Effects of overexpression of CXCL10 (cytokine-responsive gene-2) on MA-10 mouse Leydig tumor cell steroidogenesis and proliferation

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Abstract

Chemokines have been implicated in tumor growth, angiogenesis, metastasis and the host immune response to malignant cells. Infection and autoimmune disorders can reduce androgen production by Leydig cells and adversely affect spermatogenesis. Cytokine-responsive gene-2 (crg-2) (systematic name CXCL10, also known as interferon-α/IFN-α-inducible protein 10 (IP-10)) is a potent chemokine expressed predominantly by macrophages and Leydig cells in the testis. CXCL10 binds to CXCR3 receptor (a G-protein-coupled receptor) and acts via Gi protein. We have shown previously that CXCL10 is differentially expressed in normal Leydig cells, inhibited by human chorionic gonadotropin and induced by interferon-γ, interleukin-1 and tumor necrosis factor-α. The purpose of the present study was to determine the effects of overexpression of CXCL10 by transfection experiments in MA-10 cells on cell growth, CXCR3 expression, progesterone synthesis and steroidogenic acute regulatory protein (StAR D1, a key regulatory factor in steroidogenesis) gene expression. We cloned the complete CXCL10 cDNA in a mammalian expression vector with the CMV promoter, pcDNA3·1D/V5-His-TOPO, and confirmed its expression with rat CXCL10 antibody and V5 antibody. Results showed large amounts of CXCL10 protein secreted in the medium in the CXCL10 transfectants by Western blotting. The production of CXCL10 mRNA ranged from 30–50-fold more (n=6) in the transfected cells than the control cells, as determined by semiquantitative and real-time RT-PCR. 8-Br-cAMP downregulated CXCL10 mRNA expression and stimulated CXCR3 mRNA expression. Transfection of MA-10 cells with CXCL10 decreased cAMP-induced progesterone synthesis from 38·5 ± 1·7 ng/ml (1·5 × 105 cells/ml) in control cells to 23·2 ± 1·5 ng in transfected cells (P<0·01). 8-Br-cAMP (0·2 mM)-induced StAR D1 mRNA was decreased 30–40% by transfection with CXCL10. Interestingly, overexpression of CXCL10 induced the expression of its receptor CXCR3 gene, as determined by RT-PCR and fluorescence-activated cell sorter (FACS) analysis. Transfection of CXCL10 also significantly decreased insulin-like growth factor-I (IGF-I, 100 ng/ml)-induced [3H]thymidine incorporation into DNA. These data suggest that CXCL10 also inhibits MA-10 tumor cell proliferation. In conclusion, CXCL10 inhibits StAR D1 expression, decreases progesterone synthesis and inhibits cell proliferation. CXCL10 has the potential to be used in gene therapy for prostate cancer due to its antiangiogenic effect and its inhibitory effect on steroidogenesis.


Introduction

Leydig cells in the testis are responsible for the production of androgens needed for spermatogenesis and development of male characteristics. The testis is a dynamic tissue, containing many cell types that produce a variety of compounds that affect Leydig cell function. CXCL10 (cytokine-responsive gene-2) has been shown to be secreted by Leydig cells, macrophages and T cells into the microenvironment of the testis (Hu et al. 1998, Goffic et al. 2002). CXCL10 has also been shown to exhibit potent antiangiogenic and antitumor activities in vitro and in vivo (Angiolillo et al. 1995, Farber 1997). The overexpression of CXCL10 in combination with IL–12 could be used as a gene therapy to enhance tumor regression (Palmer et al. 2001).

CXCL10 belongs to a large family of chemotactic cytokines, now termed ‘chemokines’, that are expressed in...
diverse cell types wherein they regulate innate and adaptive immune responses (Murphy et al. 2000). Chemokines are chemotactic factors and growth regulators, which exert their effects through seven transmembrane-domain, G protein-coupled receptors (Farber 1997, Tamaru et al. 1998). The action of CXCL10 is mediated by its receptor CXCR3, as well as by its nonspecific binding to heparan sulfate proteoglycans and unknown novel receptors (Soejima & Rollins 2001). Ligand binding leads to rapid elevation of intracellular Ca2+ and activation of several other intracellular signaling pathways (Ganju et al. 1998).

