ABSTRACT
Each of the proinflammatory cytokines interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor (TNF) α has been identified in reproductive tissues during labor. The cellular origin of these cytokines is unclear. The aim of this study was to localize these proinflammatory cytokines in myometrium (upper and lower segment), cervix, and fetal membranes at term. Biopsies were taken from women undergoing cesarean section either before or after the onset of labor. Immunohistochemistry was used to localize each of the cytokines IL-1β, IL-6, IL-8, and TNFα. Leukocytes were localized using an antibody to CD45. In myometrium and cervix, immunostaining for IL-1β was predominantly in leukocytes. In fetal membranes, IL-1β localized to leukocytes and to the stromal cells of the decidua. In myometrium, IL-6, IL-8, and TNFα were restricted to leukocytes, which were present in greater numbers in tissue obtained during labor. In cervix, IL-6, IL-8, and TNFα localized to leukocytes and glandular and surface epithelium. IL-8 also localized to cervical stromal cells. In fetal membranes, IL-6 and TNFα were expressed by decidual stromal cells, infiltrating leukocytes, and extravillous trophoblasts. In membranes, IL-8 localized to leukocytes in the chorion but was not detected in the amnion. In fetal membranes collected at labor, IL-8 was expressed in decidual stromal cells. Infiltrating leukocytes are a major source of cytokines in uterine tissues during labor.

INTRODUCTION
The biology of human parturition remains obscure [1]. A role for inflammatory processes seems certain. Leukocytes, possibly attracted by an increase in cell adhesion molecule expression, invade the myometrium, placenta, cervix, and fetal membranes at or immediately after the onset of labor [2–4]. Coincident with these events is the production of proinflammatory cytokines, particularly IL (interleukin)-1, IL-6, IL-8, and tumor necrosis factor (TNF) α, in reproductive tissues during parturition. The majority of studies have incorporated ELISAs, RIAAs, or Western or Northern blots; such techniques quantitate cytokine production but give little information on the cells responsible for their synthesis. In a few studies, immunohistochemistry or in situ hybridization has been used to identify cytokine-producing cells in reproductive tissues during pregnancy and parturition; however, such studies have been focused on the placenta and fetal membranes, largely ignoring the myometrium and cervix [5–8].

We hypothesized that during parturition, the invading leukocytes are a source of proinflammatory cytokines in the myometrium, cervix, placenta, and fetal membranes. The purpose of this work was to test this hypothesis and then to determine alternative sources of cytokines within the pregnant human uterus.

MATERIALS AND METHODS

Subjects and Tissue Collection
Two groups of women were recruited for the study: 1) pregnant women at term (>37 wk gestation) undergoing elective cesarean section prior to onset of labor and 2) pregnant women at term undergoing emergency cesarean section during spontaneous labor (cervical dilatation >4 cm and <9 cm). The indications for emergency cesarean section in the laboring group were suspected fetal distress (n = 16), failure to progress in labor (n = 6), and undiagnosed breech presentation (n = 1). Women were excluded from the study if they were carrying multiple fetuses, if there was evidence of active infection, or if the labor had been induced. No woman had been given glucocorticoids or steroids within 48 h before delivery. The study was approved by the Research Ethics Committee at North Glasgow Hospitals University NHS Trust. Informed consent was obtained from each woman prior to inclusion in the study.

Each patient had biopsies taken either from the myometrium, cervix, or fetal membranes. Lower segment myometrial biopsies were obtained from the upper margin of the lower uterine segment incision. Additional biopsies were obtained from the upper uterine segment by dissecting a strip of myometrium from the inner aspect of the posterior uterine wall. Both upper and lower segment samples were taken in each patient (7 women prior to the onset of labor; 5 women in labor). In all myometrial biopsies, myometrium was separated from surrounding structures, i.e., endometrium and decidua, by sharp dissection. Cervical biopsies in group 1 were obtained vaginally prior to cesarean section as previously described [9]. In group 2, biopsies were obtained after delivery of the infant, the cervix being approached via the uterine incision (8 women). Biopsies of fetal membranes were full thickness and included attached decidua (10 women prior to the onset of labor; 10 women in labor). The myometrial, cervical, and fetal membrane samples were fixed in 10% buffered formalin (BDH, Poole, UK) and embedded in paraffin.

