Expression of fractalkine in the Fallopian tube and of CX3CR1 in sperm

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BACKGROUND: Fractalkine is a CX3C chemokine that has chemoattractant activity for T cells, monocytes and natural killer (NK) cells. The objective of this study was 2-fold: to evaluate (i) the presence of fractalkine in the Fallopian tube and (ii) the existence of CX3CR1 (fractalkine receptor) in ejaculated sperm. METHODS AND RESULTS: Western blot analysis revealed that fractalkine protein was detected as a 95 kDa band in the isthmus, the ampulla and the infundibulum of the Fallopian tube. Immunohistochemistry revealed positive staining of epithelial cells in the Fallopian tube. RT±PCR demonstrated that fractalkine transcripts were expressed in all parts of the Fallopian tube. RT±PCR also revealed that CX3CR1-positive cells were present in the Fallopian tube. CX3CR1-positive cells were present in the stroma of the Fallopian tube. The villi of the ciliated cells were positively stained. To determine the function of fractalkine in the Fallopian tube, we examined whether CX3CR1 was present in ejaculated sperm. RT±PCR demonstrated that CX3CR1 transcripts were expressed in the ejaculated sperm. Immunohistochemistry demonstrated positive staining of the tail of the spermatozoa. CONCLUSIONS: The present findings suggest that fractalkine in the Fallopian tube contributes to the immunodefence mechanism during fertilization and to the sperm motion in the oviduct.

Key words: CX3CR1/Fallopian tube/fractalkine/sperm

Introduction

Chemokines are small proteins that stimulate the migration of leukocytes and mediate inflammation. These proteins are classified into subgroups according to characteristic cysteine signature motifs (Baggiolini, 1998). CXC molecules, such as interleukin (IL)-8, target neutrophils and, to some degree, lymphocytes; CC molecules, such as monocyte chemotactic and activating factor (MCAF), target monocytes, lymphocytes, basophils and eosinophils with variable selectivity; and the C-chemokine seems to act only on lymphocytes (Rollins, 1997). A chemokine bearing a CX3C 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; Muehlhoefer et al., 2000). The extracellular domain of fractalkine is released into the supernatant of transfected cells as a 95 kDa glycoprotein (Bazan et al., 1997; Pan et al., 1997). In the reproductive system, this molecule is present in the seminal plasma and is associated with sperm motility (Zhang et al., 2002). We have already reported that fractalkine contributes to the immunodefence mechanism during pregnancy (Shimoya et al., 2003). The expression of fractalkine has been reported to be upregulated by inflammatory signals (Schall, 1997). Fractalkine may be responsible for the accumulation of lymphocytes in regions of inflammation. The receptor for fractalkine, named CX3CR1, has been characterized (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 cell migration and adhesion (Imai et al., 1997; Jiang et al., 1998).

In the Fallopian tube, several important steps, such as gamete transport, maturation, fertilization and early embryogenesis, take place. It follows, therefore, that the environment in the Fallopian tube represents the optimal conditions for these and other important developmental processes. In addition, the mechanisms of chemotaxis and thermotaxis of the sperm have been revealed in several studies (Bahat et al., 2003; Spehr et al., 2003). The Fallopian tube contains various factors, such as
growth factors and cytokines (Buhi et al., 1999; Ota et al., 2002). However, little is known about the chemokines in the Fallopian tube. The aim of this study was to investigate the expression of fractalkine in the Fallopian tube and to clarify the expression of fractalkine receptor (CX₃CR1) in the spermatozoa.