CXCL10 has been cloned from humans, mice and rats (Ohmori & Hamilton 1990, Vanguri & Farber 1990, Luster et al. 1995, Wang et al. 1996). The murine CXCL10 gene is known as ‘cytokine responsive gene–2’ (crg-2), whereas rat CXCL10 gene is called ‘mob-1’. The CXCL10 gene consists of four exons. The CXC in CXCL10 is CysAsnCys. The murine or rat CXCL10 protein consists of 98 amino acids, with a calculated molecular weight of 10 781 (Vanguri & Farber 1990, Wang et al. 1996). Recently, the NMR structure of CXCL10 has been determined. It consists of a series of loops and one turn of a 3–10 helix at the N-terminus, followed by three antiparallel β-strands packed against a c-terminal α-helix, with two pairs of conserved cysteines, 9–36 and 11–53, that form disulfide bonds (Booth et al. 2002).

It has been well documented that interferon (IFN)-γ and tumor necrosis factor (TNF)-α secreted by macrophages induce IP-10 (interferon-γ-inducible protein 10) expression in macrophages and T cells (Narumi 1992, Ohmori et al. 1993, Ohmori & Hamilton 1995, Sgardari et al. 1996). We have previously demonstrated that CXCL10 is induced by IFN-γ, interleukin-1β (IL-1β) and TNF-α, but inhibited by human chorionic gonadotropin (hCG) in Leydig cells, and causes inhibition of hCG-induced testosterone production. In this paper, we investigated the effect of overproduction of CXCL10 by transient transfection of MA-10 cells on expression of its receptor CXCR3, progesterone production, cell proliferation and expression of the rate-limiting step for steroidogenesis, StAR D1.

Materials and Methods

Cloning the mouse CXCL10 gene in a mammalian high-expression vector

Mouse Leydig MA-10 cells (a generous gift from Dr Mario Ascoli, Department of Pharmacology, University of Iowa College of Medicine, Iowa, IA, USA) were cultured in Waymouth's MB/752 medium containing 15% horse serum. Cells were stimulated with murine (m)IFN-γ (100 IU/ml, R&D Systems, Minneapolis, MN, USA) for 16 h, and total RNA was isolated with Trizol reagent (Life Technologies, Gaithersburg, MD, USA). RNA was treated with ribonuclease-free DNaseI (Life Technologies) to remove any contaminating DNA. The published sequence for mouse CXCL10 (GenBank accession no. J05576) was used to derive PCR primers (sense, 5′-caccat gaacccaagtgctgccgt-3′; antisense, 5′-aggagccccctttagaccttt tgt-3′), incorporating a Kozak consensus sequence (Kozak 1991). The CXCL10 gene was cloned into pcDNA3-1D/ V5-His-TOPO vector (Invitrogen, Carlsbad, CA, USA) containing CMV promoter. The resultant expression plasmid was designated as pCMV-CXCL10-V5, in which the CXCL10 with a V5 epitope of 14 amino acids was driven by a CMV promoter. The cloned gene with V5 epitope was sequenced, using T7 promoter/priming site and BGH reverse priming site, with the ABI Prism 310 autosequencer (Applied Biosystems, Foster City, CA, USA) by the Oligonucleotide Facility, University of South Carolina, to establish the direction of cloning and correctness of the sequence. Further tests were carried out by restriction mapping.

Transfection of cells with pCMV-CXCL10-V5

The pCMV-CXCL10-V5 plasmid DNA was amplified and purified with Wizard Maxipreps (Promega). Cells were transfected with 5 µg plasmid DNA in FUGENE 6 reagent (Roche Diagnostics, Indianapolis, IN, USA) for 48 h. The cells were further treated with 8-bromo-cAMP or insulin-like growth factor-I (IGF-I) for various time periods and harvested, and the medium and harvested cells were stored at –80 °C for further analysis.

