Expression of intercellular adhesion molecules ICAM-1 and ICAM-2 in human endometrium: regulation by interferon-γ

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Introduction

Cell adhesion molecules mediate crucial cell–cell interactions and play an important role in cell differentiation, in the organization of the extracellular matrix and in the recruitment and aggregation of leukocytes from the circulation (Akiyama et al., 1989; Bevilacqua, 1993). The immunoglobulin superfamily, of which intercellular adhesion molecule (ICAM)-1 and ICAM-2 are members, is the most widely distributed family of cell adhesion molecules. ICAM-1 is expressed on leukocytes, epithelial and endothelial cells, ICAM-2 is mainly found on resting endothelial cells and ICAM-3 is constitutively expressed by all resting leukocytes (Bevilacqua et al., 1994).

Cell adhesion molecules are present in human endometrium, where they may play a role in regulating leukocyte trafficking into this tissue (Tabibzadeh and Poubouridis, 1990). It is recognized that the normal endometrium has a population of leukocytes, including macrophages, T-lymphocytes and granulocytes, which are important in the physiology of the endometrium (Kamat and Isaacson, 1987; Marshall and Jones, 1988). At least a proportion of these cells is actively recruited from the circulation (Sugiura and Mizuhira, 1988). At least a proportion of these cells is actively recruited into this tissue (Tabibzadeh and Poubouridis, 1990). It is recognized that the normal endometrium has a population of leukocytes (Kamat and Isaacson, 1987; Marshall and Jones, 1988).

The factors regulating the expression of cell adhesion molecules in human endometrium remain uncertain. In other tissues, the expression of ICAM-1 can be stimulated by cytokines including IFN-γ, tumour necrosis factor (TNF)-α and interleukin (IL)-1β (Springer 1990). However, endothelial, fibroblastic and epithelial cells differ in their response to ICAM-1-inducing cytokines (Springer, 1990). Vigano et al. (1994) have demonstrated an up-regulation of ICAM-1 expression in cultured human endometrial stromal cells following exposure to IL-1β. The effect of IFN-γ on ICAM-1 expression in endometrial stromal cells has not been determined. In contrast to ICAM-1, ICAM-2 expression is normally augmented by cytokine activation (Bevilacqua et al., 1994).

The purpose of the present study was to localize ICAM-1 and ICAM-2 in human endometrium throughout the menstrual cycle, and to determine whether ICAM-1 and ICAM-2 expression in endometrial stromal cells may be regulated by IFN-γ, a cytokine found within T lymphocytes of the endometrium (Tabibzadeh, 1990, 1994; Stewart et al., 1992).
Materials and methods

Tissue collection

Endometrial biopsies with attached myometrium were obtained from 61 premenopausal women with regular menstrual cycles undergoing hysterectomy for benign disease. Informed consent was obtained from each woman and the study was approved by the Local Research Ethics Committee. Histological assessment of endometrial morphology was performed according to standard criteria (Noyes et al., 1950). The endometria were dated as menstrual (n = 7), secretory (n = 32) and proliferative (n = 22) phases of the menstrual cycle.

Isolation and separation of endometrial stromal cells and gland fragments

Biopsies of endometrium were transported to the laboratory in cold Roswell Park Memorial Institute (RPMI) 1640 medium containing 10 IU/ml penicillin and 10 µg/ml streptomycin (Life Technologies, Paisley, UK). The tissue was washed three times in RPMI medium and chopped into small pieces (1 mm³) using a sterile scalpel. Tissue pieces were placed in a sterile universal tube with 5–10 ml of 1 mg/ml type I collagenase (Sigma, Poole, UK) in RPMI 1640 medium, supplemented with penicillin and streptomycin, and incubated at 37°C for 30–45 min. Undigested tissue was removed using sterile 400 µm woven polyester mesh (Lockertex; Locker Wire Weavers Ltd, Warrington, UK) and the filtrate collected. The filtrate was passed through a 30 µm mesh from which single cells and small cell clumps were collected. Epithelial gland fragments trapped on the 30 µm mesh were back-washed using RPMI 1640 supplemented with antibiotics. The stromal cell suspension was washed twice in complete medium (RPMI 1640, 10% fetal bovine serum, 10 U/ml penicillin, 10 µg/ml streptomycin and 2 mM l-glutamine) and allowed to adhere to 25 cm² tissue culture flasks for 2 h at 37°C in 5% CO₂. Non-attached cells and erythrocytes were then removed and the stromal fibroblasts were grown to confluence in complete medium. The cells were used after the second passage before detection of prolactin (Immulite®, chemiluminescent enzyme immunoassay; DPC, Gwynedd, UK) in the culture medium.

