The **CTLA-4** gene is expressed in placental fibroblasts

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In order to elucidate the mechanisms that ensure survival of the allogeneic fetus, we are investigating the expression pattern of genes that are involved in peripheral self-tolerance in tissues at the maternal–fetal interface. Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is a negative regulator of T cell activation and may modulate peripheral self-tolerance. Previously, we reported the preferential transmission of maternally-inherited shorter alleles at a 3'-UTR microsatellite locus to liveborn children, but random transmission of paternally-inherited alleles, suggesting that CTLA-4 may be involved in the maintenance of tolerance at the maternal–fetal interface. In this report, we demonstrate that CTLA-4 mRNA and protein are indeed expressed in fetal tissues at the maternal–fetal interface throughout gestation. **Key words:** CTLA-4/fibroblasts/immunochemistry/maternal–fetal interface/peripheral self tolerance

**Introduction**

Nearly 50 years after the paradox of the fetal allograft was first proposed by Medawar (1953), the mechanisms that ensure survival of the allogeneic fetus are still poorly understood. A state of mutual tolerance between mother and fetus in normal pregnancy is indicated by the lack of a lymphocyte infiltrate and graft rejection reaction at the maternal–fetal interface (Hunt, 1994), by the transport of fetal cells into the maternal circulation and their long-term survival (Bianchi et al., 1996), and the presence of maternal cells into fetal tissues (Socie et al., 1994; Piotrowski and Croy, 1996; Bonney and Matzinger, 1997). In order to understand the immunological mechanisms that operate during pregnancy, we are investigating the expression pattern of genes that are involved in peripheral self-tolerance in tissues at the maternal–fetal interface.

**CTLA-4** is a member of the immunoglobulin gene superfamily and maps to human chromosome 2q33 (Dartvich et al., 1988; June et al., 1994). CTLA-4 knock-out mice develop severe lymphoproliferative disorders and uniformly die shortly after birth (Tivol et al., 1995; Waterhouse et al., 1995). In humans, a 106 bp allele at a dinucleotide (AT) repeat polymorphism in the 3' untranslated region (UTR) of CTLA-4 or a single base pair substitution at codon 17, which is in linkage disequilibrium with the 106 bp allele, is associated with autoimmune thyroid disease (Yanagawa et al., 1995; Donner et al., 1997) and insulin-dependent diabetes mellitus (IDDM) (Nistocò et al., 1996; Donner et al., 1997; Marron et al., 1997), suggesting that CTLA-4 or a closely linked gene plays a role in the pathogenesis of autoimmune disease. We postulated that long stretches of (AT) repeats in the 3'UTR may be associated with mRNA instability and reduced fetal survival. To test this hypothesis, we examined the transmission pattern of alleles at the CTLA-4 3' UTR ATₙ locus in couples with recurrent spontaneous abortion and their liveborn and aborted fetuses (Tsai et al., 1998). The smaller allele was transmitted from heterozygous mothers to their liveborn fetuses significantly more frequently than expected by chance (P = 0.0071), whereas transmission from heterozygous fathers to their liveborn fetuses was nearly equal to random expectations (P = 0.317). Because lymphocytes are not present in the fetal thymus until 10 weeks gestation (Adinolfi, 1989) and the pregnant uterus contains few maternal T cells (Kabawat et al., 1985), we examined the expression pattern of CTLA-4 protein and mRNA in other types of cells at the maternal–fetal interface.

**Materials and methods**

Tissue samples from normal placentas, collected at the University of Chicago at early (n = 3), middle (n = 3), and late (n = 4) stages of normal pregnancy under an approved Institutional Review Board protocol, were flash-frozen in liquid nitrogen and shipped on dry ice to Kansas City for protein staining. Sections (8 µm) of frozen placental tissues were cut and fixed in cold acetone. The tissues were rinsed in phosphate-buffered saline (PBS) containing 0.3% Tween-20 (polyoxyethylenesorbitan), then non-specific binding was blocked with 10% human serum in PBS/Tween-20. The tissues were then incubated with 5 µg/ml of mouse anti-human CTLA-4 antibody or an isotype-matched control antibody directly conjugated to phycoerythrin (Pharmagen, San Diego, CA, USA). The tissues were then mounted in medium containing glycerol–gelatin for viewing by epifluorescence.