Isolation of total RNA and comparative RT-PCR

Total RNA was extracted from samples with Trizol reagent, and treated with ribonuclease-free DNaseI to remove any contaminating DNA. The purity and yield of isolated RNA were determined by absorbance at 260 and 280 nm. The integrity of the RNA was confirmed by performing denaturing gel electrophoresis. cDNA was generated with 5 µg total RNA, 2·5 µg oligo-dT primer and mouse Moloney leukemia virus reverse transcriptase (Fisher Scientific, Suwanee, GA, USA) at 42 °C for 1 h. Total cDNA (1/20 of the reaction) was used as the template for PCR. RT-PCR of the S14 ribosomal gene (a housekeeping gene) was amplified by the primer pairs, 5′-tgctgctcagggc-3′ reverse (GenBank accession no. 25070169), giving an amplification product of 323 bp (Mamchaoui et al. 2002). The CXCR3 primers used, matched with mouse and rat GenBank sequences (accession nos. 3798731 (mouse) and 8572056 (rat), were as follows: 5′-gccctttctgagacagcagac-3′ forward and 5′-ttggctgctgttcgt-3′ reverse, yielding an amplicon of 211 bp. StAR D1 primers were selected from the aligned sequences of mouse (accession no. 26386863) and rat (accession no. 1236242) StAR D1; that is, 5′-gcagccccatccttggt-3′.
forward and 5′-tgattgtcgcggcaccc-3′, yielding an amplification product of 247 bp. PCR conditions were optimized with buffers for the PCR system (Stratagene, La Jolla, CA, USA). The amplification reaction buffer also contained 0·2 mM of each dNTP, 10 pM of each primer, cDNA template and 1·5 U pfu DNA polymerase. The amplification were carried out with PTC-200 (MJ Research, Waltham, MA, USA). Cycling programs were 94 °C for 2 min, followed by 26–40 cycles of 94 °C for 30 s, 48 °C for 30 s and 72 °C for 1·5 min, with a final extension of 72 °C for 4 min. All experiments were repeated at least three times with comparable results.

Real-time quantitative PCR

Real-time quantitative PCR amplification reactions were carried out with the MyiQ system (BioRad Laboratories, Hercules, CA, USA), which incorporates a 96-channel optical unit. The reaction mixture consisted of 1 × PCR buffer containing SYBR-Green; 3 mM MgCl₂, 100 nM of each primer, 0·2 mM of each dNTP, iTaq DNA polymerase (hot-start enzyme) 25 units/ml, 10 nM SYBR-Green and stabilizers. Fifty nanograms cDNA template were added to each reaction. The final volume was adjusted to 20 µl with H₂O. The PCR conditions were as follows: 95 °C, 8:30 min; 48 cycles of 95 °C, 0:30 min; 60 °C, 0:30 min; 72 °C, 1:00 min. To distinguish specific amplicons from nonspecific amplifications, a melt curve was generated. Standard curves were generated for S14 and StAR D1.

Progestrone assay

MA-10 cells (1 × 10⁵ cells) were seeded onto 12-well plates in six replicates, and subjected to transient transfection with 5 µg pCMV-CXCL10-V5 or pCDNA3-1 (vector) for 48 h. The medium was replaced with 1 ml assay medium (Waymouth’s MB/752 medium containing 0·1 mg/ml bovine serum albumin, pH 7·4), with or without 0·2 mM 8-Br-cAMP, and incubated for 16 h at 37 °C. The medium was collected for progesterone assay, and the cell monolayer was used for the preparation of total cellular protein. The steroids were extracted from the medium by mixing one volume of medium with 10 volumes of diethyl ether in a 12 × 75 mm glass tube. The steroid extract was separated and evaporated on a 37 °C heating block in a ventilating chemical hood. The extract was dissolved in 1 ml assay buffer, and the amount of progesterone in the extracts was measured according to the protocol of the Progesterone Enzyme Linked Immunoassay Kit (Cayman, Ann Arbor, MI, USA). The cell monolayer in each well was homogenized with lysis

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buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1% NP-40, 0.25% sodium deoxycholate, 2 µg/ml aprotinin, 1 µg/ml leupeptin and 100 µg/ml PMSF) at 4°C for 30 min, and their protein concentrations were determined with the Bicinchoninic Acid Protein Assay Kit (Pierce Biochemicals, Rockford, IL, USA).