Treatment of stromal fibroblasts with IFN-γ

Monolayer cultures of endometrial stromal fibroblasts were grown to confluence in 25 cm² tissue culture flasks (2×10⁶ cells per flask) for RNA isolation or in 8-well slide flasks (2×10⁵ cells per well) in preparation for immunocytochemistry. When the cells reached confluence, the effect of exposure to IFN-γ on ICAM-1 and ICAM-2 expression was examined. Confluent cells in the slide flasks were incubated for 24 h with recombinant IFN-γ (denoted by Dr T.Meager, NIBSC, Potters Bar, UK), at 0, 1, 10 or 100 ng/ml. Cells prepared for RNA isolation were exposed to recombinant IFN-γ (1 ng/ml) for 0, 4, 8, 16 and 24 h (n = 3 preparations).

Northern blotting

RNA was purified from cultured endometrial stromal cells (n = 3 preparations; each from the secretory phase of the cycle) or freshly isolated gland fragments (n = 10 preparations; seven from the secretory phase and three from the proliferative phase of the cycle) by solvent extraction according to the manufacturer’s instructions (TRizol™; Life Technologies, Paisley, UK). The isolated RNA was re-dissolved in diethylpyrocarbonate (DEPC)-treated distilled water and quantified by UV spectrophotometry. RNA integrity was confirmed by the presence of intact 18S and 28S ribosomal RNA bands confirmed by the presence of intact 18S and 28S ribosomal RNA bands after agarose gel electrophoresis. RNA sample loading buffer (Sigma) was added to 5 or 10 µg of total RNA and separated on 1.2% agarose gels containing 6% formaldehyde and 20 mmol/l 2-[N-Morpholino]ethane-sulphonic acid (MOPS). Gels were electro-phoresed at 60 V for 3–4 h. RNA was transferred from the gels to nylon membranes (Hybond-N; Amersham Pharmacia Biotech, St Albans, UK) in 20× SSC (3 mol/l sodium chloride; 0.3 mol/l sodium citrate, pH 7.0) and cross-linked to the membrane by UV irradiation. Membranes were pre-hybridized for 4 h at 65°C in 12 ml of 5× SSC; 5× Denhardt’s solution (50×Denhardt’s is 1% polyvinylpyrrolidone, 1% Ficoll 400 and 1% bovine serum albumin); 0.5% sodium dodecyl sulphate; 100 mg/ml boiled salmon sperm DNA. Filters were then hybridized in the same buffer overnight with the appropriate cDNA probe labelled with 32P using a random priming kit according to the manufacturer’s protocol (Amersham Pharmacia Biotech). The cDNA probe for the house-keeping gene human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1.1 kb) was purchased from Clontech Laboratories UK Ltd, Basingstoke, UK. The cDNA probes for ICAM-1 (1.8 kb) and ICAM-2 (800 bp) were donated by Dr D.Simmons, Oxford, UK. Filters were washed to a final stringency of 0.5× SSC, 0.1% sodium dodecyl sulphate at 65°C. Autoradiography was performed with Kodak X-Omat film at −70°C for 2–3 days. The intensity of the bands on the autoradiograph for ICAM-1 hybridization was compared with GAPDH and the ratio determined using the Bio-Rad Multi-Analyzer 130G/PC Version 1.1.