Materials and methods

Reagents

Goat anti-human fractalkine polyclonal antibodies and recombinant fractalkine were purchased from R&D systems (Minneapolis, MN). Normal goat IgG for use as a control in the histochemical analysis was purchased from Zymed Laboratories (San Francisco, CA). A human CX₃CR1 antibody (rat IgG2b), fluorescein isothiocyanate (FITC)-labelled human CX₃CR1 antibody (rat IgG2b) and FITC-conjugated rat IgG2b were purchased from MBL (Nagoya, Japan). Bisbenzimide Hoechst 33258 fluorochrome was purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Samples

Twelve samples of Fallopian tubes were obtained from gynaecological patients who underwent total hysterectomy and bilateral oophorectomy. The age range of the patients was 29–45 years old. Patients with complications of venereal infection were excluded from the study. For the control of the analysis, amniotic membranes were obtained from pregnant women in the third trimester. This study was approved by the local ethics committee of the Department of Obstetrics and Gynecology, Osaka University Graduate School of Medicine. Informed consent was obtained from each patient. Semen samples were obtained from three proven fertile men. The fertile men had fathered at least one child and had no recent history of venereal infection. Semen was obtained by masturbation after 5 days of abstinence. Samples were collected in a sterile container and examined within 1 h after ejaculation.

Tissue preparation for western blot analysis

The homogenizing buffer for protein extraction from the Fallopian tubes and amniotic membranes consisted of 0.5 M Tris–HCl pH 6.8, 10% sodium dodecyl sulphate (SDS), 6% β-mercaptoethanol and 1% bromophenol blue. The 0.5 g sample of Fallopian tubes and amniotic membranes were homogenized in a 2 ml volume. Homogenates were centrifuged at 4°C for 30 min at 14,000 g to remove debris. Following protein determinations, the samples were aliquoted and subjected to polyacrylamide gel electrophoresis (PAGE).

Western blot analysis of Fallopian tubes

To examine fractalkine protein in the Fallopian tubes, we performed western blotting analysis using an anti-human fractalkine polyclonal antibody. A 10 µg aliquot of oviductal protein was electrophoresed on a 15% SDS–polyacrylamide gel and transferred onto a nitrocellulose membrane (0.45 μm; Schleicher and Schuell, Dassel, Germany). The membrane was incubated with 5% dried milk protein followed by anti-human fractalkine polyclonal antibody. The primary antibody was used at a final concentration of 1.0 µg/ml. The fractalkine immunoreactivity was visualized using an enhanced chemiluminescence (ECL) western blotting analysis system (Amersham, Aylesbury, UK).

Protein assay

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

Determination of fractalkine levels in various parts of the Fallopian tubes by densitometric analysis of western blotting

To measure the fractalkine levels in various parts of the Fallopian tubes among six cases, the expression 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)].

RNA extraction

RNA was extracted from Fallopian tube and amniotic membrane samples of 0.5 g wet weight by acid guanidine thiocyanate–phenol–chloroform extraction according to the method of Chomczynski and Sacchi (1987). The concentrations of RNA were determined by their absorbance at 260 nm.

RT-PCR amplification

RT–PCR was performed using an RT–PCR high kit (TOYOBO Co., Osaka, Japan). The reaction was carried out in a mixture containing of Moloney murine leukaemia virus reverse transcriptase (RTase) and 1 µg of RNA sample in 1× RTase buffer, random hexamers and dNTP mix for 40 min at 42°C. PCR amplification was performed using the reverse transcription mixture after the incubation described above (10 µl), with sequence-specific primers for human CX₃CR1 (5’-TTGAGTACGATGATTTGGCTGA-3’/5’-GGCTTTGGCCTTTCTTGTTG-3’) (GenBank accession No. U28934) or human fractalkine (5’-ACTCTTTGCCACCTCAGC-3’/5’-TGAGACGGAAGGCACTC-3’) (GenBank accession No. U84487). PCR was carried out for 35 cycles using a thermal cycler (Perkin-Elmer/Cetus, Norwalk, CT). Each cycle consisted of denaturation at 94°C (40 s), annealing at 52°C (40 s) and extension at 72°C (40 s). Amplification using CX₃CR1-specific primers yielded a 653 bp DNA product with a sequence that matched the published sequence of the CX₃CR1 gene (Muehlhoefer et al., 2000), while amplification using the fractalkine-specific primers yielded a 597 bp DNA product that matched the published sequence of the fractalkine gene (Muehlhoefer et al., 2000). A mock reverse transcription was performed to detect possible contamination of RNA samples by genomic DNA. A 20 µl aliquot 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 by comparison with a 100 bp DNA ladder. All primers were obtained from Life Technologies (Tokyo, Japan).