Western blot analysis
The cell culture media of control and transfected cells were collected and recentrifuged to remove any insoluble material. The proteins of the media were then concentrated by precipitation with cold acetone and dissolved in ice-cold lysis buffer. The CXCL10 protein in the concentrated cell medium was detected by Western blot analysis, as described previously (Chen et al. 2003). Recombinant rat CXCL10 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a positive control. Goat antirat CXCL10 polyclonal antibody (Santa Cruz Biotechnology) and HRP-conjugated bovine antigoat secondary antibody (Santa Cruz Biotechnology) were used for blotting, and Chemiluminescence Luminol Reagent (Santa Cruz Biotechnology) was used for detection.

Fluorescence-activated cell sorter (FACS) analysis
Flow cytometry was performed with a Coulter Epics×L-MCL flow cytometer at the Flow Cytometry Core Laboratory, South Carolina Cancer Center, University of South Carolina, by the procedure described by Risberg et al. (2000). Briefly, the cells were lightly trypsinized, fixed in 70% ethanol, washed and treated with antimouse CXCR3 goat polyclonal IgG antibody (Santa Cruz Biotechnology) followed by a wash with PBS containing 1% bovine serum albumin (BSA) and incubation with fluorescein isothiocyanate-conjugated rabbit antigoat IgG (Santa Cruz Biotechnology). This was followed by a wash with PBS containing 1% BSA. The cells were resuspended in PBS and passed through a cell strainer (35 µm nylon mesh) (Fisher Scientific). The single-cell suspensions were then subjected to FACS.

[3H]Thymidine incorporation into DNA
[3H]Thymidine incorporation was measured as previously described (Yang et al. 1999). Cells were cultured in 24-well plates for 48 h. Serum-containing medium was replaced with a serum-free medium containing IGF-I (100 ng/ml) and BSA (100 µg/ml), and incubated for 48 h. [3H]Thymidine (Amersham Biosciences; 0.5 µCi/ml) was added, and incubation was continued for 6 h. Unincorporated [3H]thymidine was removed by aspirating with cold 5% trichloroacetic acid. [3H]Thymidine incorporation into cellular DNA was measured by dissolving cell material in 0.5 M NaOH and measuring radioactivity in a Wallac liquid scintillation counter (Pharmacia LKB, Gathersburg, MD, USA). All data points are the average of three experiments, and each experiment was carried out with six replicates.

Statistical analyses
Results are the means ± s.e.m. of 4–6 separate experiments. One-way ANOVA followed by the Newman–Keuls multiple comparison test was used for statistical analyses (GraphPad Prism, Version 3.0; GraphPad Software, San Diego, CA, USA). P ≤ 0.05 was considered statistically significant.
Results

Expression of CXCL10 and CXCR3 mRNAs in the transfected MA-10 cells

The expression of mRNA of CXCL10, CXCR3 and S14 was determined by RT-PCR in control and pCMV-CXCL10-V5 or vector-transfected MA-10 cells untreated/treated with 0.2 mM 8-Br-cAMP. The CXCL10 RT-PCR product, a band of 297 bp, was expressed in the control cells, but its expression increased 30- to 50-fold (n=6) in the pCMV-CXCL10-V5-transfected cells (Fig. 1B). Interestingly, overexpression of CXCL10 also increased the expression of its receptor CXCR3 gene, as is evident from Fig. 1C. S14 mRNA expression remained constant in the control and transfected cells. We reported previously that hCG downregulates CXCL10 gene expression by activating adenylate cyclase and increasing cAMP formation (Hu et al. 1998). In the present study, we found that 8-Br-cAMP downregulated CXCL10 mRNA expression and stimulated CXCR3 mRNA expression.

Analysis of CXCR3 expression by FACS

We further investigated CXCR3 expression by an alternative method, FACS. The comparative analysis of the control and CXCL10 transfected cells revealed that the transfected cells had significantly increased fluorescence intensity over the control MA-10 cells (Fig. 2). The increased binding of CXCR3 antibody suggests that CXCR3 expression is induced by CXCL10 overexpression.

