Molecular Human Reproduction Vol.10, No.11 pp. 783–792, 2004
Advance Access publication October 1, 2004 doi:10.1093/molehr/gah107

Interleukin-11 expression: its significance in eutopic and ectopic human implantation

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Embryo implantation and subsequent decidualization, trophoblast invasion and formation of a functional placenta are crucial for establishment and maintenance of pregnancy. Interleukin-11 signalling has been shown to be obligatory for adequate decidualization and trophoblast invasion in mice. Defects in IL-11 signalling in mice result in trophoblast over-invasion and fetal loss. The pathological situation of human tubal pregnancy resembles that of IL-11Rα$^{-/-}$ mice concerning these symptoms. As our interest is focused on the human early pregnancy, we compared IL-11 expression at the implantation site of ectopic tubal pregnancy (EP) to 1st and 2nd trimester of normal intrauterine pregnancies (IP), and to the normal cycling endometrium. The mRNA expression of IL-11 and IL-11Rα was analysed by semiquantitative RT–PCR. Protein expression was detected by western blotting and immunohistochemistry. IL-11Rα is expressed constitutively in all tissue specimens analysed. IL-11 is expressed predominantly during follicular and early luteal phase of the menstrual cycle. In IP, IL-11 expression peaks during the 1st trimester and declines from the beginning of the 2nd trimester onwards. In tubal abortions, IL-11 expression is reduced in comparison to vital EP and IP. Cultured primary endometrial and decidual epithelial cells were analysed for hormonal regulation of IL-11 by enzyme-linked immunosorbent assay and RT–PCR. IL-11 is up-regulated by estrogen and down-regulated by progesterone. Overall, our results indicate that in humans, IL-11 signalling is significantly involved in regulation of trophoblast invasion. In the case of tubal abortion, inadequate IL-11 signalling may therefore result in dysregulation of trophoblast invasion.

Key words: decidua/endometrium/IL-11/trophoblast invasion/tubal pregnancy

Introduction

Interleukin-11 (IL-11) is a cytokine with pleiotropic functions in different tissues. It plays an important role in tissue remodelling (e.g. induction of tissue inhibitors of metalloproteinases; stimulating tissue fibrosis), haematopoiesis (e.g. stimulating proliferation of haematopoietic stem cells and thrombopoiesis; regulating macrophage proliferation and differentiation), and inflammation (e.g. stimulation of acute phase proteins such as C-reactive protein or haptoglobin; reducing proinflammatory cytokines such as TNFα; for reviews see Du and Williams, 1997; Leng and Elias, 1997). In the mouse, IL-11 prevents graft-versus-host disease after allogeneic bone marrow transplantation (Hill *et al.*, 1998).

In particular, interleukin-11 is one of the essential molecules for implantation and early pregnancy. Homozygotic disruptions of the gene encoding IL-11Rα result in female infertility due to deficient decidualization (Bilinski *et al.*, 1998; Robb *et al.*, 1998). Finally, the resulting imbalance between decidualization and trophoblast invasion leads to decidual necrosis and fetal resorption (Robb *et al.*, 1998).

IL-11 is a member of the group of IL-6-type cytokines. Besides IL-6 itself and IL-11, ciliary neurotrophic factor (CNTF), cardiotrophin (CT-1), oncostatin M (OSM), leukaemia inhibitory factor (LIF) and cardiotrophin-like cytokine (CLC) (Elson *et al.*, 2000) belong to this group of cytokines (Heinrich *et al.*, 1998, 2003). Recently, IL-27 was also found to belong to this cytokine family (Pflanz *et al.*, 2004). First, IL-11 binds to a specific IL-11Rα subunit not involved in the intracellular signal transduction cascade. Second, this low affinity complex binds to a gp130 homodimer mediating the signal transduction as a high affinity complex.

This specific IL-11Rα subunit is expressed constitutively throughout normal pregnancy in the mouse. In contrast, IL-11 was shown to be maximally expressed during the time of decidualization in response to the implanting blastocyst. Consequently, in the mouse, IL-11 expression was concluded to be the molecular regulator of decidual proliferation and differentiation (Robb *et al.*, 1998, 2002).

Recent studies have demonstrated that IL-11Rα and gp130, the IL-6 family common signal transducer, are expressed in the human endometrium throughout the menstrual cycle (Dimitriadis *et al.*, 2000; Karpovich *et al.*, 2003). Variable expression of soluble and membrane-bound gp130 found in normal human cycle and early pregnancy decidua (Classen-Linke *et al.*, 2004) might modulate IL-11 signalling. Concerning IL-11, the regulating part of the system, there are no consistent data. IL-11 was shown to be expressed predominantly by glandular epithelial cells, but stromal cells also seem to express low levels of this cytokine. Dimitriadis *et al.* (2000) reported high IL-11 expression in the mid to late luteal phase,

whereas Cork *et al.* (2001) showed an IL-11 expression peak during the late follicular to early luteal phase. Karpovich *et al.* (2003) detected IL-11 throughout the menstrual cycle. Steroidal regulation of IL-11 has been suggested by Dimitriadis *et al.* (2000) and Leng and Elias (1997). Concerning the early pregnancy there is only one study comparing normal pregnancy and 'anembryonic pregnancy' (AP) of the 1st trimester (Chen *et al.*, 2002) in which reduced IL-11 expression in AP was found. To our knowledge, to date there has been no systematical analysis of IL-11 and IL-11Rα during the 1st and 2nd trimester of human pregnancy.

The present study examines the expression of IL-11 and its IL-11Rα during the menstrual cycle and the 1st and 2nd trimester of human pregnancy by immunohistochemistry, western blotting and RT–PCR. In addition, the 'natural human model' of dysregulated trophoblast invasion, the ectopic tubal pregnancy, was compared to normal intrauterine pregnancy. To confirm the steroidal regulation of IL-11 expression in glandular epithelial cells suggested by recent studies, we have measured IL-11 expression in primary endometrial and decidual epithelial cell cultures by RT–PCR, enzyme-linked immunosorbent assay (ELISA) and western blotting.

Materials and methods

Human tissue samples

Endometrial tissue was obtained throughout the menstrual cycle from normal fertile women undergoing hysterectomy due to benign uterine diseases in collaboration with the Departments of Gynaecology and Obstetrics of Marienhospital, Aachen; Luisenhospital Aachen; St Antonius Hospital, Eschweiler; and Medical School RWTH, Aachen. Dating of each specimen was done by menstrual history, histological examination (Noyes *et al.*, 1950) and hormonal assessment for 17β-estradiol (E_2) progesterone and LH on the day of hysterectomy by routine laboratory diagnostics. All patients had a regular menstrual cycle and did not receive hormones for ≥ 6 months before surgery. Specimens from the follicular phase (day 7–14; $n = 19$), the early luteal phase (day 15–20; $n = 8$) and the mid to late luteal phase (day 21–28; $n = 7$) were included.

Decidual tissues were collected from legal termination of normal healthy pregnancies in collaboration with the Bourgognekliniek Maastricht, The Netherlands. The informed consent of the patients was obtained. Patients included in this study were proven to be pregnant by hCG serum test and sonographic control immediately before surgery.

