Fractalkine (FRK) levels in amniotic fluid and its production during pregnancy

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Fractalkine is a new CX3C chemokine that has chemoattractant activity for T cells, monocytes and natural killer (NK) cells. Western blot analysis revealed that fractalkine protein was detected as a 95 kDa band in both the amniotic fluid and the amnion during the second and third trimesters. Immunohistochemistry using an anti-fractalkine polyclonal antibody revealed positive staining of epithelial cells in amnion and trophoblasts in both the second and third trimesters. Neonatal urine also contained detectable amounts of fractalkine. RT–PCR detected fractalkine mRNA transcripts in the amnion. To determine whether fractalkine receptor (CX3CR1)-positive cells were present in amniotic fluid and amnion, we performed RT–PCR using specific primers for CX3CR1. CX3CR1-positive cells had migrated into the amniotic fluid and the amnion. The present findings suggest that fractalkine found in amniotic fluid may contribute to the immunodefence mechanism during pregnancy.

Key words: amnion/amniotic fluid/CX3CR1/fetal urine/fractalkine

Introduction

Amniotic fluid serves to cushion the fetus, allowing musculoskeletal development and protecting the fetus from trauma. It also maintains the fetal temperature and has a minimal nutritive function. Ingestion of amniotic fluid into the lung and gastrointestinal tract by inspiration and swallowing is thought to promote the growth and differentiation of these tissues (Cunningham, et al., 2001). Amniotic fluid contains various cytokines, such as interleukin (IL)-1, tumour necrosis factor (TNF)-α and IL-6 (Baud et al., 1999; Gonzalez-Bosquet et al., 1999; Simpson et al., 1999; Pacora et al., 2000; Sato et al., 2001; Bowen et al., 2002). Some reports suggest that factors in amniotic fluid, including cytokines and proteases, mediate various adverse conditions such as preterm labour, premature rupture of membranes, and pre-eclampsia (Cox et al., 1997; Arntzen et al., 1998; Nakabayashi et al., 1998; Heikkenen et al., 2001). However, it is far from clear that these factors mediate all the complications of pregnancy. We have demonstrated that secretory leukocyte elastase inhibitor (SLPI), a defence factor against inflammation, is present in amniotic fluid (Zhang et al., 2001).

Chemokines are small proteins that stimulate the migration of leukocytes and mediate inflammation. These proteins are classified into subgroups according to characteristic cysteine (C) signature motifs (Baggiolini, 1998). CXC (cysteine–any amino acid–cysteine) molecules target neutrophils and, to some degree, lymphocytes; CC molecules target monocytes, lymphocytes, basophils and eosinophils with variable selectivity; and the C–C chemokine seems to act only on lymphocytes (Rollins, 1997). A chemokine bearing a new CXC cysteine motif has been cloned (Bazan et al., 1997; Pan et al., 1997). In contrast to other chemokines, this chemokine, named fractalkine, displays potent chemoattractant activity for T cells, natural killer (NK) cells, and monocytes but not neutrophils and is of non-haemopoietic origin (Bazan et al., 1997). Fractalkine is produced by endothelial cells and neurons and occurs as a cell surface-bound as well as a cleaved soluble protein (Harrison et al., 1999; Papadopoulos et al., 1999; Muehlhofer et al., 2000). The extracellular domain of fractalkine has been shown to be released into the supernatants of transfected cells as a 95 kDa glycoprotein, possibly by proteolysis at the dibasic cleavage site proximal to the membrane, to generate soluble fractalkine (Bazan et al., 1997; Pan et al., 1997). The expression of fractalkine in microglia, endothelial cells and fibroblastic cells has been reported to be up-regulated by inflammatory signals (Schall 1997). Fractalkine may be responsible for the accumulation of lymphocytes in regions of inflammation. The receptors V28 and RBS11 have been characterized as the human and rat receptors for fractalkines, respectively, and have been renamed CX3CR1 (Imai et al., 1997; Jiang et al., 1998). CX3CR1 is expressed on the surface of NK cells, monocytes and CD8⁺ T cells (Imai et al., 1997). CX3CR1-mediated signal transduction presumably plays a role in the migration and adhesion (Imai et al., 1997; Jiang et al., 1998).

The aim of this study was to quantify fractalkine concentration in amniotic fluid and to investigate the production of fractalkine in the amniotic fluid during pregnancy. To determine whether CX3CR1-positive cells migrated into the amniotic fluid and the amnion, we also examined the existence of CX3CR1-positive cells in the amniotic fluid and the amnion.
Materials and methods

Reagents
Goat anti-human fractalkine polyclonal antibodies and recombinant fractalkine were purchased from R&D Systems (Minneapolis, MN, USA). Normal goat IgG for use as a control in the histochemical analysis was purchased from Zymed Laboratories (San Francisco, CA, USA).