Immunohistochemistry
Immunohistochemistry was performed on paraffin-embedded myometrial, cervical, and fetal membrane samples using antibodies against IL-1β, IL-6, IL-8, TNFα, and CD45, as detailed in Table 1.

Sections 5 μm thick were cut from the paraffin-embedded tissues, mounted on silane-coated slides, heated to 60°C for 35 min, deparaffinized in xylene, and rehydrated in a graded alcohol series. Endogenous peroxidase activity was quenched using 0.5% hydrogen peroxide in methanol. Sections were washed in PBS, and antigen was retrieved by microwaving in a pressure cooker (Lakeland Plastics Ltd., Cumbria, UK) at full power for 5 min in citrate buffer (10 mM, pH 6.0).

All sections to be incubated with antibodies against ILs were washed in PBS with 0.1% saponin (Sigma-Aldrich Co. Ltd., Poole, Dorset, UK) then blocked with 20% rabbit/20% human serum. Sections to be incubated with CD45 were washed in PBS then blocked in 20% horse/20% human serum for 30 min at room temperature. Sections were then incubated for 16 h at 4°C with the primary antibody (Table 1) diluted either in 2% rabbit serum with 0.1% saponin (cytokines) or in 2% horse serum (CD45). Sec-
lations were washed in either PBS/0.1% saponin or PBS alone and then incubated for 30 min with biotinylated rabbit anti-goat (Vector Laboratories, Peterborough, UK) (cytokines) or biotinylated horse anti-mouse (Vector) (CD45) diluted 1:200 in 2% rabbit serum in PBS/0.1% saponin or 2% horse serum in PBS both with 5% human serum added. Sections were washed in PBS/0.1% saponin or PBS alone and then incubated with avidin DH/biotinylated horseradish peroxidase H reagent (Vector) in PBS for 30 min before final washing. The antigen was localized using 1 mg/ml diaminobenzidine tetrahydrochloride (Sigma-Aldrich), 0.2% hydrogen peroxide in 50 mM Tris HCl, pH 7.6, and appeared as a brown end product. Sections were counterstained with Harris hematoxylin (Sigma-Aldrich). Negative controls included slides incubated without the primary antibody and sections incubated with a mouse monoclonal antibody against IgG1 Aspergillus niger glucose oxidase (DAKO), an enzyme that is neither present nor inducible in mammalian tissues. Tonsillar tissue was used as a positive control for all primary antibodies used. The specificity of each of the anti-cytokine antibodies had previously been verified by the manufacturer using ELISAs and Western blotting.

To identify decidua and trophoblast, immunohistochemistry was performed with serial paraffin-embedded sections of fetal membranes using antibodies directed against vimentin and cytokeratin, respectively. A polyclonal antibody isolated from calf lens (Euro-path, Cornwall, UK) (cytokines) or biotinylated horse anti-mouse (Vector) (CD45) diluted 1:200 in 2% rabbit serum in PBS/0.1% saponin or 2% horse serum in PBS both with 5% human serum added. Sections were washed in PBS/0.1% saponin or PBS alone and then incubated with avidin DH/biotinylated horseradish peroxidase H reagent (Vector) in PBS for 30 min before final washing. The antigen was localized using 1 mg/ml diaminobenzidine tetrahydrochloride (Sigma-Aldrich), 0.2% hydrogen peroxide in 50 mM Tris HCl, pH 7.6, and appeared as a brown end product. Sections were counterstained with Harris hematoxylin (Sigma-Aldrich). Negative controls included slides incubated without the primary antibody and sections incubated with a mouse monoclonal antibody against IgG1 Aspergillus niger glucose oxidase (DAKO), an enzyme that is neither present nor inducible in mammalian tissues. Tonsillar tissue was used as a positive control for all primary antibodies used. The specificity of each of the anti-cytokine antibodies had previously been verified by the manufacturer using ELISAs and Western blotting.

**RESULTS**

In the myometrium and cervix, IL-1β was located predominantly in leukocytes (Fig. 1, a and b). Weak staining for IL-1β was observed within myocytes in the myometrium. There was a greater density of positively staining leukocytes in biopsies taken after the onset of labor than in those obtained before labor. In fetal membranes, IL-1β localized to leukocytes and the stromal cells of the decidua (Fig. 1c). There was little IL-1β staining in cervical biopsies taken before the onset of labor (Fig. 1a). In the laboring cervical samples, the abundant leukocytes stained positively for IL-1β (Fig. 1b).