Gestational age was determined by the duration of amenorrhoea, analysis of individual cycle data and by the caput–rump length (CRL) determined sonographically. Only proven fertile women (without any spontaneous abortion and at least one child) who had not received any exogenous hormones within the last 3 months prior to pregnancy were included in this study. Termination was obtained by vacuum aspiration. Fragments of decidua basalis (with trophoblast) and parietalis (without trophoblast) were taken. Specimens were collected from 1st (5–12 weeks after the last menstrual period; $n = 81$) and 2nd (13–17 weeks after the last menstrual period; $n = 36$) trimester of pregnancy and were identified as decidua parietalis and decidua basalis by histological examination and cytokeratin staining of the trophoblast as described before (von Rango *et al.*, 2001).

Tubal tissues from ectopic pregnancies (EP) were obtained from surgical termination of extrauterine tubal pregnancy in cases when tubectomy could not be avoided. Tissue specimens ($n = 3$) were cut in slices of ~5–6 mm. Alternating slides were frozen for further protein or RNA isolation or fixed in formaldehyde and embedded in paraffin to define the implantation site by immunohistochemical staining of cytokeratin as described previously (von Rango *et al.*, 2001), and for further immunohistochemical analysis. Besides these specimens, tissues retrieved from the archives of the Department of Pathology and the Department of Anatomy II were used for immunohistochemical detection of IL-11. All specimens were derived from the 7th to 9th week of pregnancy.

Not all types of analysis could be performed with all specimens included within the study. The exact number for each special part of the study is given within the Materials and methods section.

Our research on these specimens was approved by the Ethics Committee of the University of Aachen School of Medicine.

Cell culture

Epithelial cell culture was performed as described in detail previously (Classen-Linke *et al.*, 1997, 1998). The tissue, either endometrial tissue of the follicular phase or decidual tissue of early pregnancy, was minced under a laminar flow in ~1 mm^3 fragments and digested by shaking for 1 h in 0.125% type I A collagenase (470 IU/mg; Sigma, Germany) at 37°C in Dulbecco's modified Eagle's medium/Ham's F-12 without Phenol Red (ccPro, Germany) containing 1% streptomycin, penicillin and fungizone and 10% charcoal-treated steroid hormone-free fetal calf serm (ccPro).

Stromal and epithelial cells were separated by two filtration steps. The epithelial cells and smaller glandular fragments were seeded with a density of ~$2–5 \times 10^5$ cells/cm^2 on a transparent Biopore membrane with a membrane pore size of 0.4 mm (Millicell CM-filters, 12 mm; Millipore, Germany) coated with the extracellular matrix Matrigel (Becton Dickinson, Germany) diluted 1:3 with culture medium without any additions in a dual-chambered Millicell system. From one endometrial/decidual specimen, 12 inserts were seeded, each containing the same number of cells (2×10^5 cells counted before cell culture with the 'Neubauer haemocytometer' using Trypan Blue dye exclusion for only live-cell counting). The isolated cells were cytocentrifuged on glass slides and their purity was checked by immunohistochemical staining of cytokeratin as described by von Rango *et al.* (2003a). In addition, in exemplary cases epithelial cells were cultivated on Lab-Tek slides (Nunc, USA) and stained for cytokeratin after 8–10 days of culture. Purity of epithelial cells determined by this staining was $\geq 95\%$ as shown earlier by immunohistochemical staining of isolated and cultured cells and by electron microscopy (Classen-Linke *et al.*, 1997; von Rango *et al.*, 2003a).

Endometrial and decidual cell cultures were obtained from ten different donors (five from menstrual cycle, five from early pregnancy) and subdivided into three groups: group 1 (four inserts) was treated only with E_2 (Sigma; 10^{-8} mol/l); group 2 (four inserts) with E_2 (10^{-8} mol/l) in combination with medroxyprogesterone acetate (MPA; Sigma), a metabolically stable progestin, in a concentration of 10^{-6} mol/l, both diluted in ethanol (<0.1%); group 3 (four inserts) was cultured without any hormone supplementation. In two cases of primary decidual cell culture—due to the low cell number obtained during isolation—only culture groups 1 and 2 could be investigated. Every 2 days the culture medium was changed. The apical and basal medium was collected after 8 days of culture and frozen in aliquots before the IL-11 concentration was measured by ELISA.

Immunohistochemistry

Briefly, 5 mm thick sections were mounted on 3-amino-propyl-tri-ethoxy-silane (APES)-coated glass slides, deparaffinized with xylol and rehydrated. For immunohistochemical detection the Vectastain universal Elite ABC kit (Vector Laboratories) was used.

For antigen retrieval, sections were incubated in 0.05% Trypsin/0.1% $CaCl_2$ for 15 min at room temperature. Afterwards they were placed in 0.3% H_2O_2/methyl alcohol for 30 min to block endogenous peroxidase activity, washed in phosphate-buffered saline (PBS)/0.1% bovine serum albumin (BSA), and non-specific binding sites were blocked with normal horse serum supplied with the kit according to the manufacturer's instructions. Sections then were incubated with the monoclonal primary antibody against IL-11 (clone 22616.1; R&D Systems; 1:40 in PBS/1.5% BSA overnight, 4°C). Afterwards the slides were washed in PBS/0.1% BSA and incubated at room temperature with the second antibody (anti-rabbit and anti-mouse; made in horse) provided by the Vector Kit according to the manufacturer's instructions. Following three washes in PBS/0.1% BSA, slides were placed in streptavidin–peroxidase conjugate supplied with the kit. Antibody binding was detected by immersion of the sections for 5–10 min in chromogen solution Vector Blue (Vector SG substrate Kit; Vector Laboratories). Finally, slides

were washed in PBS and deionized water and mounted in glycerolgelatin without additional counterstaining.

For negative controls, normal mouse IgG was used instead of primary antibody at the same protein concentration as the specific antibody.

Decidua basalis and decidua parietalis were identified by immunostaining with an anti-pan cytokeratin antibody (detecting cytokeratin 8, 18 and 19) as described earlier (von Rango *et al.*, 2001). All tissue specimens from one patient were stained for cytokeratin and two randomly chosen specimens of both decidua basalis and decidua parietalis were included in the study. Staining was done two to three times for each tissue fragment (25 endometrial specimens; 69 decidual specimens; 3 EP abortions 5 EP viable tubal pregnancies).

The staining intensity for IL-11 in the tissue sections was scored by two independent investigators. The predominantly epithelial staining was analysed gradually: no staining = 0, weak staining and/or single cells strongly stained = 1, moderate staining and/or <50% cells strongly stained = 2, strong staining of >50% cells = 3. The mean value of each specimen was included for data analysis.

Protein isolation

Endometrial and decidual tissue fragments were snap-frozen immediately after surgery in isotonic ammonium hydrogen carbonate [0.15 mol/l $NH_4(HCO_3)$]. For protein isolation, the samples were quickly thawed at 37°C and homogenized using an Elwein potter by adding 400–800 µl ammonium buffer containing the protease inhibitor cocktail complete TM mini (Roche, Germany). After homogenization, the cells were centrifuged (10 000 *g* 10 min 4°C) and the supernatant containing mainly cytoplasmic proteins was frozen in aliquots for further processing. The total protein concentration of each sample was measured by a Bio-Rad DC protein assay (Bio-Rad Laboratories, Germany).