Expression of CXCL10 protein in transfected MA-10 cells

After 72 h of cell culture, the culture medium from control and transfected cells was collected, concentrated, dissolved in the loading buffer and separated by gel electrophoresis under reducing conditions. Western blotting of the medium from CXCL10-transfected cells revealed large bands with electrophoretic mobility slightly slower than that of the recombinant mouse CXCL10 (Fig. 3). This could be explained by the fact that the transfection was
carried out by the plasmid containing CXCL10 plus V5 antigen and His6. Blotting with V5 antibody revealed large bands in the transfected MA-10 cells, but not in the control or rCXCL10.

**Effect of overexpression of CXCL10 on progesterone synthesis and StAR D1 expression**

We evaluated the effect of overexpression of CXCL10 on the steroidogenesis in mouse MA-10 Leydig tumor cells, which produce progesterone, but not testosterone in response to cAMP stimulation (Ascoli 1981). Treatment of cells with 0.2 mM 8-Br-cAMP significantly increased progesterone production by 30–40-fold over that of the control cells (Fig. 4). Progesterone secretion by MA-10 cells ranged from 23.2 ± 1.5 ng in transfected cells to 38.5 ± 1.7 ng/ml (1.5 × 10^5 cells/ml) after 24 h of stimulation by 8-Br-cAMP (0.2 mM). Thus, the overexpression of CXCL10 resulted in significant inhibition of progesterone production by 40% (Fig. 4).

We further evaluated the effect of overexpression of CXCL10 on StAR D1 expression in order to study the possible mechanism involved in the effect on 8-Br-cAMP-induced steroidogenesis. We used real-time quantitative RT-PCR, which measures directly the accumulation of the PCR product during the log-linear phase of the reaction and reduces post-PCR manipulations (Bustin 2000, Olney et al. 2002). Fig. 5A shows a melt curve for StAR D1, with one specific product, distinguishing it from nonspecific products and primer dimers. Standard curves of the fluorescence intensity vs log concentration of mRNA for StAR D1 and S14 were plotted. Fig. 5B shows a representative standard curve for StAR D1. Fig. 5C shows that StAR D1 expression was dramatically induced by 8-br-cAMP (0.2 mM). The CXCL10-transfected cells showed a 30–40% decrease in cAMP-induced StAR D1 mRNA levels.

**Effect of overexpression of CXCL10 on IGF-I-stimulated cell proliferation**

Control and CXCL10-transfected cells were stimulated with IGF-I (100 ng/ml) for 48 h, and [3H]thymidine incorporation into the DNA was measured. IGF-I stimulated [3H]thymidine incorporation by 1.5- to 2-fold of the control cells. The incorporation was suppressed by 30–40% by the overexpression of CXCL10 in the transfected cells (Fig. 6). The data suggest a significant decrease in cell proliferation in IGF-I-stimulated cells because of the overexpression of CXCL10.
Discussion

We have utilized a CMV expression system developed by Invitrogen to overexpress mouse CXCL10 in mouse Leydig tumor MA-10 cells and demonstrate that overexpression of CXCL10 inhibits steroidogenesis and cell growth. The efficiency of this system has been reported previously by Pind et al. (2002). They showed that the overexpression of 3-phosphoglycerate dehydrogenase in pCDNA3-1 vector with CMV promoter caused a dramatic increase in the enzymatic activity in baby hamster kidney (BHK)-21 cells (Pind et al. 2002). Since the CXCL10 protein is produced and secreted from the eukaryotic cells in this study, it is expected to be comparable to its native function.