Samples
Amniotic fluids were obtained from 20 pregnant women in the second trimester and 20 pregnant women in the third trimester at the time of genetic amniocentesis and amniocentesis for fetal lung maturity. Ten samples of amnion were obtained at delivery from pregnant women who were in the second trimester or at term and were without medical complications. Those samples obtained in the second trimester were as a result of legal abortions. Human placentas were collected from normal pregnant women at the time of legal abortion in the second trimester and Caesarean section in the third trimester. The mean gestational ages of the second and third trimesters were 17 ± 0.9 and 38 ± 1.2 weeks respectively. Neonatal urine samples were obtained using urine collection bags within 6 h after birth. All babies were born without medical complications. Five babies were born by vaginal deliveries and five babies were born by Caesarean section. This study was approved by the local ethics committee of the Department of Obstetrics and Gynecology, Faculty of Medicine, Osaka University. Informed consent was obtained from each patient. Amniotic fluid was centrifuged (700 g for 10 min) to separate the cells from the supernatant. The supernatant was then stored at −20°C until assayed. The cell pellet was stored at −80°C until it was processed to obtain RNA.

Tissue preparation for Western blot analysis
The homogenizing buffer for protein extraction from the amnion consisted of 0.5 mol/l Tris-HCl (pH 6.8), 10% sodium dodecyl sulphate (SDS), β-mercaptoethanol, and 1% bromophenol blue. The amnion was homogenized in a 2 ml volume. Homogenates were centrifuged at 4°C for 30 min at 14 000 g to remove debris. Following the determination of protein concentrations, the samples were aliquoted, and subjected to polyacrylamide gel electrophoresis (PAGE).

Western blot analysis of amniotic fluids and amnion
To detect fractalkine protein in the amniotic fluid and amnion, we performed Western blot analysis using an anti-human fractalkine polyclonal antibody. Samples containing amniotic fluid (10 µl) or ~10 µg of amnion protein were electrophoresed on 15% SDS–polyacrylamide gels and transferred onto nitrocellulose membranes (0.45 µm; Schleicher and Schuell, Dassel, Germany). The membranes were incubated with 5% dried milk protein to block nonspecific binding sites and then incubated with primary antibodies raised against human fractalkine. After washing, the membranes were incubated with secondary antibodies conjugated with horseradish peroxidase and the fractalkine immunoreactivity was visualized using an ECL Western blotting analysis system (Amersham, Aylesbury, UK).

Protein assay
Protein levels were determined using Bio-Rad (Hercules, CA, USA) Protein Determination Reagent according to the method of Bradford (1976).

Determination of fractalkine titre in the amniotic fluid by densitometric analysis of Western blots
To measure the titre of fractalkine in the amniotic fluid, the level of fractalkine protein was quantified and analysed using the NIH Image software program developed and provided by the Research Services Branch (RSB) of the National Institute of Mental Health (NIMH). Intra- and inter-assay variabilities of the fractalkine titres were 5.0–9.5 and 5.0–9.0% (means 7.5 and 7.0) respectively. The detection limit was 0.1 µg/ml.

RNA extraction
RNA was extracted from cells in the amniotic fluid and amnion samples of 0.5 g wet weight by acid guanidine thiocyanate–phenol–chloroform extraction according to the method of Chomczynski and Sacchi (1987).

RT–PCR amplification
RT–PCR was performed using an RT–PCR high kit (Tatoyo Co., Tokyo, Japan). The reaction was carried out in a mixture containing M-MLV (Maloney murine leukaemia virus) RTase (reverse transcriptase) and ~200 ng RNA sample in 1× RTase buffer, random hexamers, and dNTP mix for 40 min at 42°C. PCR amplification was performed using the RT mixture after the incubation described above (10 µl), with sequence-specific primers for human CX3CR1 (5′-TTGAGTACGATGATTGGCTGA-3′/5′-GGCTTTGGCTTTC-TTGTTG-3′) (GenBank accession number U82934), for human fractalkine (5′-ACTCTTGGCCACCTCAAGC3′/5′-TTGGAGACCGGAGGACCT-3′) (GenBank accession number U84487) or GAPD (also known as G3PDH) (5′-ACACAGTCTCCATGCATAAC-3′/5′-TCCACACCCCTCGTTGCTGA-3′).

PCR was carried out for 35 cycles using a thermal cycler (Perkin–Elmer/Cetus, Norwalk, CT, USA). Each cycle consisted of denaturation at 94°C (40 s), annealing at 52°C (40 s), and extension at 72°C (40 s). For amplification of GAPD, 25 cycles of 94°C for 40 s, 52°C for 40 s, and 72°C for 40 s were performed. Amplification using CX3CR1-specific primers yielded a 653 bp DNA product with a sequence that matched the published sequence of the CX3CR1 gene (Muethlohofer et al., 2000), while amplification using the fractalkine-specific primers yielded a 597 bp DNA product according to the published sequence of the fractalkine gene (Muethlohofer et al., 2000). RT was performed with total RNA without reverse transcriptase (a mock RT sample) to detect possible contamination of RNA samples by genomic DNA. Twenty microlitres of a 50 µl PCR mixture was electrophoresed on a 1% agarose gel.
and stained with ethidium bromide, and amplified products were visualized by UV illumination. Molecular sizes were estimated using a 100 bp DNA ladder. All primers were obtained from Life Technologies (Tokyo, Japan).