Immunocytochemistry

Biopsies of endometrium with attached myometrium were either snap-frozen in liquid nitrogen and stored at −70°C, or fixed in 10% neutral buffered formalin (BDH, Poole, UK) and embedded in paraffin. ICAM-2 distribution was examined by ICC in cryosections (n = 30) and in cultured stromal fibroblast cells (n = 3 preparations). ICAM-1 was immunolocalized using three different antibodies. Two of these were monoclonal and employed on cryosections (n = 30) and one was polyclonal and used on paraffin sections (n = 24) (Table I). The monoclonal antibody, BBIG-I1 (R&D, Abingdon, UK) was used to determine ICAM-1 expression in cultured stromal fibroblast cells.

Immunocytochemistry on cryosections

Sections 5 µm thick were cut from the frozen tissue and mounted on silane-coated slides. The sections were fixed in acetone for 10 min,
Figure 1.

Figure 2.
washed in Tris-buffered saline (TBS), and placed in 0.5% hydrogen peroxide in methanol for 30 min at room temperature. The sections were then washed as before, pre-incubated with 20% (w/v) normal goat serum (SAPU, Carluke, UK) in TBS for 30 min at room temperature, and then incubated for 16 h at 4°C with the primary antibody diluted in 1.5% horse serum (Table I). Sections were washed in TBS before incubation for 1 h with biotinylated goat anti-mouse immunoglobulin (Dako Ltd, High Wycombe, UK), diluted in 2% normal goat serum and 1.5% normal human serum. The sections were thoroughly washed again, then incubated for 30 min with streptavidin–horseradish peroxidase (Dako) in TBS before final washing. The antigen was localized using 1 mg/ml diaminobenzidine tetrahydrochloride (DAB), 0.02% H2O2 in 50 mM Tris–Cl, pH 7.6, and appeared as a brown end-product. Negative controls included sections incubated without the primary antibody and sections incubated with a mouse monoclonal antibody against IgG1 Aspergillus niger glucose oxidase (Dako Ltd), an enzyme which is not expressed in mammalian cell systems.

**Immunocytochemistry on paraffin-embedded tissue**

Sections 8 µm thick were cut from the paraffin-embedded tissues and mounted on silane-coated slides, heated to 60°C for 35 min, deparaffinized in xylene and rehydrated in a graded alcohol series. The antigen was retrieved by microwaving the sections in a pressure cooker (Lakeland Plastics Ltd, Cumbria, UK) at full power for 5 min in citrate buffer (10 mM, pH 6.0). The polyclonal ICAM-1 antibody (Table I) was used in a dilution of 1/1000, and immunocytochemistry was carried out using a goat IgG Vectastain ABC kit (Vector, UK) according to the manufacturer’s instructions. T-lymphocytes were localized within endometrial lymphoid cell aggregates in consecutive paraffin sections using a polyclonal antibody against the cell surface antigen CD3 (Table I). Negative controls included slides incubated without the primary antibody and slides incubated with non-immune goat serum (SAPU), in place of the primary antibody.

**Immunocytochemistry on cultured stromal fibroblast cells**

After incubation with recombinant IFN-γ (0, 1, 10 or 100 ng/ml) the medium was removed from the cells, the wells were gently rinsed with phosphate-buffered saline (pH 7.4) and then the cells were fixed in ice-cold acetone for 10 min at 4°C. The wells were allowed to dry for 5 min, the upper chambers of the slide-flasks were peeled away and the slides processed for immunocytochemistry as described above.

The specimens were photographed with colour slide film (Figures 1 and 2, Ektachrome 64T, Kodak, Rochester, NY, USA), or with monochrome film (Technical Pan, Kodak) and a blue filter to maintain the observed contrast (Figure 3).

