A combination of interleukin-6 and its soluble receptor impairs sperm motility: implications in infertility associated with endometriosis

Souichi Yoshida1, Tasuku Harada, Tomio Iwabe, Fuminori Taniguchi, Masahiro Mitsunari, Nobuhiro Yamauchi, Imari Deura, Sayako Horie and Naoki Terakawa

Department of Obstetrics and Gynecology, Tottori University School of Medicine, Yonago 683-8504, Japan
1To whom correspondence should be addressed. E-mail: souichi@grape.med.tottri-u.ac.jp

BACKGROUND: We previously reported that the level of interleukin (IL)-6 is increased in the peritoneal fluid of women with endometriosis. This study was undertaken to assess the effects of IL-6 and soluble IL-6 receptor (sIL-6R) on in vitro sperm motility. METHODS: Sperm (n = 20) were cultured with IL-6 or sIL-6R, or with a combination of both. After 24 h cultures, sperm motility was evaluated using a computer-assisted semen analysis system. Gene and protein expressions of IL-6, IL-6 receptor (IL-6R), and glycoprotein 130 (gp130) were examined in sperm by RT–PCR analysis and western blot analysis. RESULTS: Addition of IL-6 or sIL-6R individually to the culture media had no affect on sperm motion. However, adding a combination of IL-6 and sIL-6R dose-dependently reduced the percentage of motile and rapidly moving sperm. Adding anti-IL-6R antibody abolished these adverse effects. Sperm expressed the gp130 gene and protein, but not IL-6 or IL-6R. CONCLUSIONS: A combination of IL-6 and sIL-6R may be associated with gp130 expressed in the sperm and reduce sperm motility. IL-6 and sIL-6R may contribute to the pathogenesis of endometriosis-associated infertility.

Key words: glycoprotein 130/interleukin-6/soluble interleukin-6 receptor/sperm motility/peritoneal fluid

Introduction

Endometriosis, characterized by the presence and growth of endometrial cells outside the uterus that cause pelvic pain, is often associated with infertility. Infertility can occur even in the early stages of endometriosis when adhesions or anatomical distortion are not yet present. Although the pathogenesis of endometriosis-associated infertility is poorly understood, a number of authors have suggested possible causes, such as tubal occlusion due to adhesions (Haney, 1991), a defect in granulosa cell steroidogenesis (Harlow et al., 1996), occurrence of luteinizised unruptured follicles (Mio et al., 1992), an inadequate luteal phase (Hirschowitz et al., 1987), and association of auto-antibodies (Fernandez-Shaw et al., 1993). Moreover, accumulated data suggest that the peritoneal microenvironment may contribute to endometriosis-associated infertility and to the pathogenesis of endometriosis (Harada et al., 2001).

The volume of peritoneal fluid (PF) is significantly elevated in infertile women with endometriosis compared to those without endometriosis (Harada et al., 1997a). PF is reported to be associated with infertility in several ways, e.g. inhibiting sperm motion (Drudy et al., 1994; Aeby et al., 1996), sperm acrosome reaction (Arunugam, 1994), mouse embryo growth (Prough et al., 1990), sperm binding to the zona pellucida (Coddington et al., 1992), and ciliary action in the human Fallopian tube (Lyons et al., 2002). Since the PF of infertile women with endometriosis contains abundant cytokines and growth factors, these factors may exert those detrimental effects on the reproductive process and possibly contribute to endometriosis-associated infertility (Harada et al., 2001).

Interleukin-6 (IL-6), a pleiotropic cytokine produced by a variety of cell types, plays a pivotal role as a mediator of numerous physiological and pathogenic processes (Tagoh et al., 1989). It has also been suggested that IL-6 has important functions in reproductive physiology, including the regulation of ovarian steroid production, folliculogenesis, and early events related to implantation (Gorospe et al., 1992; Ray et al., 1997). IL-6 exerts these effects to bind cognate membrane receptor complexes, consisting of an 80kDa ligand binding subunit [glycoprotein (gp) 80: interleukin-6 receptor; IL-6R] and a 130kDa signal transducing protein (gp130). IL-6R binds IL-6 with low affinity and must associate with gp130 for high affinity binding and signal transduction to occur (Jacobs et al., 1992). A 55kDa soluble form of IL-6R (soluble IL-6 receptor; sIL-6R), which is proteolytically cleaved from IL-6R, exists in body fluid. It can enhance the bioactivity of IL-6 by direct binding of the IL-6/sIL-6R complex to gp130 (Rose-John et al., 1994).

