Down-regulation of Expression of Interleukin-6 and its Receptor Results in Growth Inhibition of MCF-7 Breast Cancer Cells

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Abstract. Interleukin-6 (IL-6) plays an important role in the neoplastic process through its action on cancer cell adhesion, motility, proliferation, tumor-specific antigen expression, and thrombopoiesis. IL-6 exerts its activity by binding to a high affinity receptor complex consisting of two membrane glycoproteins: the 80 kDa IL-6 a-receptor subunit (IL-6R) and the 130 kDa signal-transducing protein (GP130). In the present study, MCF-7 breast cancer cells were cultured with human IL-6 and IL-6 soluble receptor (sIL-6R). MCF-7 cells were also treated with either antibodies specific to human IL-6 and IL-6R, or synthetic antisense oligodeoxynucleotides (ODNs) targeted to IL-6 and IL-6R genes. Cell growth was measured, and it was found that human IL-6 and sIL-6R did not significantly increase the proliferation of MCF-7 cells. When IL-6 produced by the MCF-7 cells was bound by rabbit anti-human IL-6 antibody, there was a significant dose-dependent inhibition of cell proliferation. IL-6 and IL-6R antisense ODNs caused a marked and specific decrease in IL-6 and IL-6R mRNA and proteins, respectively. Both IL-6 and IL-6R antisense ODNs significantly inhibited the proliferation of MCF-7 cells, but the inhibitory effect of IL-6R antisense ODN was greater than that of IL-6 antisense ODN (IC50: IL-6R: 1 μM; IL-6: 5 μM, 72-hour incubation). Addition of exogenous IL-6 partially reversed the growth inhibition caused by IL-6 antisense ODN but not the growth inhibition caused by IL-6R antisense ODN. In conclusion, IL-6 plays an important role in maintaining the growth of MCF-7 breast cancer cells. These results suggest careful modulation of IL-6 and IL-6R expression of cells as a potential approach for breast cancer therapy.

Interleukin-6 (IL-6) is a multipotent cytokine that plays an important role in immune responses, inflammation, bone metabolism, reproduction, arthritis, aging and neoplasia (1). It has been shown that serum levels of IL-6 are a prognostic factor in various types of cancer (multiple myeloma, Hodgkin’s lymphoma, renal cell carcinoma, bladder carcinoma, ovarian cancer, esophageal squamous cell carcinoma, and breast cancer (2-12)). A high concentration of serum IL-6 is associated with aggressive tumor types, and poor disease-free and overall survival. IL-6 is involved in cancer progression through the decrease in cancer cell adhesion and increase in cancer cell motility, induction of thrombopoiesis, increase in cancer cell proliferation, as well as stimulation of tumor-specific antigen expression (13-16). It has also been reported that breast cancer cells treated in vitro with IL-6 express higher levels of a breast cancer antigen (CA15-3) and carcinoembryonic antigen (CEA) (16).

IL-6 exerts its activity through binding to a high affinity receptor complex consisting of two membrane glycoproteins: the 80 kDa IL-6 binding receptor protein (IL-6R) and the 130 kDa signal-transducing protein (GP130). IL-6R has a 55 kDa isoform, the soluble IL-6R (sIL-6R). sIL-6R may develop as a result of proteolytic cleavage and release of the membrane-bound form of IL-6R. No naturally occurring mRNA encoding a truncated form of the IL-6R has been reported. IL-6R is not capable of inducing signal transduction directly, but must form a hexameric IL-6:IL-6R complex to be fully activated and induce cell signaling. The sIL-6R also forms a fully active hexameric IL-6:sIL-6R:GP130 complex which induces cell signaling (17-19).

In order to further demonstrate the role of IL-6 in the growth of breast cancer cells, we treated MCF-7 cells with specific antibodies that bind IL-6 and IL-6R proteins, and antisense ODNs that inhibit gene expression of IL-6 and IL-6R. Inhibition of cell growth, and inhibition of IL-6 and IL-6R gene expression and protein secretion were determined.
Materials and Methods

Human recombinant IL-6, human recombinant sIL-6R, rabbit polyclonal anti-human IL-6 IgG (IL-6Ab), mouse monoclonal anti-human IL-6R IgG1 (IL-6RAb), and rabbit IgG and mouse IgG controls were purchased from Sigma (St. Louis, MO, USA). The human sIL-6R was produced by cloning the cDNA of the IL-6R and introducing a stop codon into the cDNA sequence immediately preceding the transmembrane-bound form of the receptor. The CyteElisa™ IL-6 kit and the Pelikine Compact™ soluble human IL-6 receptor ELISA kit were obtained from SYTImmune Science, Inc. (College Park, MD, USA) and Research Diagnostics, Inc. (Flanders, NJ, USA), respectively.

Antisense oligodeoxynucleotides (ODNs). ODNs were synthesized by GeneLab, Louisiana State University (Baton Rouge, LA, USA). All ODNs were completely phosphorothioated and purified by HPLC. The antisense ODN sequence 5’-TCTGGGGGTTACT-3’ (IL-6AS) is specific for the second exon of the IL-6 gene (20) and was designed to target IL-6 mRNA. The antisense ODN sequence 5’-ACGGCCAGCATGC-3’ (IL-6AS) flanking the translation initiation codon of the IL-6R cDNA (21) was created to target IL-6R mRNA. Complementary sense ODNs, 5’-AGTACCCCCAGGA-3’ (IL-6S) and 5’-GCATGCTGGCCGT-3’ (IL-6RS), and random sequence (RS) 5’-ATACACCATACGT-3’ were used as controls.