**Figure 1.** Immunolocalization of intercellular adhesion molecule (ICAM)-1 in human endometrium and myometrium using a polyclonal antibody (R&D, UK). ICAM-1 was identified with variable expression on the apical surface of the glandular (A) and luminal (B) epithelium (arrows) throughout the menstrual cycle, and on endometrial stromal cells throughout the menstrual cycle. Stromal cell expression of ICAM-1 was increased in menstrual specimens (C). Immunostaining for ICAM-1 was identified within endometrial lymphoid cell aggregates (D), where it co-localized with CD3 in consecutive sections (E). ICAM-1 was expressed on the endometrial and myometrial vascular endothelium (F). (A), (D) and (E) are secretory phase endometrium, (B) and (F) are late proliferative and (C) is menstrual phase endometrium. The negative controls (see text) exhibited no reactivity. Scale bars = 50 µm.

**Figure 2.** Expression of intercellular adhesion molecule (ICAM)-2 in (A) secretory phase endometrium and (B) proliferative phase myometrium. ICAM-2 expression was restricted to the vascular endothelium, and was not detected in the luminal and glandular epithelium, the endometrial stromal cells, the vascular smooth muscle and the lymphoid aggregates. The negative controls (see text) exhibited no reactivity. Scale bars = 50 µm.

### Results

#### Immunolocalization of ICAM-1 and ICAM-2 in vivo

ICAM-1 was identified with variable expression on the apical surface of the luminal and glandular epithelium throughout the menstrual cycle (Figure 1A and B). The distribution of ICAM-1 was similar in both cryostat sections and formalin-fixed, paraffin-embedded tissue, and with each of the three ICAM-1 antibodies employed. Expression on the apical surface of the glandular epithelium was most marked using the polyclonal ICAM-1 antibody (R&D). Whereas ICAM-1 was identified in patches in endometrial stromal cells throughout the menstrual cycle, stromal expression in the functionalis was increased in menstrual specimens (Figure 1C). This finding was consistent with each of the three ICAM-1 antibodies employed. ICAM-1 expression was widespread within the endometrial lymphoid aggregates in the basalis, where its distribution co-localized with CD3 in consecutive sections (Figure 1D and E). As expected, immunostaining for ICAM-1 was present throughout the vascular endothelium of the endometrium and myometrium throughout the menstrual cycle (Figure 1F). The negative control slides for ICAM-1 showed no immunostaining.

ICAM-2 expression was restricted to the vascular endothelium of the endometrium and myometrium and expression did not alter throughout the menstrual cycle (Figure 2A and B). This molecule could not be detected in the luminal and glandular epithelium, the endometrial stromal cells and the lymphoid aggregates. The negative control slides for ICAM-2 showed no immunostaining.

#### Immunocytochemistry on cultured stromal fibroblasts

Endometrial stromal cells cultured in RPMI medium alone (Figure 3B) showed limited expression of ICAM-1. This expression was increased after incubation with 1, 10 or 100 ng/ml of IFN-γ (Figure 3C). ICAM-2 antigen was not detectable in stromal cells either before or after incubation with IFN-γ.

#### Northern blotting

Northern blotting confirmed the presence of ICAM-1 mRNA in isolated endometrial glands from five of 10 specimens.
ICAM-1 and ICAM-2 are expressed in normal human endometrium, (Tabibzadeh and Poubouridis, 1990; Trejdosiewicz, 1997). Northern blotting of isolated endometrial glandular and surface epithelial cells (Tabibzadeh et al., 1994) have shown a constant expression of ICAM-1 in the glandular or luminal epithelium, and stromal staining for ICAM-1 was rare except at menstruation when it seemed to be upregulated. In contrast, others (Tabibzadeh and Poubouridis, 1990; Tabibzadeh et al., 1994) have shown a constant expression of ICAM-1 within both endometrial glandular cells and stromal cells throughout the menstrual cycle. These conflicting observations were tested in the present study by employing three different ICAM-1 antibodies (monoclonal and polyclonal) and examining both cryostat and formalin-fixed, paraffin-embedded tissue sections. This combination of techniques was used to ensure that both minimum epitope denaturation and preservation of morphology were achieved (Southgate and Trejdosiewicz, 1997). Northern blotting of isolated endometrial glands and cultured endometrial stromal cells provided an independent method of verifying the expression of ICAM-1. The present study confirms that ICAM-1 is expressed within glandular and surface epithelial cells (Tabibzadeh et al., 1994). However, immunolocalization of ICAM-1 in endometrial glandular cells was variable, and in some cases absent. Differing intensities of ICAM-1 staining were noted both within the same histological specimen and when biopsies from different patients were compared. Consistent with this finding, ICAM-1 mRNA was detected in only five of 10 endometrial glandular preparations examined, where ICAM-1 mRNA was identified in five.