Cytokine protein detection by ELISA

A commercially available ELISA kit was used to detect IL-11 (R&D Systems, USA; sensitivity 8.0 pg/ml; intra-assay variance <5%; inter-assay variance <6.5%). The cytokine concentrations obtained were above the quoted sensitivity of the ELISA kit. Each measurement was done in duplicate according to manufacturer's instructions. Supernatants of the epithelial cell culture ($n = 5$ in case of decidua; $n = 5$ in case of endometrium) were measured for IL-11. Cytokine protein concentrations are given as pg/ml.

Sodium dodecyl sulphate–disc polyacrylamide gel electrophoresis and western blotting

This was performed under reducing conditions (5% mercaptoethanol) according to Laemmli (1970). Gradient gels 8.3–16.6% were used to separate the proteins according to their molecular weight. The separated proteins were transferred to a polyvinylidene difluoride membrane (Immobilon P; Millipore, Germany; pore diameter 0.45 µm) by a semidry electroblotting procedure (2 mA/cm^2) for 40 min. The membranes were blocked at 4°C overnight in TBS (5 mmol/l Tris-buffered saline; pH 7.6) + 0.1% Tween + 5% milk powder. The detection of the immobilized proteins was performed by incubating for 1 h at room temperature with the antibody against IL-11 (same clone as used for immunohistochemistry) diluted 1:500 in TBS/0.1% Tween/1% milk powder followed by 1 h incubation with a horseradish peroxidase-conjugated secondary antibody (goat anti-mouse IgG; Santa Cruz, USA; 1:5000 in PBS/0.1% Tween). Immunoreactive proteins were detected by chemiluminescence using the enhanced chemiluminescence (ECL) kit (Amersham Pharmacia Biotech, Germany) following the manufacturer's instructions. Western blots were analysed semiquantitatively by Image Master Labscan 1D ELITE software (Amersham). IL-11 migrates as a 23 kDa band (Leng and Elias, 1997).

RNA extraction and reverse transcription to cDNA

RNA was extracted from the cultured cells with the High pure RNA isolation kit (Roche) according to the manufacturer's instructions. RNA from tissues was isolated by the RNeasy Mini Kit (Qiagen; Hilden, Germany). Afterwards the RNA was cleaned by DNAse treatment using the DNA-free Kit (Ambion, UK). Reverse transcription was performed using AMV reverse transcriptase (Roche). The reaction mixture [10 mmol/l Tris; 50 mmol/l KCl pH 8.3; 5 mmol/l $MgCl_2$; deoxynucleotides, 1 mmol/l each; oligo dT primer, 0.08 µg/µl; RNAse inhibitor from human placenta (Roche) 2.5 IU/µl; AMV 16 IU/µl] contained 500 ng total RNA. The reverse transcription programme was: primer annealing for 10 min at 25°C; reverse transcription for 1 h at 42°C; heating to 99°C to denature AMV, and cooling at 4°C. The cDNA obtained was stored at −70°C until further use.

Oligonucleotide primers

Oligonucleotide primers (synthesized by MWG Biotec, Germany) for IL-11 and IL-11-receptor α (IL-11Rα) were designed from the published nucleotide sequences: IL-11 (M57765) forward: ACTGCTGCTGCTGAAGACTCGGCTGT (position 709–734), reverse: ATGGGGAAGAGCCAGGGCAGAAGTCTGT (position 1030–1003), fragment size 322 bp; Il-11Rα (U32324) forward: GTTCTTGGCCTCAGTGATTCC (position 1276–1296), reverse: CACACGTTCCTTGAGCAGAAC (position 1546–1525), fragment size 271 bp; Cyt C (M10546) forward: CGTCACAGCCCATGCATTTG (position 2382–2401), reverse: GGTTAGGTCTACGGAGGCTC (position 2649–2630), fragment size 269 bp; S26 (NG003189) forward: AATGGTCGTGCCAAAAAGGG (position 122–141), reverse: TTACATGGGCTTTGGTGGGG (position 429–448), fragment size 327 bp. The resulting cDNA has an intron-overlapping sequence to yield a longer product in the case of amplification of genomic DNA.

Cytokine mRNA analysis by RT–PCR

The cDNA (decidua $n = 28$; endometrium $n = 25$; EP $n = 3$; cell culture endometrium ($n = 4$); cell culture decidua ($n = 5$) were amplified in a PCR reaction (100 µl total volume) containing 1 × PCR buffer (10 mmol/l Tris; 50 mmol/l KCl; 1.5 mmol/l $MgCl_2$ pH 8.3), 0.2 mmol/l dNTP, 0.1 µmol/l each of sense and antisense primers, 1 µmol/l digoxigenin-11-dUTP (Dig dUTP, Roche), and 2.5 IU Taq-polymerase (Roche). After a preheating phase of 5 min at 94°C, the following amplification protocol was used: 1 min at 94°C, 1 min at the annealing temperature (IL-11: 69°C, 37 cycles in the case of tissue RNA, 29 cycles in the case of RNA form cultured cells; IL-11Rα: 60°C, 36 cycles; Cyt C: 57°C, 20 cycles; S26: 60°C, 25 cycles), and 1.5 min at 72°C for primer extension (IL-11Rα, 45 s). A final extension time of 10 min at 72°C was chosen. PCR products were visualized by ethidium bromide staining on a 1.2% or 1.5% high melting point agarose gel. Discrimination between positive and negative signals was optimized for each RT–PCR by a cycle test to define the linear amplification range. All investigations were performed in duplicate. Quantification of the RT–PCR products was performed using Image Station 440 (Kodak, Germany) giving optical density (OD) values corrected for background staining (NetOD). The housekeeping gene Cytochrome C oxidase subunit 1 (Cyt C) as well as the ribosomal protein S26 were used as an internal control to normalize the amount of cDNA used as template within the PCR reaction of the cytokines. To correct the OD values for minimal differences among the Cyt C (S26) signals, NetOD values for the cytokines were referred to the Cyt C (S26) signal [NetOD cytokine/NetOD Cyt C (S26)] resulting in relative OD values for each cytokine. The mean of the resulting relative OD levels for Cyt C and S26 normalization was used for data analysis.

The identity of the amplified products was confirmed by non-radioactive sequencing (SEQLAB, Germany) and sequence analysis using the blast program (www.ncbi.nlm.nih.gov/BLAST/).

Quantification and statistical analysis

Statistical analysis was performed with GraphPad PRISM Version 3.00 for Windows (GraphPad Software, USA). Mean and SEM were calculated and data were tested to conform to a normal distribution as assessed by Kolmogorov–Smirnov test. Differences between the three groups of different hormonal treatment of cultured epithelial cells were analysed using one-way analysis of variance (ANOVA). Bonferroni's post tests analysed differences between each pairs of these three groups. $P < 0.05$ was accepted as statistically significant.