Our results showed significant amounts of CXCL10 protein secreted in the medium in the CXCL10 transfectants, as estimated by Western blotting. Interestingly, CXCL10 overexpression caused a significant increase in expression of its receptor CXCR3. The mechanism by which receptor expression could be increased by its ligand is intriguing. Recently, Goldberg-Bittman et al. (2004) reported that treatment with recombinant human CXCL10 upregulated the expression of CXCR3 in human breast adenocarcinoma cell lines. A number of reports suggest that activation by a ligand could initiate the activation of transcriptional factors and induce its receptor expression (Wang et al. 2000, Zang et al. 2000, Lisignoli et al. 2002, Nickerson et al. 2003). Keratinocyte growth factor (KGF) treatment enhanced KGF receptor (KGFR)
gene expression in MCF-7 breast cancer cells (Zang et al. 2000). Furthermore, Zang et al. demonstrated that KGFR is involved in mediating the KGF-induced motility response in MCF-7 cells. If KGF is an early signal in the progression to a metastatic phenotype, KGFR may provide a useful therapeutic target to prevent metastatic development in breast cancer (Zang et al. 2003).

It has been observed previously that human colon epithelial cells stimulated with stromal cell-derived factor 1α (SDF-1α) and macrophage inflammatory protein (MIP)-1α or MIP1β, which are the chemokine ligands for CXCR4 or CCR5, upregulated production of the CXC chemokines IL-8 and GROα. Each of these chemokines binds to CCR5 to elicit autocrine or paracrine effects (Dwinell et al. 1999). In addition to their membrane localization, CXCR4 and CCR5 were observed intracellularly. Such intracellular stores of chemokine receptor may redistribute to the cell membrane in response to stimuli (Dwinell et al. 1999). Increases in the expression of the chemokine receptors CCR1 and CCR3 in monocytoïd cells have been demonstrated in treatment with recombinant IFN-α by RT-PCR and flow cytometry techniques (Zella et al. 1999).

Our data showed that CXCL10 overexpression inhibited IGF-I-induced cell proliferation. Our results are in agreement with the previous report that CXCL10 inhibits endothelial cell proliferation (Luster et al. 1995). Romagnani et al. (1999) have shown that CXCL10 is highly effective in the inhibition of primary human microvascular endothelial cell proliferation. Recently, it has been demonstrated that the growth of CXCL10-transduced melanoma cells was markedly diminished compared with the parental or null-transduced cells. These findings suggest that CXCL10 gene therapy might be effective in patients with cancer (Feldman et al. 2002). Previously, it has been shown that CXCL10 and MIG mimic the antitumor effects of IL-12 (Kanegane et al. 1998, Tannebaum et al. 1998, Palmer et al. 2001). Neutralizing antibodies to CXCL10 and MIG partially abrogated IL-12-mediated regression in the RENCA model (Tannebaum et al. 1998).

CXCL10 is expressed in a wide variety of cell types in response to administration of lipopolysaccharide (LPS) (Ohmori & Hamilton 1990, Vanguri & Farber 1990), IFN-γ and TNF (Ohmori et al. 1993, Luster 1998). Any nucleated cell is capable of producing at least some chemokines constitutively or upon activation (Gangur et al. 2002). A majority of chemokines with proinflammatory activities is induced upon cellular activation by inflammatory stimuli (Moser & Loetscher 2001).

In the present study, overexpression of CXCL10 decreased 8-Br-cAMP-stimulated progesterone synthesis and inhibited StAR D1 expression. Previously, we have reported that CXCL10 antisense oligonucleotides enhanced basal and hCG-induced testosterone formation in rat Leydig cells (Hu et al. 1998). This suggested that endogenous CXCL10 has an inhibitory effect on Leydig cell steroidogenesis. CXCL10 is expressed in rat Leydig cells and may have paracrine and autocrine effects on testicular function.

In summary, we have shown that overexpression of CXCL10 suppresses IGF-I-induced cell proliferation, 8-Br-cAMP-induced steroidogenesis and StAR D1 expression. Although further studies are necessary to elucidate the potential mechanisms of the effects of CXCL10 on the expression of CXCR3 and StAR D1, the regulatory effect of CXCL10 on steroidogenesis may contribute to the development of androgen-modulating therapeutic strategies in prostate cancer.

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References


Zella D, Barabitskaja O, Casareto L, Romero F, Secchiero P, Reitz MS Jr, Gallo RC & Weichold FF 1999 Recombinant IFN-α (2b) increases the expression of apoptosis receptor CD95 and chemokine receptors CCR1 and CCR3 in monocytoid cells. Journal of Immunology 163 3169–3175.

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