Semiquantitative PCR of fractalkine and CX3CR1 mRNA were performed to assess the amounts of PCR products after 25–40 cycles of amplification. PCR products were digested with Taq I to confirm that they were authentic fractalkine transcripts. PCR products were digested with Hpa II to confirm that they were authentic CX3CR1 transcripts.

Immunohistochemical staining of fractalkine in the amnion and placenta

To determine the localization of fractalkine in the amnion and placenta, we performed immunohistochemical staining using an avidin–biotin peroxidase complex method kit (OminiTags Universal Streptavidin/Biotin Affinity Immunostaining Systems, Lipshaw, Pittsburg, PA, USA). Fresh frozen sections of the samples were incubated in 0.3% hydrogen peroxide to block endogenous peroxidase and then incubated with 2% goat IgG to minimize non-specific binding. The thickness of the tissue was 7 μm. The appropriately diluted goat polyclonal anti-fractalkine antibody (R&D Systems) or goat IgG for the control was applied at room temperature and left for 1 h. After rinsing with phosphate-buffered saline solution, the sections were further incubated for 30 min with biotin-labelled goat anti-mouse IgG, followed by the addition of avidin–peroxidase complex at 4°C for 30 min. Peroxidase activity in the sections was visualized with 0.1% 3,3-diaminobenzidine-tetrahydrochloride containing 0.02% hydrogen peroxide in 0.1 mol/l Tris buffer (pH 7.2). The slides were counterstained with Mayer’s haematoxylin.

Statistical analysis

Statistical analysis was conducted using Welch’s t-test, and P < 0.05 was considered significant. The values given represent the means ± SEM.

Results

To determine whether fractalkine protein was present in amniotic fluid, we performed Western blot analysis. As shown in Figure 1A, fractalkine protein was detected as a 95 kDa band in the amniotic fluid. To determine the fractalkine concentration in amniotic fluid, we analysed the densitometric intensity of Western blot signals using the software ‘NIH Image’ to semiquantify fractalkine levels in the amniotic fluid. The mean fractalkine titre in the amniotic fluid of pregnant women in the second trimester was 6.5 ± SEM μg/ml and
that in the third trimester was $5.5 \pm \text{SEM} \mu\text{g/ml}$. To determine the source of fractalkine in the amniotic fluid, we examined whether fractalkine was present in the amnion. As shown in Figure 1B, fractalkine protein was detected in the amnion as a 95 kDa band by Western blot analysis. RT–PCR was performed to examine the expression of fractalkine mRNA in the amnion during pregnancy. Figure 1C shows that fractalkine transcripts were present in the amnion in both the second and third trimesters. The PCR products were digested with Taq I to confirm that they were derived from authentic fractalkine gene transcripts. The 597 bp DNA product was digested to 311 and 269 bp fragments, as expected (data not shown). RT–PCR was operating in the linear range of amplification and control of GAPD gene showed equivalent RNA in the starting reactions (data not shown). To identify the origin of this large amount of fractalkine, we performed immunohistochemical staining of sections of the amnion in both the second and third trimesters, using an anti-fractalkine polyclonal antibody. Epithelial cells in the amnion showed that epithelial cells of the amnion in the second and third trimesters were intensely stained, indicating that these epithelial cells were one of the main sources of fractalkine in amniotic fluid. Trophoblasts in both the second and third trimesters were also intensely immunoreactive. In the present study, neonatal urine was also found to contain a detectable amount of fractalkine. As fetal urine is considered to be an important source of amniotic fluid and modifies the amniotic fluid composition, we speculated that placenta-derived fractalkine in the fetal circulation may be excreted into the amniotic fluid by urination. It is possible that the relative amounts of fractalkine derived from the amnion and urine affects the fractalkine concentration in amniotic fluid during pregnancy.

Amniotic fluid has bacteriostatic and bactericidal properties. Various studies have demonstrated that amniotic fluid of pregnant women contains a high level of cytokines (Keelan et al., 1997; Arntzen et al., 1998; Baud et al., 1999; Gonzalez-Bosquet et al., 1999; Simpson et al., 1999; Pacora et al., 2000; Sato et al., 2001; Bowen et al., 2002). We have previously reported that placental cells produced various cytokines during placental infection (Shimoya et al., 1998, 1999). Several inflammatory mediators enhance the fractalkine mRNA levels and the production of fractalkine (Muelhoefer et al., 2000). These findings suggest that several cytokines in amniotic fluid might induce fractalkine in the amniotic cavity. Amniotic fluid has an important role in fetal lung function and maturation. Elevated amniotic fluid TNF-α levels are associated with respiratory distress syndrome (RDS) and prenatal exposure to TNF-α is a risk factor for
Fractalkine regulation during pregnancy

Identification and molecular characterization of fractalkine receptor CX3CR1, which mediates both leukocyte migration and adhesion. Cell, 91, 521–530.


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