Fibroblasts isolated from the chorionic villi of human placentas at 8, 10 and 19 weeks of gestation characterized as described earlier (Fatt, 1991) were gifts to J.S.H. from M.Fatt, University of Texas, Houston Medical School. The fibroblasts were grown in Dulbecco's

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minimal essential medium (DMEM) supplemented with 10% fetal calf serum and antibiotics (Sigma Chemical Co, St Louis, MO, USA). Fibroblasts were harvested at confluency by brief exposure to trypsin. The cells were either washed, pelleted and shipped to Chicago on dry ice for extraction of RNA or seeded into Lab-Tek tissue culture chamber slides (Naperville, IL, USA) and tested by immune cytochemistry as described above. Total RNA was extracted from activated lymphocytes (positive control), from cultured mesenchyme cells obtained by chorionic villus sampling, and placental fibroblasts using PureScript (Genta, MN, USA). cDNA was made using oligo (T)n primers for reverse transcription–polymerase chain reaction (RT–PCR) (Perkin-Elmer, NJ, USA). cDNA was PCR-amplified using two sets of primers: (i) primers CTLA4cDNAF (5'-GGC TTG CCT TGG ATT TCA G-3') and CTLA4cDNAR (5'-TCA CAT TCT GGC TCT GTT GG-3') span exons 1 through 5 and amplify a 633 bp product in cDNA and a >5.7 kb in genomic DNA; and (ii) CTLA4cDNAF and CTLA4IIR (5'-GTA GGT TGC CGC ACA GAC TT-3') span exons 1 and 2 and amplify a 265 bp product in cDNA and an ~2.3 kb product in genomic DNA (Figure 2b).

Results

In placentas from all stages of gestation, numerous stromal cells expressed immunoreactive CTLA-4 whereas trophoblast cells and endothelial cells were negative (Figure 1A). No staining was observed when the isotype-matched control antibody was substituted for anti-CTLA-4 (Figure 1B). Expression was detected in first, second, and third trimester placentas.

Consistent with the immunohistochemistry results, CTLA-4 mRNA was present in cultured mesenchyme. CTLA-4 cDNA amplified with primers spanning exons 1 and 2 and spanning exons 1 through 5, yielding fragments of 633 bp and 265 bp respectively, and confirming that CTLA-4 is transcribed in mesenchyme cells as well as in stimulated T cells (Figure 2). Because T cells and natural killer (NK) cells are essentially absent from placenta (Kabawat et al., 1985), we examined other cells of mesenchymal origin for CTLA-4 mRNA expression. Indeed, CTLA-4 mRNA (Figure 2a) as well as CTLA-4 protein (Figure 1C) was detected in cultured placental fibroblast cells indicating that these cells express the CTLA-4 gene throughout pregnancy.

Discussion

These studies demonstrate for the first time that CTLA-4 is expressed on non-haematopoetic fetal cells at the maternal–fetal interface. In additional experiments we failed to identify CTLA-4 protein in human foreskin fibroblasts (ATCC RL-2097, passage 4), suggesting that expression in this lineage may be restricted to the maternal–fetal interface (data not shown). The identification of CTLA-4 protein and mRNA in placental fibroblast cells and the previous report that this locus is associated with recurrent miscarriage (Tsai et al., 1998) suggest a role for CTLA-4 in the maintenance of pregnancy. It should be noted that studies using the CTLA-4 knock-out mice may not have addressed the role of CTLA-4 during pregnancy because these animals die at 2–3 weeks of age and are normally bred as heterozygotes.

Based on our previous work (Tsai et al., 1998), we postulated
that CTLA-4 had an immunological function at the maternal–fetal interface. However, the expression of CTLA-4 in placental fibroblasts, which are not in contact with maternal tissues and are in an environment that is devoid of T cells, suggests that this protein may serve a non-immunological function in pregnancy. This is not uncommon in placenta. For example, the inflammation-associated cytokines, tumour necrosis factor (TNF) and interleukin (IL)-6, have non-immunological roles in pregnancy as regulators of hormone synthesis by placental cells (Li et al., 1992; Neki et al., 1993; Pedersen et al., 1995). Similarly, CTLA-4 may have a novel, and as yet unknown, function during pregnancy.

Associations between maternally-inherited CTLA-4 alleles and miscarriage (Tsai et al., 1998) further suggest that aberrant or reduced expression of CTLA-4 in fetal tissues at the maternal–fetal interface may be a potentially common aetiological factor in human miscarriage. Thus, understanding the function of CTLA-4 in pregnancy may elucidate a common genetic cause of pregnancy loss and suggest new approaches to treating the most common disorder in pregnancy.

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References


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