Within the myometrium, IL-6 was restricted to a subpopulation of leukocytes. In the cervix, IL-6 was localized to a subpopulation of leukocytes and to glandular and surface epithelium (Fig. 1, d and e). The multilobed appearance of these leukocytes suggests that they are neutrophils. In fetal membranes, IL-6 was expressed by decidual stromal cells, mesenchymal cells, infiltrating leukocytes, and extravillous trophoblast (Fig. 1f). As with IL-1β, the density of positively staining leukocytes appeared greater in laboring than in nonlaboring myometrium and cervix (Fig. 1, d and e).

In the myometrium, IL-8 immunostaining was observed in infiltrating leukocytes in laboring tissues. These cells were not seen in nonlaboring tissues (Fig. 1, g and h). In fetal membranes, IL-8 localized to leukocytes in the chorion, but these cells were absent from the amnion (Fig. 1i).

**DISCUSSION**

IL-1 is a proinflammatory cytokine. Two isoforms have been identified: IL-1α and IL-1β; IL-1β predominates in human tissues. IL-1 is secreted by a variety of cell types, including inflammatory cells, endothelial cells, fibroblasts, and smooth muscle cells. IL-1 promotes the adhesion of neutrophils, monocytes, T cells, and B cells by enhancing the expression of adhesion molecules such as intercellular adhesion molecule 1. IL-1 increases the synthesis of prostaglandins and stimulates release of proteases such as collagenase. Each of these processes is known to occur in association with parturition. IL-1β mRNA and protein have been demonstrated in the myometrial lower segment using reverse transcription polymerase chain reaction (RT-PCR) [10] and ELISA [11], respectively. More IL-1β protein was seen in tissues from advanced than in those from early labor [11]. However, myometrial concentrations of the IL-1 receptor (measured by Western blot) decline as labor progresses [12]; thus, the role of IL-1 in the process of labor is unclear. In our study, both myocytes and leukocytes expressed IL-1β. These data support our hypothesis that invading leukocytes are a source of IL-1β. Further work is required to determine which of these cell types is the major contributor to the increase in IL-1β production observed during labor.

IL-1β has previously been demonstrated in fetal membranes, and a variety of studies have indicated greater concentrations in membranes [13] and in amniotic fluid [14] in samples taken after than in those taken prior to the onset of labor. In contrast to other reports, we failed to find IL-1β staining in the amnion [5, 7], although our results are consistent with those showing that mRNA for IL-1β is absent from the amnion and confined to the chorion laeae in cells that are decidual in origin [5]. An alternative explanation is that low levels of IL-1β are present in the amnion but that our immunohistochemical technique was insuffi-
IMMUNOLOCALIZATION OF CYTOKINES DURING LABOR

FIG. 1. Immunolocalization of proinflammatory cytokines in human myometrium, cervix and fetal membranes. Cervical biopsies taken before the onset of labor (a) show little staining for IL-1β. Cervical biopsies collected during labor (b) show IL-1β within abundant leukocytes. In fetal membranes (c), IL-1β is localized to leukocytes and the stromal cells of the decidua (arrows). In cervical biopsies from nonlaboring (d) and laboring (e) tissue, IL-6 was restricted to a subpopulation of leukocytes, the density of which appeared greater in laboring tissue. Fetal membranes (f) show IL-6 in decidual stromal cells and extravillous trophoblast. In myometrium, IL-8 was not identified in nonlaboring (g) myometrium but was present within leukocytes in laboring (h) myometrium. Fetal membranes collected during labor (i) show IL-8 in decidual stromal cells and infiltrating leukocytes but not in amnion. TNFα staining was absent in nonlaboring myometrium (j). Laboring cervix showing TNFα in leukocytes (k). In fetal membranes, TNFα is present in decidual stromal cells and extravillous trophoblast (l). The blue background is hematoxylin counterstain. Bars = 50 μm.

Ciently sensitive to detect it. We have shown that leukocytes that invade the fetal membranes during parturition produce IL-1β, in agreement with results of other published studies [5, 7].