Results

IL-11 system during the menstrual cycle

Endometrial tissue specimens were divided into three groups: (1) follicular phase (cycle day 7–14); (2) early luteal phase (cycle day 15–20) when—in case of pregnancy—the blastocyst migrates from the Fallopian tube to the uterus and implants; (3) mid–late luteal phase (cycle day 21–28)—after implantation in case of pregnancy—when predecidualization of the endometrium takes place. IL-11 protein as revealed by immunohistochemistry was found predominantly within the glandular epithelial cells (Figure 1). By western blotting the specificity of the antibody used for immunohistochemistry was confirmed by detecting one IL-11 protein band at 23 kDa (Figure 2) in an exemplary specimen from the follicular phase (day 9) of the normal menstrual cycle. IL-11 was not detected in human serum or in cell culture medium control.

Protein expression was maximal in the follicular phase and early luteal phase. Thereafter it declined rapidly (Figures 1 A–C, 3A). In contrast, mRNA expression reached a maximum between cycle days 15 and 20 and declined only slightly during the luteal phase, not reaching the low level of the follicular phase (Figure 3B). IL-11R mRNA was constitutively expressed, showing only a slight decrease from follicular phase on (Figure 3C).

IL-11 system during pregnancy

As in the endometrium during the menstrual cycle, IL-11 was located predominantly within the glandular epithelium as shown by immunohistochemistry (Figure 1D–F). There was no difference in the protein expression pattern between decidua basalis and decidua parietalis. Protein as well as mRNA levels of IL-11 did not vary significantly between pregnancy weeks 5 and 6 and mid-luteal phase endometrium (compare Figures 3A, B and 4A, B). Afterwards there was a strong increase of IL-11 mRNA and protein within the pregnancy weeks 7–8 (Figure 4A, B). Thenceforward, IL-11 decreased, reaching the lowest levels during the 2nd trimester.

IL-11R a seemed to have a biphasic expression, increasing at the beginning of the 1st trimester, rapidly declining until the transition from 1st to 2nd trimester and increasing again during the 2nd trimester until the latest time-point analysed at the 16th week of pregnancy. However, these differences were not very pronounced (Figure 4C).

IL-11 in epithelial cell culture

Cultured primary epithelial cells isolated from follicular phase endometrium and 1st trimester decidua were cultured without hormones (control) or supplemented with E_2 alone or E_2 + MPA [$n = 3$ decidual cultures (two additional cultures were performed without the no hormone control); $n = 5$ endometrial cultures; Figure 5]. Absolute values of endometrial and decidual IL-11 protein expression were in the same order of magnitude ranging from 30 to 650 pg/ml. Due to inter-individual differences between the primary cell cultures from different women, data (obtained by RT–PCR and ELISA) from each single woman were normalized on cells cultured with E_2 alone (defined as 100%) to compare the hormonal regulation of IL-11 between the different cell culture experiments.

Protein and mRNA expression was high in endometrial cell cultures with E_2 alone. In epithelial cells cultured without hormone supplementation, IL-11 expression was slightly reduced. In cells supplemented with E_2 and MPA, IL-11 expression was significantly down-regulated (Figure 5A, B). In case of decidual epithelial cells, mRNA expression of cells cultured without hormone was similar to cells cultured with E_2, whereas MPA supplementation reduced IL-11 mRNA expression as in the endometrial epithelial cells. Protein expression of decidual epithelial cells showed the same pattern as endometrial epithelial cells (Figure 5A, B). These data were confirmed by western blotting (Figure 5C).

IL-11 in intrauterine versus ectopic tubal pregnancies

All EP specimens derived from 7 to 9 weeks of pregnancy. Therefore, they were compared with the corresponding group of decidual specimens. The expression of both IL-11 and IL-11Rα seems to differ between IP and EP. Especially IL-11 mRNA expression seems to be reduced (Figure 6B, C). Hence, due to the limited number of specimens the conclusion to be drawn from these data is limited and it is not possible to subdivide the EP group into viable and non-viable pregnancies.

Immunohistochemical analysis was possible to perform on archival material (Figure 1G, H). Therefore, more specimens were included within the study and it was possible to subdivide the cases of EP into viable EP ($n = 5$) and EP abortion ($n = 3$). This was done according to Kemp *et al.* (1999) who established a method to distinguish with Doppler sonography between vital EP and tubal abortion just before the expulsion of the fetal material from the Fallopian tube. Vital EP and IP show similar IL-11 staining, whereas in EP abortion protein expression of IL-11 is reduced.

Discussion

Successful blastocyst implantation and subsequent invasion of fetal trophoblast cells require a precisely regulated sequence of signals exchanged between mother and fetus. IL-11 signalling seems to be obligatory for establishment of pregnancy. Mice lacking an intact gene for IL-11Rα are infertile (or subfertile) due to defective decidualization resulting in stronger trophoblast invasion and finally necrotic loss of the fetus (Bilinski *et al.*, 1998; Robb *et al.*, 1998). In our study we show that, in the human also, IL-11 signalling may play a significant role.

Hormone-dependent expression of IL-11 in endometrium and decidua

IL-11 was found by immunohistochemistry to be predominantly expressed in epithelial cells. IL-11 protein secretion of cultured epithelial cells was up-regulated by E_2 and down-regulated under the influence of E_2 + MPA after 8 days of cell culture. Comparably, during the menstrual cycle the immunohistochemical staining of epithelial cells for IL-11 was high in the follicular (estrogen-dominated) phase and declined during the early to mid-luteal phase which is regulated by progesterone. The expression of mRNA of IL-11 was prolonged until mid-luteal phase. This might be important in the case of embryo implantation when IL-11 expression has to rise quickly. IL-11 protein expression may be translationally regulated during this time of the normal menstrual cycle.

In early pregnancy, IL-11 expression increases during the first weeks, under the influence of estradiol which rises markedly compared to progesterone levels, and declines afterwards, when the influence of progesterone predominates.

In tubal EP, IL-11 is expressed predominantly in vital specimens, whereas in tubal abortion expression was strongly reduced.

Our data on IL-11 expression during the menstrual cycle confirm the earlier study by Cork *et al.* (2001) in so far as they describe an intense immunohistochemical staining of the epithelial cells