Discussion

This study demonstrates that the cell adhesion molecules ICAM-1 and ICAM-2 are expressed in normal human endometrium. ICAM-1 is localized to the luminal and glandular epithelial cells, the endometrial stromal cells and the endothelial cells of the endometrial vasculature. The expression of ICAM-1 in the endometrial stroma is up-regulated at the time of menstruation and, in cultured endometrial stromal cells, by IFN-γ. ICAM-2 expression is restricted to the uterine vascular endothelium.

Although others have investigated ICAM-1 distribution in human endometrium, (Tabibzadeh and Poubouridis, 1990; Tawia et al., 1993; Tabibzadeh et al., 1994), there is little agreement between the findings of both groups (Table II). In summary, Tawia et al. (1993) did not observe ICAM-1 in the glandular or luminal epithelium, and stromal staining for ICAM-1 was rare except at menstruation when it seemed to be upregulated. In contrast, others (Tabibzadeh and Poubouridis, 1990; Tabibzadeh et al., 1994) have shown a constant expression of ICAM-1 within both endometrial glandular cells and stromal cells throughout the menstrual cycle. These conflicting observations were tested in the present study by employing three different ICAM-1 antibodies (monoclonal and polyclonal) and examining both cryostat and formalin-fixed, paraffin-embedded tissue sections. This combination of techniques was used to ensure that both minimum epitope denaturation and preservation of morphology were achieved (Southgate and Trejdosiewicz, 1997). Northern blotting of isolated endometrial glands and cultured endometrial stromal cells provided an independent method of verifying the expression of ICAM-1. The present study confirms that ICAM-1 is expressed within glandular and surface epithelial cells (Tabibzadeh et al., 1994). However, immunolocalization of ICAM-1 in endometrial glandular cells was variable, and in some cases absent. Differing intensities of ICAM-1 staining were noted both within the same histological specimen and when biopsies from different patients were compared. Consistent with this finding, ICAM-1 mRNA was detected in only five of 10 endometrial glandular preparations. This variable expression of ICAM-1 within the endometrial glands may account for the observations of Tawia et al. (1993). Furthermore, Tabibzadeh et al. (1994) described a fine granular immunoreactivity for ICAM-1 within...
the cytoplasm of glandular cells, whereas in the present study the strongest immunostaining for ICAM-1 within glandular epithelial cells was localized to the apical surface.

Similar to Tawia et al. (1993), we observed an increased expression of ICAM-1 in endometrial stromal cells in menstrual specimens. Menstruation has been likened to an ischaemic condition since vasocstriction with subsequent dilatation occurs in the endometrial vasculature during menstruation (Markee, 1940). An increased expression of cell adhesion molecules, including ICAM-1, is associated with the tissue damage that occurs following ischaemia and reperfusion, although the mechanism of action remains uncertain (Frenette and Wagner, 1996). In animal models of myocardial ischaemia–reperfusion injury, anti-ICAM-1 antibodies have been shown to reduce the accumulation of leukocytes and subsequent infarct size (Ma et al., 1992; Yamazaki et al., 1993), while mice lacking ICAM-1 have less severe brain damage following middle cerebral artery occlusion than normal mice (Frenette and Wagner, 1996). An increased expression of extravascular ICAM-1 within the peri-menstrual endometrium might facilitate the accumulation of large granular lymphocytes within the endometrial stroma (Bulmer, 1994) and contribute to the glandular fragmentation and endothelial damage which characterise the menstrual phase (Tabibzadeh, 1996).