We previously reported that levels of IL-6 in PF were significantly higher in infertile women with endometriosis...
and that its soluble receptor also exists in PF (Harada et al., 1997a). Although the main source of IL-6 had been considered to be macrophages, our previous study demonstrated that IL-6 is produced in endometriotic stromal cells at levels similar to that in macrophages (Tsudo et al., 2000).

Accordingly, we hypothesize that IL-6 and sIL-6R may be responsible for the reported adverse effects on reproductive processes of PF from patients with endometriosis. In this study, we focused on the effects of IL-6 and sIL-6R on in vitro sperm motility. In addition, we also examined the expressions of IL-6, IL-6R and gp130 in sperm.

Materials and methods

Semen samples and washing of sperm

Semen samples were obtained from 20 normozoospermic fertile volunteers who met the diagnostic criteria of the World Health Organization (WHO) standards (1992) and abstained from coitus for 3 days. Informed consent was obtained from all subjects. The semen samples were processed by the Percoll (Sigma Co., USA) gradient technique (three layers: 40, 70 and 90%) to separate seminal plasma and to remove cell contamination. The supernatant was removed, and the pellet was resuspended in 2 ml human tubal fluid medium (HTF; Irvine Scientific, USA). The tube was centrifuged at 500 g for 10 min. A swim-up migration was then performed by placing a layer of 1 ml serum-free fresh medium over the sperm pellet. The tube was then incubated for 45 min at 37°C under an atmosphere of 5% CO₂ in air. The supernatant was gently aspirated and sperm concentration and motility evaluated. A part of the supernatant was centrifuged again, then the sperm pellets were snap-frozen, and stored at -80°C for RNA or protein extraction procedures described below.

Effects of IL-6 and sIL-6R on sperm motility

All 20 sperm samples were diluted with serum-free HTF medium with various concentrations of IL-6 (0–10 ng/ml, recombinant human IL-6; Genzyme, USA) with or without sIL-6R (50 ng/ml; recombinant human IL-6 soluble receptor; R&D Systems, USA). The final concentration of sperm was adjusted to 5 × 10⁶/ml, and 0.5 ml aliquots placed in 6 ml culture tubes (Falcon; Becton Dickinson UK Ltd). A monoclonal antibody against IL-6R (5 μg/ml; monoclonal anti-human IL-6 receptor antibody; Genzyme/Techne, USA) was used to neutralize the specific effects of IL-6 and sIL-6R. Each sample was incubated at 37°C under a humid atmosphere of 5% CO₂ in air. A part of the aliquots was evaluated by computer-assisted sperm motion analysis (CASA; version 10 HTM-IVOS, Hamilton Thorne Research, USA) system at 24 h. At the same time, the sperm motility on the control samples was also assessed.

RT–PCR

Total RNA was extracted from the sperm by the guanidium thiocyanate method according to the manufacturer’s instructions (Isogen; Nippon Gene Co. Ltd, Japan). Reverse transcription of RNA from sperm into cDNA and PCR amplification were performed using the Gene Amp RNA PCR Core Kit (Perkin Elmer, USA). The reverse transcription of RNA to cDNA was performed with 2.5 U/μl of MuLV reverse transcriptase as follows: 10 min at 30°C, 20 min at 47°C, 5 min at 99°C and 5 min at 4°C. PCR amplification was carried out in 25 μl MgCl₂ solution (2 mmol/l), 10 × PCR buffer II (1 ×), DI water (65.5 ml) containing 0.1% DEPE, and Ampli Taq DNA polymerase (2.5 U/100 ml). Samples were amplified for 32 cycles of denaturation (30 s at 94°C), annealing (30 s at 60°C), synthesis (1.5 min at 72°C), and primer extension of 5 min at 75°C after each cycle.

For PCR analysis, previously described specific primers for human IL-6R and human gp130 were used: IL-6R (sense): 5'-CATTGCGCATTTGTCAGTTTCC-3' and IL-6R (anti-sense): 5'-AGTACTGTGTGGTCTGAGTT-3'; and gp130 (sense): 5'-ACAGATGGAAGGTTGGAGAT-3' and gp130 (anti-sense): 5'-AGATGACATGCGTGAAGACC-3' (Yamazaki et al., 1996; Yoshioka et al., 1999). The distances between primers, including the primers, were 251 and 423 bp respectively. PCR products were resolved on 2% agarose gel with a small molecular weight DNA marker (X 174 digested with Hae III).