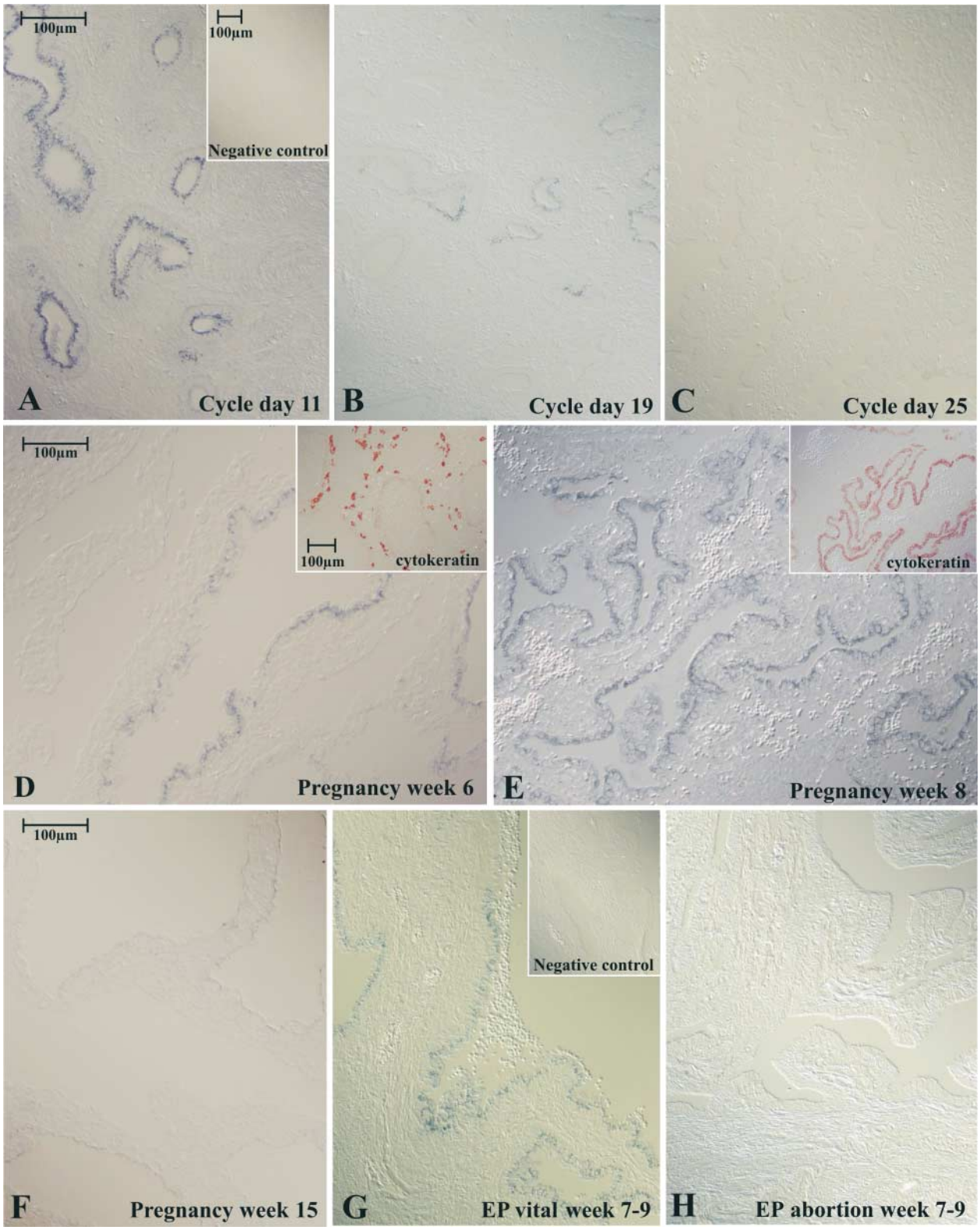


Figure 1. Immunohistochemical detection of interleukin (IL)-11 in human endometrium, decidua and ectopic tubal pregnancy. Tissue specimens from normal menstrual cycle (**A**–**C**), human decidua (**D**–**F**) and vital tubal pregnancy (**G**) as well as tubal abortion (**H**) were analysed for IL-11 expression by immunohistochemistry. IL-11 staining was found predominantly in the glandular epithelial cells. IL-11 was found during proliferative (**A**) and early luteal (**B**) phase, whereas in mid–late luteal phase (**C**) the glands were only very weakly stained. During early pregnancy, a strong increase of IL-11 staining was shown in the glandular epithelia (**D** and **E**). Inserts in **D** and **E**: cytokeratin staining defines decidua basalis (insert **D**) and decidua parietalis (insert **E**). There was no difference in IL-11 staining between decidua basalis and parietalis from one woman. IL-11 expression declines from the beginning of the second trimester onwards (**F**). Vital tubal pregnancies (**G**) showed more intense IL-11 staining than tissues from tubal abortion (**H**).

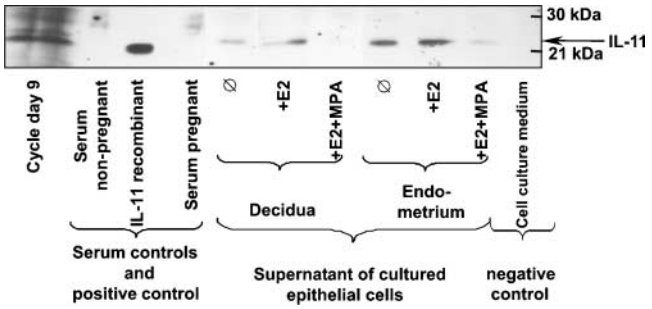


Figure 2. Detection of interleukin (IL)-11 protein by western blotting. Using the same antibody clone as for immunohistochemistry, IL-11 protein was detected by western blotting in an exemplary tissue specimen of the proliferative phase (day 9). Neither pregnant nor non-pregnant serum contained detectable amounts of IL-11 protein. Secretion pattern of cultured epithelial cells confirmed the RNA data (see Figure 5) whereas control cell culture medium was negative.

and maximal expression during the late follicular and early luteal phase. Quite apart from that, these authors locate IL-11 also in stromal cells and detect IL-11 until the late luteal phase. This may be due to the fact that cryosections were used in that study, whereas we used formaldehyde-fixed paraffin-embedded tissues. Epithelial staining of IL-11 throughout the cycle with low levels in the stroma was shown by Karpovich *et al.* (2003) on cryosections too. Another group described the most intense immunohistochemical staining of IL-11 during the late luteal phase in almost all cell populations with the highest intensity in decidualized stromal cells (Dimitriadis *et al.*, 2000) and in decidua from 8 to 9 weeks of pregnancy (Dimitriadis *et al.*, 2003). The differences from our data may be due to the longer time of fixation in comparison with our specimens, the lack of antigen retrieval prior to IL-11 staining and probably a different dilution of the first antibody.

Chen *et al.* (2002) did not detect significant levels of IL-11 during the normal menstrual cycle, but showed a predominantly glandular staining during the early pregnancy. These data were confirmed by real-time PCR. The predominant glandular staining and the increased immunoreaction in early pregnancy are in line with our immunohistochemical data, which we confirmed by RT–PCR and ELISA (data not shown).

Constitutive expression of IL-11Rα

IL-11Rα is expressed constitutively during the menstrual cycle as well as during the 1st and early 2nd trimester of pregnancy. These data are in accordance with the observations of other groups who analysed IL-11Rα by immunohistochemistry (Cork *et al.*, 2001; Karpovich *et al.*, 2003) or RT–PCR (Dimitriadis *et al.*, 2000; Chen *et al.*, 2002). Thereby, IL-11Rα expression was shown predominantly in stromal cells, whereas epithelial cell populations showed little IL-11Rα expression, suggesting that IL-11, which is expressed predominantly by the epithelial cells, may influence the IL-11Rα-expressing stromal cells.

As our cell culture data have shown a steroid-dependent expression of IL-11, the influence of IL-11 on the stromal cells should vary during the menstrual cycle. In fact, it has been shown that the up-regulation of stromal proliferation by IL-11 was restricted to cells derived form endometrium between days 16 and 22 (Karpovich *et al.*, 2003). This is consistent with the main detection of IL-11 in our study from the follicular phase until the early to mid-luteal phase.