Under normal conditions, ICAM-1 is present in tissues at low levels and is markedly up-regulated by factors including IFN-γ (Bevilacqua et al., 1994). This up-regulation is largely controlled at the mRNA level, with an increased surface expression on endothelial cells identified after 4 h (Springer, 1990). This previously observed time course is consistent with the present study of ICAM-1 mRNA expression in cultured endometrial stromal cells exposed to IFN-γ. The primary source of IFN-γ within the endometrium is T-lymphocytes, located in the lymphoid aggregates in the stratum basalis, the glandular epithelium and throughout the stroma of basal and functional layers (Stewart et al., 1992). A recent study has demonstrated that endometrial polymorphonuclear leukocytes also produce IFN-γ (Yeaman et al., 1998). Some studies have indicated that uterine natural killer cells and macrophages may also produce this cytokine (Robinson et al., 1985; Pestka and Langer, 1987), but within the normal endometrium these cells are not immunoreactive for IFN-γ (Stewart et al., 1992).

The expression of ICAM-2 in human endometrium and myometrium has not previously been reported. ICAM-2 is constitutively expressed on endothelial cells although it can be expressed on a variety of non-endothelial cell types including most leukocytes (Springer, 1990). We have demonstrated that, within the endometrium and myometrium, ICAM-2 expression is restricted to the endothelial cells of the endometrial and myometrial vasculature. In contrast to ICAM-1, the expression of ICAM-2 is not known to be modified by inflammatory cytokines and, consistent with this, we were unable to stimulate its production in cultured endometrial stromal cells exposed to IFN-γ. The constitutive expression of ICAM-2 on the endometrial and myometrial endothelium is likely to provide a ready surface for β2 integrin-dependent adhesion of leukocytes including B- and T-lymphocytes, monocytes and granulocytes (Bevilacqua, 1993).

Expression of ICAM-1 on the apical surface of the luminal and glandular epithelium suggests a role for this molecule in embryo implantation. Various cell adhesion molecules and extracellular matrix ligands, on both the preimplantation embryo and the endometrial cell surface, have been proposed as candidates involved in the embryo–endometrial interaction, (Aplin, 1996, for review). Human embryos and fertilized oocytes, but not unfertilized oocytes, are known to secrete a factor which up-regulates the expression of cell adhesion molecules (Check et al., 1995). Ozornek et al. (1997) have recently demonstrated that the human preimplantation embryo is capable of producing IFN-γ in vitro, and that production is greatest between days 3 and 4 which would extrapolate to the time in vivo just before implantation. Taken together, these studies suggest that cell adhesion molecule expression on the endometrial surface epithelium could be regulated by IFN-γ secreted by the preimplantation embryo.

A further role for ICAM-1 in the control of embryo implantation has been proposed from studies on first trimester decidua. ICAM-1 has been localized to the decidual stromal cells and endothelial cells in first trimester human pregnancy, where it has been postulated to play a crucial role in recruiting uterine natural killer cells from the peripheral blood (Marzusch et al., 1993; Ruck et al., 1994). These leukocytes may contribute to the control of cytotrophoblastic invasion of the placental bed (King and Loke, 1991).

In summary, the widespread distribution of ICAM-1 in human endometrium throughout the menstrual cycle suggests a variety of roles for this molecule. These roles include the regulation of leukocyte trafficking into the endometrium from...
the vasculature, and the maintenance of glandular and stromal integrity. It is tempting to speculate that the localization of ICAM-1 on the apical surface of the luminal epithelium suggests an involvement of this molecule in embryo implantation, but further work is required to confirm this hypothesis. It is likely that IFN-γ, produced by T-lymphocytes within the endometrium and perhaps also by the preimplantation embryo, regulates the expression of ICAM-1. In contrast, the restricted and constitutive expression of ICAM-2 on the vascular endothelium suggests that the role of this molecule is to regulate leukocyte invasion of the stroma.

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References