Immunohistochemical staining of fractalkine in the Fallopian tubes

To determine the localization of fractalkine in the Fallopian tube, we performed immunohistochemical staining by using an avidin–biotin peroxidase complex method kit (OminiTags Universal Streptavidin/Biotin Affinity Immunostaining Systems, Lipshaw, Pittsburg, PA). Paraffin sections of the Fallopian tube were incubated in 0.3% hydrogen peroxide to block endogenous peroxidase and covered with 2% goat IgG to minimize non-specific binding. The 1000-fold diluted goat polyclonal anti-fractalkine antibody (R&D Systems) or control pre-immune goat serum for the control was applied at room temperature and left for 1 h. After the sections were rinsed with phosphate-buffered saline (PBS) solution, they were incubated further for 30 min with biotin-labelled goat anti-mouse IgG, and then with avidin–peroxidase complex at 4°C. 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 brown colour was determined to be positive. The slides were counterstained with Mayer’s haematoxylin.
Immunohistochemical staining of CX3CR1 in the Fallopian tubes

The method for the determination of the localization of CX3CR1 in the Fallopian tube was as described above for fractalkine, except that a 1000-fold diluted goat polyclonal anti-CX3CR1 antibody from MBL (Nagoya, Japan) was used.

Preparation of motile sperm

Semen specimens were obtained after 5 days of abstinence. After liquefaction at room temperature, the semen was examined to determine the sperm count and motility using a Makler Counting Chamber (Seti-Medical Instruments, Haifa, Israel). The absence of leukospermia (polymorphonuclear cells >10/µl) in the collected samples was verified. Motile sperm were obtained by the swim-up method in addition to the Percoll gradient method (World Health Organization, 1999).

Immunohistochemical staining of CX3CR1 in the sperm

To determine the localization of CX3CR1 in the spermatozoa, we performed fluorescence staining with anti-CX3CR1 antibody. The spermatozoa were washed twice with PBS and co-incubated with FITC-labelled anti-human CX3CR1 (rat IgG2b) and control IgG2b for 1 h at 37°C in 5% CO2. The spermatozoa were washed three times with PBS and suspended in 95% ethanol for 10 min. An aliquot was dried on a glass slide. The slide was incubated with 0.8 µg/ml bisbenzimide Hoechst 33258 fluorochrome (H33258) for 2 min. The slide was rinsed with distilled water and observed with an Olympus BH2 epifluorescence microscope (Tokyo, Japan) with an excitation maximum of 490 nm and an emission maximum of 520 nm to detect FITC staining.

Statistical analysis

The data were subjected to one-way ANOVA using the Statview statistics package (Abacus Concepts, Inc., Berkeley, CA). *P < 0.05 was considered significant.

Results

To detect fractalkine protein in the Fallopian tubes, we performed western blot analysis. As shown in Figure 1A, fractalkine protein was detected as a 95 kDa band in all parts of the Fallopian tube. To determine fractalkine levels in various parts of the Fallopian tubes, we analysed the densitometric intensity of the bands observed in western blot analysis using the software ‘NIH image’. The fractalkine levels in various parts of the Fallopian tubes of six samples were analysed by western blot analysis. The intensity of fractalkine protein in the ampulla of the tube (lane 4) looked stronger than that in the infundibulum (lanes 5 and 6). However, there were no significant differences in the expression levels of fractalkine in the isthmus, ampulla and infundibulum among six cases (*P > 0.05). Fractalkine protein was present at similar levels in the isthmus, ampulla and infundibulum.

