C or A allele containing oligonucleotide sequences using nuclear extracts from LPS treated or untreated trophoblasts and in the presence or absence of varying amounts of Sp1 antibody. Data from this analysis are shown in Fig. 5 and present a very interesting scenario. LPS induced a DNA–protein complex that was weakly present in nuclear extract from LPS untreated cells and disrupted by Sp1 antibody. This was unique to the –592 sequence containing the A allele (Fig. 5a). With the –592 sequence containing the C allele, no noticeable change was observed with LPS treatment or Sp1 antibody. These data suggest that LPS induces a repressor that binds to A allele sequences and involves regulation by Sp1.

Discussion

Promoter polymorphisms have been described that influence transcriptional, phenotypic, and functional characteristics of a spectrum of genes.^{25,26} For the human IL-10 gene promoter, polymorphic changes at three well characterized sites, –1082, –819, and –592, are thought to contribute to dysregulated IL-10 production and to the onset and severity of autoimmune, neoplastic, and infectious disorders.^{17–21} We and others have demonstrated in both human and mouse pregnancy models that IL-10 is a critical molecule for successful pregnancy outcome.^{4,11,12,27–31} Moreover, the placental expression of IL-10 is compromised in conditions such as spontaneous abortion, preterm birth, and preeclampsia with minimum effects in circulating PBMCs.^{12,14,15} It was thus plausible that differential regulation as a result of polymorphisms in the IL-10 promoter in specific cell types may lead to poor IL-10 production in the placental microenvironment, particularly in response to intrauterine microbial infections. The current studies validate this hypothesis.

We examined the promoter activity of three constructs that harbored the canonical haplotypes encompassing the –1082, –819, and –592 sites giving rise to either the GCC, ACC, or ATA haplotype (see Fig. 1). Screening of DNA from patients with a history of RSA revealed heterozygous or homozygous allelic variants as defined by unique restriction patterns (Fig. 1). This helped us to avoid generating heterozygous constructs for evaluation of transcriptional potential under varying conditions and in different cell types. EMSAs were used to correlate transcriptional activity in trophoblast cells and THP1 monocytic cells in response to the inflammatory trigger, LPS. Our data are intriguing in showing that LPS leads

to differential repression of transcriptional activation of the IL-10 promoter by the ATA haplotype only in trophoblasts cells, but not in THP1 cells. Importantly, this transcriptional repression was found to be associated with LPS-mediated induction of a protein complex involving the Sp1 transcription factor (Fig. 5). Thus, these data show that polymorphic changes may alter the utilization of promoter haplotypes in a cell-specific and environment-specific manner.

As already mentioned, placental expression of IL-10 in conditions such as spontaneous abortion, preterm birth, and preeclampsia is down-regulated. However, the nature of the molecular mechanisms governing IL-10 production was incompletely understood. Based on these observations, we propose that IL-10 gene promoter polymorphic changes account for some of these clinical observations. The ATA haplotype change may subserve a fail-safe function. In the absence of inflammatory/infectious settings, this haplotype may constitutively augment IL-10 production to program a successful, term pregnancy. However, microbial infections or other inflammatory conditions may threaten maternal and fetal health. In this scenario, repressed IL-10 production may trigger the inflammatory cascade and result in unscheduled delivery.¹⁴ Our data thus suggest that polymorphic changes in the IL-10 promoter and unscheduled induction of Sp1 activity in trophoblasts may provide clues to the underlying mechanisms for adverse pregnancy outcomes.

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Fig. 1.

Construction of IL-10 promoter haplotype-based reporter gene expression vectors. (a) The promoter region encompassing the -1082, -819, and -592 sites harboring homozygous or heterozygous genotypes at these sites was PCR amplified from genomic DNA using forward and reverse primers (see Materials and Methods) containing Nhe1 and Xho1 restriction sites, respectively. The genotypic changes were confirmed by site-directed DNA sequencing. The G/A, C/T, and C/A genotypes have been identified at the -1082, -819, and -592 sites, respectively. (b) An example is shown for homozygous or heterozygous genotypic changes at the -819 site. If C/C allele is present at this position, Mae III enzyme will restrict it, whereas a change to A/A will confer resistance to cutting by this enzyme. A 758-bp fragment was PCR amplified from lymphocyte DNA samples from patients with a history of recurrent spontaneous abortion (RSA) and digested with MaeIII. This reaction gave rise to four DNA bands on an agarose gel. The smaller size bands in this reaction run as a doublet, giving an appearance of a three band profile (see lanes 3, 5, 8, and 9). In case of homozygous change to A/A, a higher molecular weight band and the doublet were observed (lane 4), whereas the heterozygous change led to a mixed profile seen in lanes 1, 2, and 7. U denotes undigested DNA and E represents enzyme digested DNA.