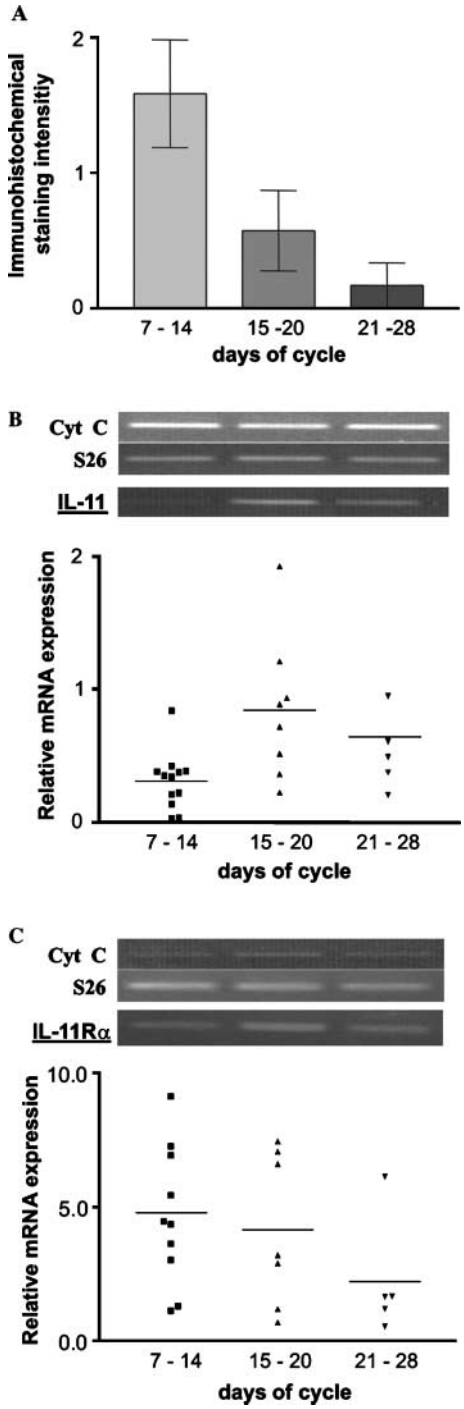
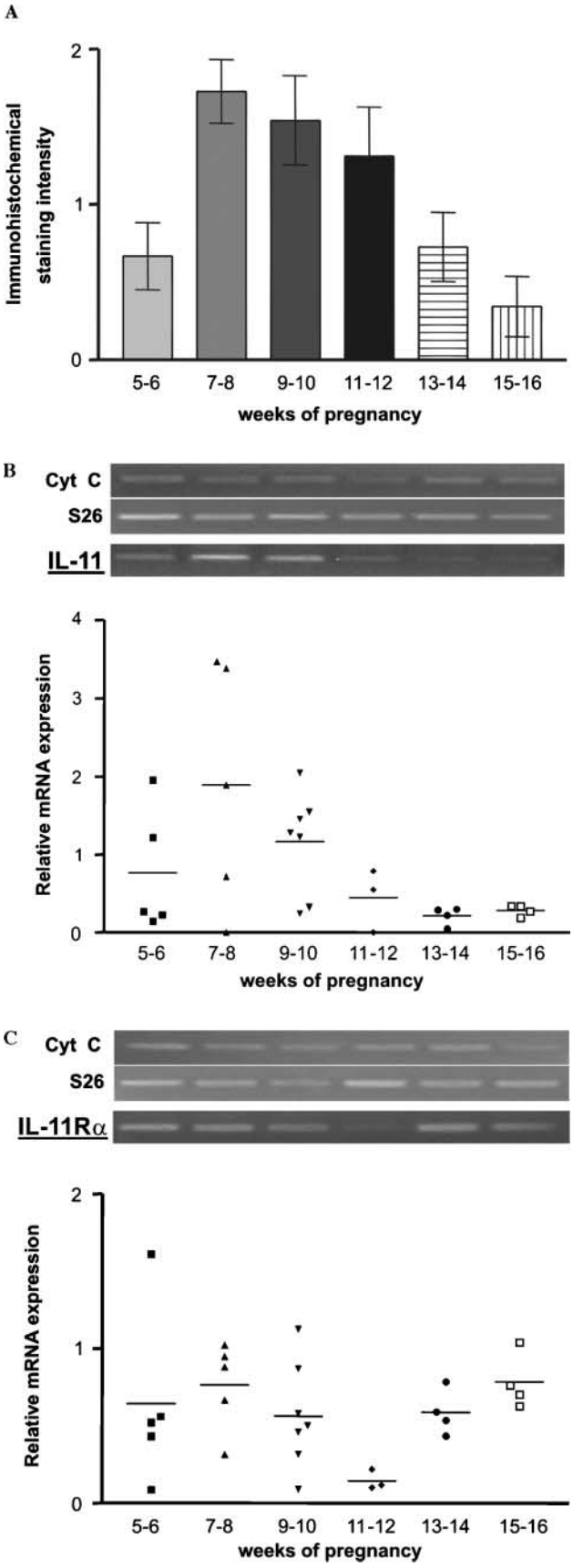


Figure 3. Interleukin (IL)-11 and IL-11Rα mRNA expression during the non-conceptive menstrual cycle. (**A**) Analysis of immunohistochemical IL-11 staining. Expression was most intense during the proliferative phase and declined thereafter until the late luteal phase. (**B**) Relative IL-11 mRNA expression, determined by RT–PCR normalized to cytochrome oxidase C subunit 1 (Cyt C) as well as the ribosomal protein S26 showed an expression peak during the time of a possible implantation. (**C**) Expression of IL-11Rα mRNA as shown by RT–PCR only slightly changed during the menstrual cycle.

Significance of IL-11 in humans compared to mice

Mice lacking IL-11Rα (IL-11Rα$^{-/-}$) are infertile due to defective decidualization. The blastocyst implantation is normal (in contrast to LIF$^{-/-}$ mice) but the post-implantation decidual reaction is inadequate, leading to a small haemorrhagic decidua.



The trophoblast invasion and proliferation seems to be increased compared to wild type (WT) mice, probably due to the lack of the limiting effect of an adequate decidua. These effects are similar to trophoblast placed in ectopic sites (Kirby and Cowell, 1968; for review see Robb *et al.*, 2002). Reduced capillary permeability, and in consequence reduced blood flow, leads to embryo necrosis and resorption (Robb *et al.*, 1998). This pathological situation results from a maternal defect, as IL-11Rα$^{-/-}$ embryos are able to implant and develop in WT mice.

Two phases of decidual reaction are distinguished in mice: a primary decidual reaction in the peri-implantation phase, which is avascular and may be compared to the predecidualization taking place in the human endometrium around the time of a possible implantation. In fact, epithelial IL-11 was shown to advance the progesterone-induced decidualization of human stromal cells (Dimitriadis *et al.*, 2002). The secondary decidual reaction after blastocyst implantation forms the placenta, including blood vessels. During this second decidualization phase in WT mice, an increase of IL-11 expression was found, peaking at day 5.5–7.5 (Robb *et al.*, 1998), respectively day 8.0–9.5 (later on not shown; Bilinski *et al.*, 1998). Taking into account stages of the embryological development (Theiler, 1972; Moore and Persaud, 1996), the corresponding phase of human pregnancy starts in the 5th–6th week (day 5.5–7.5), respectively 6th–7th week (day 7.5–9.5) when we find IL-11 expression to increase. Hence, as IL-11Rα is constitutively expressed in humans as in mice (Robb *et al.*, 1998), IL-11 may be the regulating effector in both species.