RT–PCR was then performed to examine the expression of the fractalkine gene in the Fallopian tube. Figure 1B shows that fractalkine transcripts were present in all parts of the Fallopian tube. To identify the origin of fractalkine, we performed immunohistochemical staining of sections of the Fallopian tube using an anti-fractalkine polyclonal antibody. The epithelial cells in the Fallopian tube were stained (Figure 2). The intensity of staining in the infundibulum of the Fallopian tube was slightly weaker than that in other parts. Both the ciliated and non-ciliated epithelial cells were stained (Figure 2G). The positive staining seemed to be mainly nuclear. To determine whether CX3CR1-positive cells were present in the Fallopian tube, we performed RT–PCR using specific primers for CX3CR1. As shown in Figure 3A, RT–PCR demonstrated the presence of CX3CR1 mRNA expression in the Fallopian tube. The expression of CX3CR1 mRNA in the spermatozoa was also examined by RT–PCR. As shown in Figure 3B, CX3CR1 mRNA was detected in the spermatozoa.

The localization of CX3CR1-positive cells in the Fallopian tube is demonstrated in Figure 4. There were several cells in the stroma of the Fallopian tube stained with CX3CR1 antibodies (Figure 4A and B). In addition to the positively stained cells in the stroma of the Fallopian tube, the villi of ciliated epithelial cells were also positively stained (Figure 4C). To determine...
whether CX3CR1 molecule was present on spermatozoa, we performed immunocytochemical staining. About half of spermatozoa were stained by anti-CX3CR1 antibody conjugated with FITC in Figure 5A. As shown in Figure 5C, immunocytochemical analysis using an anti-CX3CR1 antibody conjugated with FITC demonstrated positive staining of the tails of the spermatozoa. To demonstrate the localization of CX3CR1 we used bisbenzamide Hoechst 33258 fluorochrome. The localization in the sperm head is shown in Figure 5B and D.

Discussion

Human semen contains a certain amount of fractalkine, and an association between sperm motility and the fractalkine concentration in the seminal plasma has been observed (Zhang et al., 2002). Recently, several studies revealed the mechanism of chemotaxis and thermotaxis of the sperm (Bahat et al., 2003; Spehr et al., 2003). Isobe et al. (2002) demonstrated that mRNAs for the RANTES (regulated on activation normal T expressed and secreted chemokine) receptors CCR-1 and CCR-5 are present in human sperm. RANTES has a chemotactic effect on human sperm but does not affect sperm motility (Isobe et al., 2002). In the present study, we demonstrated that CX3CR1 mRNA and protein were present in sperm. Taken together, these findings indicate that fractalkine might play a role in maintaining the motility of spermatozoa and regulating sperm chemotaxis.

Human oviductal cells produce factors that are important for the maintenance of sperm motility in vitro (Yao et al., 2000). Several studies using animal models demonstrated that the oviduct had important roles in the fertilization process, such as the penetration of eggs by sperm, capacitation of the sperm and the reservoir for spermatozoa during the period from mating to ovulation (Hunter, 1984; Smith et al., 1987; Parrish et al., 1989; Boatman and Magnoni, 1995). In the isthmus of the Fallopian tube, spermatozoa bind to the oviduct epithelium. This interaction may represent part of the in vivo capacitation process (Smith et al., 1987). Because the fractalkine receptor (CX3CR1) is present on the surface of sperm, fractalkine in the isthmus might play an important role in maintaining the motility of spermatozoa and their ability to undergo the acrosome reaction while sperm are stored until ovulation.

Fractalkine is one of the chemokines with a CX3C cysteine motif. In the present study, we demonstrated the expression of fractalkine protein and mRNA in the Fallopian tube. The present findings suggest that fractalkine is constitutively present in all parts of the Fallopian tube. Our immunohistochemical analysis using anti-fractalkine polyclonal antibody showed that the epithelial cells of the Fallopian tube were stained, suggesting that these epithelial cells are the main source of fractalkine in the Fallopian tube. The reason why the