The PCR products were transferred to a nylon membrane (Sartor, Germany) using a vacuum blotter with 0.4 mol/l NaOH and 1 mol/l NaCl. The DNA on the membrane was fixed with a UV cross-linker. The membranes were hybridized with internal probes of IL-6R (5'-ACAAGCATGCATCCTGG-3') or gp130 (5'-CCAGATCCTTG-AAAG-3'). The biotin-labelled product on the membrane was detected with a Sinvite kit (Sumitomo Metal Industry Co., Japan). The membrane was treated with streptavidin–alkaline phosphatase, followed by chemiluminescence detection. The membrane was exposed to X-ray film for 15 min at room temperature (Iwabe et al., 1998).

Western blot analysis

IL-6, IL-6R and gp130 protein expressions in sperm were examined by means of western blot analysis as previously described in detail (Yoshida et al., 2002). Briefly, the frozen sperm pellets were lysed with lysis buffer containing 50 mmol/l Tris–HCl (pH 7.6), 150 mmol/l NaCl, 0.1% SDS, 1 mmol/l dithiothreitol, and 1 × Complete Protease Inhibitor Cocktail (Boehringer Mannheim, Germany). The lysate was centrifuged and supernatant was prepared. Protein concentration of the supernatant was measured by Bradford assay. Eighty micrograms of protein samples were resolved by electrophoresis on a 4–20% gradient polyacrylamide gel. These separated protein samples were then electroblotted onto a nitrocellulose membrane before blocking in 2.5% skimmed milk for 60 min. The membrane was then probed with anti-IL-6R (1:1000) or anti-gp130 (1:1000) antibodies, followed by chemiluminescence detection. The membrane was exposed to X-ray film for 15 min at room temperature (Iwabe et al., 1998).

Statistical analysis

Results were analyzed using two-way analysis of variance, followed by Student’s paired sample t-test in order to compare the values obtained with various treatments versus the values of control. The data were expressed as means ± SEM. P < 0.05 was accepted as statistically significant.

Results

Effects of IL-6 and/or sIL-6R on sperm motility

To evaluate the effects of IL-6 and sIL-6R on sperm motility, we examined Percoll-purified sperm fractions adjusted to 5 × 10⁶/ml (motility: 91.6 ± 3.7%; and percentage rapid cells: 66.8 ± 6.9%, n = 20). Neither IL-6 (10 ng/ml) alone nor sIL-6R (50 ng/ml) added alone to the culture medium
had an effect on sperm motion. An antibody against IL-6R also exerted no effect on sperm motility (Figure 1A). In contrast, as shown in Figure 1B, the addition of 0.1–10 ng/ml IL-6 and 50 ng/ml sIL-6R together adversely affected sperm motility at 24 h culture. This combination of IL-6 and sIL-6R also caused a profound decrease in the percentages of rapidly motile cells in a dose-dependent manner (n = 12). Adding the anti-IL-6R antibody abolished these adverse effects. *P < 0.05, **P < 0.001 versus control.

Expression of IL-6, IL-6R, and gp130 in sperm

Because the addition of IL-6 or sIL-6R alone had no effect, but, when combined, significantly reduced sperm motility, we examined the gene expression of IL-6, IL-6R and gp130 in sperm by RT–PCR. A representative result of RT–PCR is shown in Figure 2A. Only a product of the expected size (423 bp) of gp130 was detected. The identity of the PCR-amplified cDNA fragment of gp130 was verified using southern hybridization with the labelled internal probe (Figure 2B). Furthermore, protein production of gp130 in sperm was detected by western blot analysis (Figure 2C). However, the IL-6 and IL-6R protein expressions were not observed (data not shown).

Discussion

The present study demonstrates that the addition of combined IL-6 and sIL-6R significantly reduced the percentage of motile and rapidly moving sperm. On the other hand, the addition of IL-6 or sIL-6R alone in the culture media failed to adversely affect sperm motility. Sperm expressed only gp130, and did not show the IL-6 and IL-6R expression, suggesting that the combination of IL-6 and sIL-6R may associate with gp130 and exert its effect on sperm.

For most cytokines (such as IL-2 and TNFα), soluble receptors have antagonistic activity, precluding their ligand binding to specific membrane receptors. In contrast, sIL-6R is unique in exerting an agonistic action with its ligand, resulting in subsequent signal transduction. Because gp130 is expressed ubiquitously on various cells, IL-6 can exert its biological effect in the presence of sIL-6R, even though effector cells lack IL-6R on their plasma membrane, as demonstrated by negative control of thyroid function in cultured human thyroid follicles (Yamazaki et al., 1996). The IL-6/sIL-6R complex associates with the constitutively expressed gp130 on IL-6R-negative cells to induce homodimerization of gp130, leading to a cellular response. Thus, our findings suggest an underlying mechanism in which IL-6 increases in the PF of patients with endometriosis, combines with sIL-6R that also exists in PF, and the combined
IL-6/sIL-6R associate with the gp130 expressed on IL-6R-negative sperm. Consequently, combined IL-6/sIL-6R may exert adverse effects on sperm motility.