Robb *et al.* (1998) postulated that in response to a blastocyst signal, IL-11 expression in the primary decidual zone was initiated, even though IL-11 expression was also found following an artificial decidualization. The fact that in the case of oil-induced decidualization the expression of IL-11 was prolonged, supports the idea of a coordinated embryo–maternal dialogue in the case of IL-11 regulation.

This suggests that the decline in IL-11 expression after the early–mid-luteal phase which we found in normal human cycles may be due to the lack of a signal by an implanting blastocyst. Protein and mRNA levels of IL-11 are similar in mid-luteal phase endometrium and pregnancy weeks 5–6. Hence, in the case of pregnancy, when a blastocyst is actually present, IL-11 expression may not decline. High values of mRNA, especially during the early and mid-luteal phase when IL-11 protein is already depleted during the non-conceptive menstrual cycle, may either be due to the fact that secreted IL-11 is no longer detectable in epithelial cells by immunohistochemistry, or may indicate a translational regulation of IL-11 at this time of the menstrual cycle. This might be important in the case of implantation as there is a rapid increase of IL-11 expression during pregnancy weeks 7–8. This increase and the high levels of IL-11 until week 12 of pregnancy may assure the adequate decidualization process. With the beginning of the 2nd trimester, when the invasive capacity of the trophoblast declines, IL-11 expression also decreases. From these data and the pathological effects in IL-11Rα$^{-/-}$ mice, it can be suggested that the IL-11-mediated decidualization limits trophoblast invasion and 'guides' the invasion to the normal populations of intravascular and interstitial trophoblast.

Figure 4. Interleukin (IL)-11 and IL-11Rα mRNA expression during the 1st and 2nd trimester of pregnancy. (**A**) Analysis of immunohistochemical IL-11 staining. IL-11 expression increased rapidly during the first weeks of pregnancy. With the beginning of the second trimester, IL-11 expression declines. Analysis of IL-11 RNA expression (**B**) confirmed these data. (**C**) Expression of IL-11 receptor as shown by RT–PCR changed only slightly during this time.

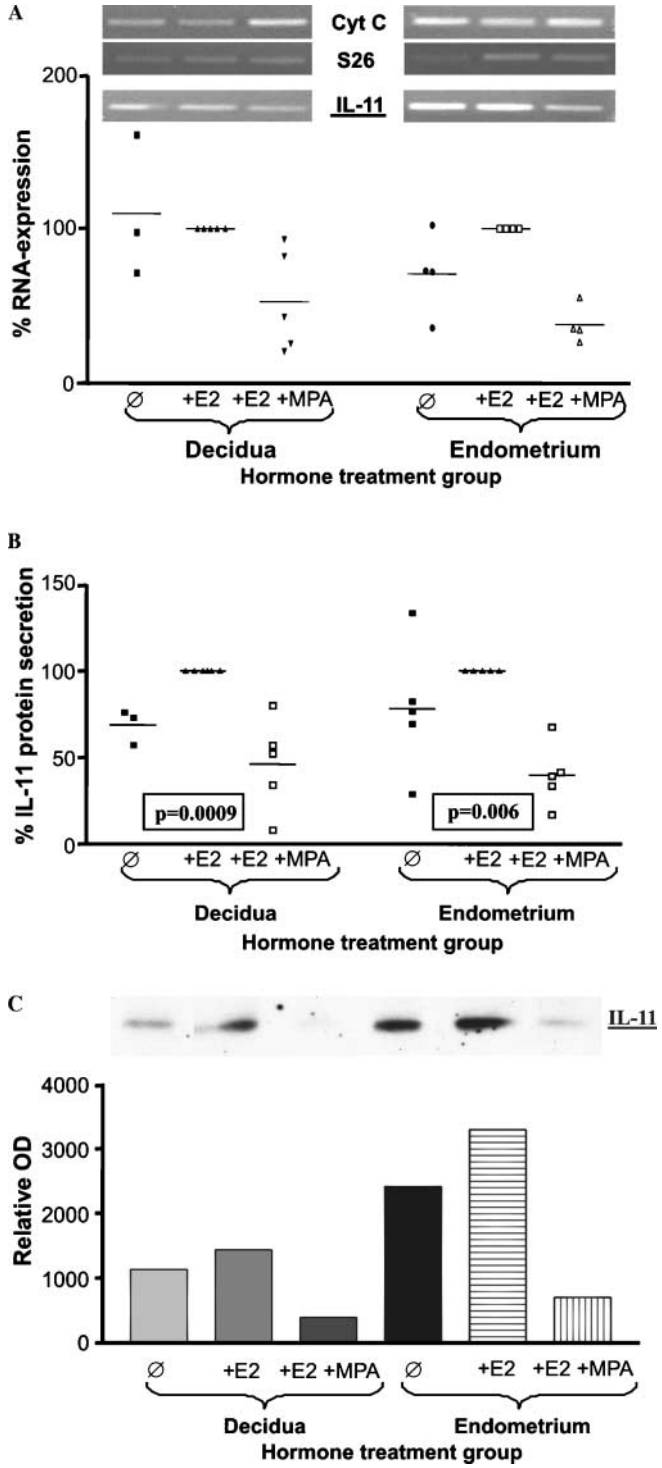


Figure 5. Hormonal regulation of interleukin (IL)-11 in endometrial and decidual epithelial cells. Hormonal influence on IL-11 expression and secretion was studied on cultured endometrial and decidual primary epithelial cells. RNA expression analysed by RT–PCR (**A**), protein secretion by ELISA (**B**) and western blotting (**C**) showed highest IL-11 values under 17-estradiol (E_2) alone. Culturing without hormones declined IL-11 expression and supplementation with E_2 and medroxyprogesterone acetate reduced IL-11 expression and secretion markedly. *P*-Value is given for analysis of the three groups of decidual or endometrial cell culture by analysis of variance.