There was little IL-1β staining in the cervix, except in leukocytes invading at the onset of labor. Our results indicate that either cervical leukocytes or the tissues of the fetal membranes could be responsible for the increase in IL-1β observed in cervicovaginal fluid after the onset of labor [15, 16].

IL-6, like IL-1, is a proinflammatory cytokine known to be expressed by stimulated leukocytes. We demonstrated IL-6 in infiltrating leukocytes in the upper and lower segment myometrium, cervix, and fetal membranes. In a previous study, term human myometrium released IL-6 in culture, and both preterm and term myometrium contained IL-6 mRNA [10]. The results of our study suggest that leukocytes are the main source of this IL-6. Not all CD45-positive cells stained positively for IL-6. Morphological examination of IL-6- and CD45-positive cells suggested that they are neutrophils; however, further work is required to confirm this. Potential changes in IL-6 concentration during labor remain to be determined. Because invading leukocytes are a major source of IL-6 and because the concentration of invading leukocytes is dramatically increased following the onset of labor, one might expect labor to be associated with an increase in myometrial IL-6 concentrations.

We demonstrated IL-6 expression in the amnion and chorion in agreement with published reports [5]. Staining of serial sections with vimentin, cytokeratin, and CD45 demonstrated that the cells expressing IL-6 within the chorion include decidual cells, extravillous trophoblast, and leukocytes. Any or all of these cells could be responsible for the increase in IL-6 production observed in fetal membranes and amniotic fluid following the onset of labor [13, 14].

An increase in IL-6 concentrations in cervicovaginal fluid has been observed during labor at term and preterm, and measurement of cervicovaginal IL-6 has been suggested as a method of predicting delivery in women in suspected preterm labor [17]. In the cervix, IL-6 was localized to glandular and surface epithelium. We are not aware of any studies localizing IL-6 within the pregnant cervix. However, our results indicating IL-6 localization in superficial and glandular epithelium are in agreement with findings of IL-6 in the nonpregnant cervix [18]. Because the invading leukocytes expressed IL-6, these cells may be the source of the
increased levels of IL-6 protein and mRNA observed in association with cervical ripening [19].

The proinflammatory cytokine IL-8 attracts leukocytes and stimulates adhesion molecule expression and neutrophil activity. Changes in IL-8 concentrations during pregnancy have been studied extensively. IL-8 production (measured by ELISA) is greater in laboring than in nonlaboring term myometrium and increases further with increasing cervical dilation [11, 20]. Using RT-PCR, IL-8 mRNA was identified in laboring but not in nonlaboring myometrial samples [10]. Whether production of IL-8 from fetal membranes also increases in association with labor is more controversial; increases have been demonstrated in some studies [13] but not in others [20, 21]. More intense staining for IL-8 receptor types I and II has been demonstrated in amnion obtained after the onset of labor compared with that in amnion obtained before labor [22]. In none of these studies was the cell type responsible for IL-8 production identified. In the present study, we have determined that cells within the myometrium and fetal membranes that expressed IL-8 also expressed CD45. IL-8 expression was not identified in any other cell types. Neutrophils, macrophages, and to a lesser extent T and B lymphocytes are known to invade the myometrium and fetal membranes in large numbers at the time of parturition [2]. The data presented here suggest that these infiltrating leukocytes are the sole source of the major increase in myometrial IL-8 production occurring at the time of labor.

The effects of IL-8 within reproductive tissues depend not only on IL-8 concentration but also on the concentration of its receptor. IL-8 receptor mRNA declines in the myometrium in association with labor, possibly as a result of increased IL-8 concentrations [12]. In contrast, increased amniotic fluid concentrations of IL-8 receptor are found in association with preterm labor in the absence of infection [23]. The net effects of IL-8 in parturition may vary therefore within different reproductive tissues.

The role of IL-8 in cervical ripening is well established; it has been suggested that IL-8 is the mediator of cervical ripening at least in humans [24]. IL-8 mRNA and protein synthesis increase in parallel with cervical ripening [19]. In the present study, we localized IL-8 staining to squamous and glandular epithelial cells and to stromal cells, in agreement with previously published data [19]. In addition, identification of leukocytes using CD45 showed that IL-8 is also present in the invading inflammatory cells. Further work is required to determine the relative contributions of native cervical cell types and invading leukocytes to the increased levels of IL-8 seen in association with parturition.