Fig. 2.

Haplotype-based IL-10 promoter activity in primary term trophoblasts and THP-1 cells. The GCC, ACC, and ATA constructs as described in Fig. 1 were assessed for transcriptional activity by transient transfection as described in Materials and Methods. The Luciferase activity was quantified and expressed as relative expression versus empty vector in primary trophoblast cells (panel a) and THP-1 cells (panel b). The expression activity is shown at baseline (no treatment) and following LPS treatment of cells. Data represent at least 3–5 experiments. Note the differences in the two axes.

Fig. 3.

EMSA with nuclear extracts from primary trophoblasts and THP-1 cells using probes corresponding to sequences encompassing -1082 (G or A), -592 (C or A), or Sp1 consensus motif. Three panels, free probe, primary trophoblast, and THP-1 represent the -1082 (lanes 1 and 2, 6 and 7, 11 and 12), the -592 (lanes 3 and 4, 8 and 9, 13 and 14), and the Sp1 consensus (lanes 5, 10, 15) sequences. The -1082 and -592 lanes represent G (lanes 1, 6, 11) or A (lanes 2, 7, 12) and C (lanes 3, 8, 13) or A (lanes 4, 9, 14) genotypes, respectively. The free probe is not visible because the panels only represent DNA-protein complexes. Note the EMSA differences between primary trophoblasts and THP-1 cells. There are two critical bands that represent specific DNA-protein complexes. Nuclear extract from primary term cytotrophoblasts, the faster migrating complex is prominent, whereas the slower migrating complex is prominent with nuclear extract from THP1 cells. The -592 sequences are the binding site with nuclear extract from trophoblast cells, whereas THP1 nuclear extract gives a similar profile with both the -1082 and -592 sequences. The DNA-protein complex profile with the consensus Sp1 binding sequences also differs between primary trophoblasts and THP1 cells.

Fig. 4.

The -1082 site DNA-protein complex does not involve participation of Sp1 in primary trophoblasts as demonstrated by lack of supershift by Sp1 antibody. EMSA and supershift were performed as described in Materials and Methods. Both the -1082 A (lanes 1-4) and -1082 G (lanes 5-8) probes were used. Lanes 1 and 5 represent free probes. Nuclear extracts were isolated from LPS untreated and treated primary trophoblasts. Lanes 2 and 6 represent nuclear extract from untreated cells, whereas lanes 3 and 7 contained nuclear extract from LPS-treated cells. Supershift with Sp1 antibody was performed with nuclear extract from LPS-treated cells (lanes 4 and 8). Note no supershifted bands were observed in the presence of Sp1 antibody.

Fig. 5.

LPS treatment of primary trophoblasts induces Sp1 involvement in DNA–protein complexes at the –592A sequences, not at the –592C sequences. EMSA and supershift were performed as described in legend to Fig. 4. Panels a and b represent EMSA with –592A and –592C probes (see Methods and Materials). Lanes 1–5 represent free probes (lane 1), nuclear extract from untreated cells (lane 2), nuclear extract from LPS-treated cells (lane 3), nuclear extract LPS + 1 μ L of Sp1 antibody (lane 4), and nuclear extract LPS + 5 μ L of Sp1 antibody. Note that Sp1 antibody abolished the faster moving band observed with the –592A probe.

Table I**Primer Sets for EMSA**

Position/Allele Sequence	
-1082A	Forward 5' ctactaaggcttctttgggaaggggaagtagggataggta3'
	Reverse 5' tcgataacctatccctacttcccctcccaagaagccttag3'
-1082G	Forward 5' ctactaaggcttctttgggagggggaagtagggataggta3';
	Reverse 5' tcgataacctatccctacttcccctcccaagaagccttag3'
-592A	Forward 5' catcctgtgaccccgctgtacttagg 3'
	Reverse 5' tcgaagagactggcttcctacagtacag3'
-592C	Forward 5' catcctgtgaccccgctgtccttagg3'
	Reverse 5' tcgaagagactggcttcctacaggacag3'