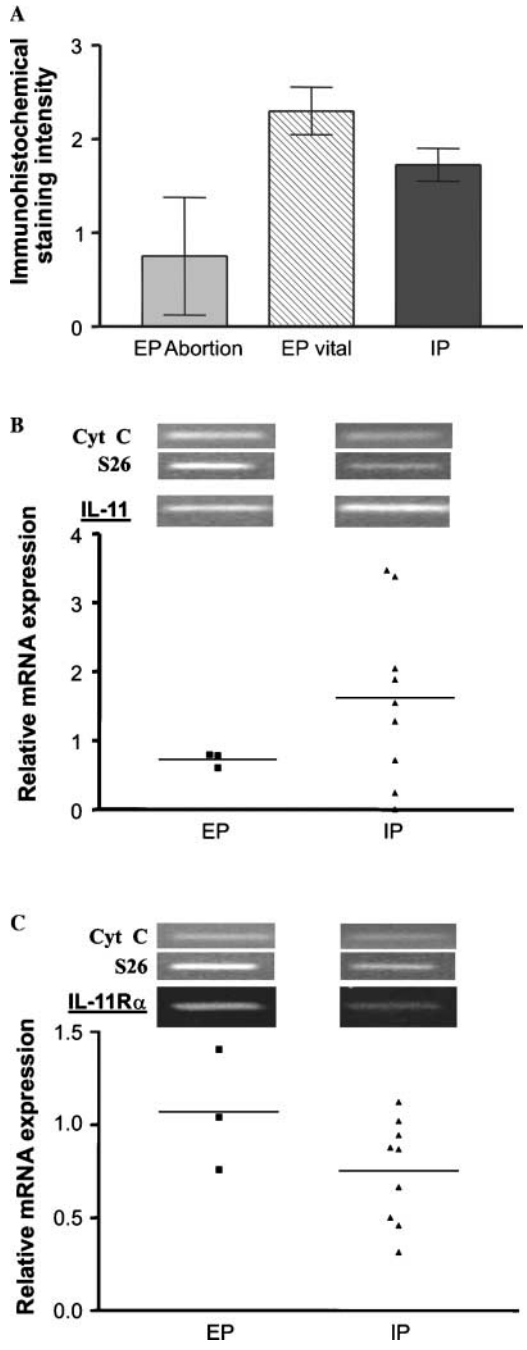


Figure 6. Interleukin (IL)-11 in intrauterine versus ectopic tubal pregnancies (EP). (**A**) Vital tubal pregnancies, tubal abortions, and normal healthy intrauterine pregnancies (IP) were compared for their IL-11 expression. Whereas staining at the vital tubal implantation site was comparable to the IP, IL-11 staining in tubal abortion was reduced. (**B**, **C**) RT–PCR analysis could be performed only between EP and IP without discriminating between viable and non-viable tubal pregnancies. IL-11 seemed to be reduced in EP (**B**), but unfortunately the number of tissue specimens was very small. IL-11 receptor was not remarkably changed (**C**).

Consequences of disturbed IL-11 signalling in the human

A 'natural' human model for dysregulation of trophoblast invasion is the ectopic tubal pregnancy. As in the IL-11R$\alpha^{-/-}$ mouse, trophoblast invasion is increased in this pathological case. More extended trophoblast cell columns (Goffin *et al.*, 2003), broad overlap of the proliferative and invasive phenotype (Kemp *et al.*, 2002) and reduced apoptosis in extravillous trophoblast cells (von Rango *et al.*, 2003b) are found. Therefore, we analysed IL-11 expression in these specimens. IL-11Rα expression was similar in IP and EP. IL-11 mRNA expression seems to be reduced in ectopic tubal

pregnancies. However, due to the small number of EP specimens suitable for RNA isolation, the significance of the data is limited. Using archival material for the immunohistochemical analysis, it was shown that in vital tubal pregnancy the staining of IL-11 is similar to the intrauterine situation, whereas in tubal abortion IL-11 staining was reduced.

The lack of IL-11 in tubal abortions may be a sign of dysregulated decidual development and trophoblast invasion. As in IL-11R$\alpha^{-/-}$ mouse, the trophoblast invasion may not be limited and not guided to the adequate intravascular sites. This may result in fetal death analogous to the findings in IL-11R$\alpha^{-/-}$ mouse. However, in vital cases of tubal pregnancy IL-11 was expressed, perhaps because the dysregulation had not yet been started. The different proliferative and invasive behaviour of the trophoblast, probably induced by the different tubal microenvironment, may result in a lack of an adequate signal to induce and/or maintain IL-11 expression. Subsequently the embryo–maternal dialogue will be dysregulated, resulting in fetal abortion. Hence, an early beginning of this dysregulation will result in fetal abortion (~70% of tubal pregnancies), whereas a late start or the lack of any dysregulation will result in the rupture of the tubal wall induced by a vital tubal pregnancy (~30% of tubal pregnancies). This hypothesis is supported by data showing reduced IL-11 expression in anembryonic pregnancies (Chen *et al.*, 2002).

IL-11 signalling and decidual immunology

As the invading fetal cells are semi-allogeneic to the mother, immunological implications are widely discussed in human early pregnancy. IL-11Rα was found to be expressed on several leukocyte subtypes (Bozza *et al.*, 2001; Curti *et al.*, 2001) and IL-11 was shown to influence their cytokine expression (Schwertschlag *et al.*, 1999; Curti *et al.*, 2001). In addition, IL-11 modulates macrophage activation (Schwertschlag *et al.*, 1999) and may regulate natural killer (NK) cell development (Aiba and Ogawa, 1998).

This might be also important for ectopic tubal pregnancy, as NK cells are lacking there (von Rango *et al.*, 2001) and IL-11 seems to be down-regulated in most cases (this study). In human endometrium, strong stromal staining for IL-11Rα was found (Cork *et al.*, 2002; Karpovich *et al.*, 2003), not differentiating between decidual cells and leukocytes. We found IL-11Rα to be expressed on isolated leukocytes (data not shown) using RT–PCR. Hence, IL-11 may be involved in balancing the immune response between maternal tolerance, guidance and limitation of trophoblast invasion. In fact, IL-11 was shown to prevent graft-versus-host disease after allogeneic bone marrow transplantation in the mouse (Hill *et al.*, 1998).

In conclusion, we demonstrate in this study that IL-11 is present in the menstrual cycle during the preimplantation phase. In normal menstrual cycle, but not in pregnancy, IL-11 expression is reduced during the luteal phase. In early pregnancy, IL-11 increases and expression remains high until the end of the 1st trimester and declines at the beginning of the 2nd trimester. In EP, IL-11 expression was found nearly exclusively in vital specimens, whereas in tubal abortions it was found to be reduced. These data suggest that IL-11 is one of the pivotal factors for the regulation of trophoblast invasion. It may be involved in the correct guidance of the invading cells into the spiral arteries. In ectopic tubal pregnancy, dysregulated IL-11 expression may be involved in a series of reactions leading to inadequate trophoblast invasion. Further functional *in vitro* studies on the influence of IL-11 on cytokine secretion, migration and activation of decidual leukocytes, as well as on trophoblast migration, may lead us to further insights into this elaborated network of communication between the mother and her fetus.

Acknowledgements

The authors wish to acknowledge the excellent technical assistance of Tina Breuer, Rick Kamps, Diana Seelis and Kerstin Ziob. Thanks are due to Prof. Kusche (Marienhospital Aachen), Dr Crommelingk (Luisenhospital Aachen), Prof. Karl (St Antonius Hospital Eschweiler) Prof. Rath (RWTH Medical School Aachen) and their teams for their support of this work. We thank Frans Bocken, Gabie Raven and all colleagues at the Bourgognekliniek Maastricht for the excellent cooperation. This work was supported by the START program (Project 24/2001/2002 of the School of Medicine, RWTH Aachen)

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Submitted on July 7, 2004; resubmitted on August 19, 2004; accepted on August 19, 2004