TNF is known to be secreted by a variety of leukocytes, principally macrophages. TNF secretion has also been demonstrated in some cell lines, including smooth muscle cells and fibroblasts. Within the myometrium, we demonstrated TNFα in only a small subpopulation of leukocytes. The morphology of these leukocytes suggests that they are macrophages; however, staining for surface antigens is required to confirm this hypothesis. Other studies on human pregnant myometrium have demonstrated either low levels of TNFα (using enzyme immunoassay) [10] or levels below the limit of detection (using ELISA) [20]. Using RT-PCR, small amounts of TNFα mRNA were identified in the myometrium from 2 of 5 laboring women [10].

Within the cervix, we localized TNFα to infiltrating leukocytes and cervical epithelial cells. We are not aware of any other data localizing TNFα production within the pregnant cervix. In our study, only a subpopulation of leukocytes expressed TNFα. These leukocytes had the morphology of macrophages, but further work is required to confirm their surface antigen expression.

Previous studies have shown that TNFα is released from fetal membranes and is elevated in both vaginal secretions and amniotic fluid in labor [14, 16, 25]. Using immunohistochemistry, TNFα was localized to CD11b-positive cells (a leukocyte subpopulation) and to decidual cells in membranes [7].

The function(s) of these proinflammatory cytokines in the process of parturition remains obscure. Assuming that they serve a specific purpose rather than being the by-product of another pathophysiological event, likely effects include stimulation of uterine activity, either directly or via an increase in prostaglandin production, attraction of leukocytes, and tissue remodelling.

There is some support for the hypothesis that these cytokines stimulate uterine contractions. Both IL-1 and TNFα stimulate arachidonic acid release and prostaglandin production in human myometrial cells, thus stimulating myometrial contractions and ripening of the uterine cervix [26, 27]. Additionally, IL-1 potentiated oxytocin-induced myometrial contractions in an in vitro system [26].

Cytokines themselves might mediate the leukocyte attraction that occurs at the time of parturition. Cytokines might mediate leukocytic infiltration, either directly (in the case of IL-8) or via upregulation of cell adhesion molecules (IL-1 and TNFα). We and others have demonstrated a massive upregulation of adhesion molecule expression at the onset of labor [4]. Additionally, amniotic fluid IL-1, IL-6, and TNFα concentrations are correlated with the degree of leukocytic infiltrate in the placenta and membranes [28]. These data raise the possibility that a positive feedback mechanism exists whereby proinflammatory cytokines attract leukocytes into reproductive tissues at the time of parturition. The leukocytes themselves also generate proinflammatory cytokines, attracting more leukocytes.

Proinflammatory cytokines may be important in tissue remodelling. IL-1 and TNFα upregulate production of matrix metalloproteinase-9 in human myometrial smooth muscle cells and may therefore play a role in the tissue remodelling that occurs during parturition and immediately thereafter [29]. TNFα also stimulates matrix metalloproteinase production in human fetal membranes [30, 31]. Support for a role for TNF in tissue remodelling and rupture within the fetal membranes was provided by a recent study demonstrating an association between polymorphism of the promoter of the TNFα gene and increased risk of preterm premature rupture of the membranes [32].

We localized production of each of the inflammatory mediators IL-1β, IL-6, IL-8, and TNFα in the myometrium, fetal membranes, and cervix before and after labor. The leukocytic infiltrate that occurs in these tissues is a rich source of inflammatory cytokines. Further work, using fluorescence-activated cell sorting or laser scanning cytometry could quantify the relative contribution of inflammatory cells and other cell types to cytokine production at the time of parturition. Assuming that future results confirm the data obtained in the present study, we anticipate that strategies aimed at reducing the inflammatory infiltrate or inhibiting cytokine production in these cells might be effective in the treatment of preterm labor.

REFERENCES

2. Thomson AJ, Telfer JF, Young A, Campbell S, Stewart CT, Cameron IT, Greer IA, Norman JE. Leukocytes infiltrate the myometrium during human parturition: further evidence that labour is an inflammatory process. Hum Reprod 1999; 14:229–236.