Figure 2. Immunohistochemical staining of fractalkine-producing cells in the Fallopian tube. The cells in the Fallopian tube were stained by the avidin–biotin complex method with a goat polyclonal anti-fractalkine antibody (A, C and E) or control goat serum (B, D and F). (A and B) The isthmus of the Fallopian tube. (C and D) The ampulla of the Fallopian tube. (E and F) The infundibulum of the Fallopian tube. The epithelial cells in the Fallopian tube were stained. (G) High magnification of the ampulla of the Fallopian tube.
Figure 3. (A) CX3CR1 mRNA expression in the Fallopian tube. Agarose gel electrophoresis of RT–PCR-amplified CX3CR1 cDNA. Lane 1: cDNA from the isthmus of the Fallopian tubes of a woman with myoma uteri (case 1). Lane 2: cDNA from a mock RT sample of the isthmus of the Fallopian tubes of case 1. Lane 3: cDNA from the ampulla of the Fallopian tubes of case 1. Lane 4: cDNA from a mock RT sample of the ampulla of the Fallopian tubes of case 1. Lane 5: cDNA from the infundibulum of the Fallopian tubes of case 1. Lane 6: cDNA from mock RT sample of the infundibulum of the Fallopian tubes of case 1. Lane 7: cDNA from the control sample of the amniotic membranes. Lane 8: cDNA from a mock RT sample of the control sample of the amniotic membranes. (B) CX3CR1 mRNA expression in the spermatozoa. Motile sperm were obtained by the Percoll gradient method and swim-up method. Agarose gel electrophoresis of PCR-amplified DNA of CX3CR1. cDNA from the spermatozoa of donor 1 (lane 1), donor 2 (lane 2) and donor 3 (lane 3). Lane 4: cDNA from the control sample of the amniotic membranes.

Figure 4. Immunohistochemical staining of CX3CR1-positive cells in the Fallopian tube. The cells in the Fallopian tube were stained by the avidin–biotin complex method with a rat monoclonal anti-CX3CR1 antibody (A–C) or control goat serum (D–F). (A and D) The ampulla of the Fallopian tube at low magnification. (B and E) The stroma of the ampulla of the Fallopian tube at high magnification. (C and F) The epithelium of the ampulla of the Fallopian tube at high magnification.

Figure 5. Fluorescence staining of CX3CR1 in the spermatozoa. (A and C) The spermatozoa were stained with the FITC-conjugated anti-CX3CR1 antibody. (A) Low and (C) high magnification. (B and D) Bisbenzimide Hoechst 33258 fluorochrome staining. (C) Low and (D) high magnification. The arrows represent the positive staining in the tail of spermatozoa.
positive staining seemed to be mainly nuclear is unknown. Further investigations would be necessary to reveal this. The Fallopian tubes have bacteriostatic and bactercidal mechanisms that protect against infection through the Fallopian tubes into the peritoneal cavity. It is well known that inflammatory cytokines are increased at inflammation sites. Several inflammatory mediators enhance the fractalkine mRNA levels and the production of fractalkine (Muelhhofer et al., 2000). Large numbers of leukocytes in the Fallopian tube are associated with oviductal infection. In the present study, we detected the presence of CX3CR1 mRNA in the cells of the Fallopian tube. Immunohistochemistry revealed that CX3CR1-positive cells were present in the Fallopian tube. Fractalkine has an important role in the inflammatory response. Several infections such as salpingitis. The villi of the ciliated cells were demonstrated that the epithelial cells of the Fallopian tube presence of CX3CR1 mRNA in the cells of the Fallopian tube. This molecule might contribute to homeostasis of the immunodefence system and the inflammatory response (Cockwell et al., 2001; Nishimura et al., 2002). Such lymphocytes might contribute to the immunodefence system in the oviduct. Fractalkine might protect the Fallopian tube epithelium against infection in the oviduct. This molecule might contribute to homeostasis of the immunodefence system in the oviduct in cases of salpingitis, for example in clamhymia infection. Further investigations will be necessary to examine the relationship between the fractalkine level and genital tract infections such as salpingitis. The villi of the ciliated cells were also positively stained by CX3CR1 antibody. These results demonstrated that the epithelial cells of the Fallopian tube might be regulated by fractalkine in the autocrine mechanism.

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