Furthermore, our data corroborate previous reports demonstrating that IL-6 levels in seminal plasma or cervical mucus were significantly higher in infertile patients compared with the fertile control patients, and that its levels in seminal plasma negatively correlated with sperm motility (Gruschwitz et al., 1996; Naz and Butler, 1996). Since the seminal plasma contains sIL-6R (Dousset et al., 1997), increased IL-6 in seminal plasma and cervical mucus associate with sIL-6R, and then the binding of IL-6/sIL-6R complex to gp130 presented in sperm may result in the impairment of sperm function.

For the association of sperm motility and seminal plasma IL-6 concentrations, some authors reported different results, suggesting that IL-6 levels in seminal plasma did not correlate with sperm motility (Dousset et al., 1997; Kocak et al., 2002; Friebe et al., 2003). The reasons for the controversy are not evident but may reflect the effects of other factors contained in seminal plasma. Seminal plasma contains various factors that stimulate or maintain sperm motility, such as carnitine (Gurbuz et al., 2003), as well as inhibitory factors. The balance of those factors could regulate sperm motions in the seminal plasma. We speculate that the effects of the factors other than IL-6 may be responsible for the controversy.

Seminal plasma reportedly contains similar levels of IL-6 with PF (Eggert-Kruse et al., 2001). In contrast, concentrations of IL-6 were much higher in cervical mucus of infertile women than in PF of patients with endometriosis (Naz and Butler, 1996). Indeed it seems that the sperm contact with cervical mucus may be the most significant in vivo. The exposure to elevated IL-6 levels in PF of patients with endometriosis and in the Fallopian tubes subsequent to contact with cervical mucus may further impair the sperm motility. Additionally it is speculated that the seminal plasma, which contains the protective factors of sperm motility, has been removed from the fallopian tubes, and then IL-6 contained in PF diffusing down the fallopian tubes may affect sperm motions at lower concentrations than cervical mucus.

In the present study, we observed an inhibition of sperm motility at a concentration of ~0.1 ng/ml of IL-6. The median IL-6 concentration in PF of patients with endometriosis (measured in our previous study) was lower than levels used in the present study (Harada et al., 1997a). Thus, this study has only a limited ability to determine the cause of endometriosis-associated infertility for all patients. However, in general, half of endometriosis patients are reported to be infertile. The inhibition of sperm motility by IL-6 may be involved in the infertility of at least some patients with endometriosis who have highly elevated levels of IL-6 in PF.

PF diffusing into the tubal and endometrial environment may affect sperm and their interaction with the oocyte and embryo development. Many authors have demonstrated that the PF of patients with endometriosis has detrimental effects on several steps in the reproductive process. On the other hand, various substances, including interleukins, were reported to increase in the PF of infertile women with endometriosis (Ramey et al., 1993; Harada et al., 2001). Recently, some of these substances are believed to be a cause of endometriosis-associated infertility. In fact, substances such as IL-1 (Sueldo et al., 1990), tumour necrosis factor-α (Eisermann et al., 1989), endometrial placental protein 14 (Oehninger et al., 1995), antibodies to transferrin and α2-HS glycoprotein (Pillai et al., 1998) were reported to adversely affect sperm motility and function and/or embryo development.

We previously reported that IL-6 was increased in the PF of patients with endometriosis and correlated with the extent of active endometriotic lesions (Harada et al., 1997a). We also demonstrated that IL-6 was produced by endometriotic stromal cells at levels similar to those of peritoneal macrophages from patients with endometriosis (Tsudom et al., 2000). One study demonstrated that the addition of IL-6 to culture medium reduced the blastocyst formation rate of mouse embryos, suggesting that increased IL-6 in the PF of endometriosis patients may contribute to infertility (Harada et al., 1997b).

Thus, the concept that IL-6 may contribute to endometriosis-associated infertility has been conjectural so far. The present study supports earlier studies that demonstrated the inhibitory effects of PF of patients with endometriosis on sperm motility (Aeby et al., 1996) and functions (Coddington et al., 1992; Arumugam, 1994), and it supports the contribution of IL-6 to endometriosis-associated infertility by adversely affecting sperm motility. From our series of studies on IL-6, we conclude that IL-6 may contribute to the pathogenesis of endometriosis-associated infertility.

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