Cell culture. The MCF-7 human breast cancer cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). MCF-7 cells were maintained in alpha modification of Minimum Essential Medium Eagle (αMEM) supplemented with 10% fetal calf serum, 1 mM glutamine and 0.05 mg/ml gentamicin (Life Technologies Inc., Frederick, MD, USA) in 5% CO2 at 37˚C. Cells were removed from cell culture flasks or plates by trypsin-EDTA digestion, counted with trypan blue staining of viable cells, and pelleted by centrifugation. Cell pellets were used to isolate RNA. The concentrations of IL-6 and IL-6R in the culture media were quantitated by the CyteElisa™ IL-6 kit (SYTImmune Science, Inc., College Park, MD, USA) and the Pelikine Compact™ soluble human IL-6 receptor ELISA kit (Research Diagnostics, Inc., Flanders, NJ, USA), respectively, according to the manufacturer’s protocol. In brief, the supernatants from MCF-7 cultures were added to wells which were pre-coated with murine monoclonal IL-6 or IL-6R antibodies. IL-6 or IL-6R specific rabbit anti-human polyclonal antibodies were then added to the 96-well plate and incubated for 3 hours at room temperature. After washing, the wells were covered with goat anti-rabbit polyclonal antibody conjugated to alkaline phosphatase and incubated for another 45 minutes at room temperature. After washing again, the color-generating solution was added to the plate and the optical density (OD) of the liquid in each well was measured. The standard curve demonstrated a linear relationship between the OD and the IL-6 and IL-6R concentrations. Using the standard curve, the concentrations of IL-6 and IL-6R in the media were determined. The amount of IL-6 and IL-6R per 1×10^6 MCF-7 cells was calculated. The experiments were repeated three times independently.

Reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR was employed to measure the mRNA levels of IL-6 and IL-6R in MCF-7 cells. Cellular RNA from 2×10^5 to 5×10^5 cells was isolated by the quaniudinium isothiocyanate method using a Glass Max RNA Micro Isolation Kit (Life Technologies). The RNA was quantitated by spectrophotometry at a wavelength of 260 nm (Beckman Co.). One microgram of denatured RNA was used for each reverse transcriptase (RT) reaction. The RT mixture included 50 mM Tris-HCl (pH 8.3), 40 mM KCl, 1 mM (DTT), 6 mM MgCl2, 0.5 mM dNTP, 0.1 mM Oligo(dT), 100 µg/ml (BSA) and 0.25 units AMV reverse transcriptase (Life Technologies). The reaction mixture was incubated at 37˚C for 60 min. PCR primers for target and control genes were synthesized by GeneLab, Louisiana State University, Baton Rouge, Louisiana. The primers used for amplification of the fragment of IL-6 cDNA (628 bp) were 5’-ATGACACTCTTCTCCA CAAG-3’ (upstream) and 5’-AGAGCCCTCCAGGT GAGACT-3’ (downstream); the primers for amplification of IL-6R (251 bp) were 5’-ATTGCCATGTGTC TGGAGT-3’ (upstream) and 5’-TAGTCTGT TATGGCTGTATCC-3’ (downstream) (22). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal standard and the primers used for GAPDH were 5’-TGAAGGTCCGAGT CAACGGATTGTG-3’ (upstream) and 5’-CATGTCGGCCCAT AGGCCACAC-3’ (downstream). PCR was performed in a total volume of 25 µl with the primers for IL-6, IL-6R and GAPDH. Each PCR cycle included: denaturation at 94˚C for 0.5 minutes, annealing at 65˚C for 0.5 min and extension at 72˚C for 1.5 min in an Ome-E DNA Thermal PCR instrument (National Labnet Co., Woodbridge, NJ, USA). After 35 cycles, there was a final elongation step at 72˚C for 10 min. The PCR products were run on a 2% agarose gel and stained with ethidium bromide, and the band densities were evaluated with Eagle Eye II Still Video System (Stratagene Cloning System, La Jolla, CA, USA). The experiments were repeated three times independently.

Statistics. Differences in proliferation rates, mRNA levels and protein secretion between cells were evaluated for statistical significance by Student’s t-test. Differences were considered statistically significant when p<0.05.
Results

Effects of exogenous IL-6 and IL-6R protein on MCF-7 cells. Previously, investigators have demonstrated in the T47D breast carcinoma cell line that IL-6 simultaneously inhibits cell proliferation and increases cell migration (23). However, we found that human IL-6 had no significant effect on the growth of MCF-7 cells, even when the concentration was increased to 1,000 ng/ml (Figure 1). A slight inhibition of growth of MCF-7 cells with 1,000 ng/ml of IL-6 was observed, but the difference was not statistically significant (p>0.05, paired t-test, compared to control). In agreement with the findings for IL-6, the sIL-6R also did not affect the growth of MCF-7 cells, even when the concentration was increased to 1,000 ng/ml (p>0.05, paired t-test, compared to control) (Figure 1).

Growth inhibition of MCF-7 cells caused by specific IL-6Ab. In the present study, MCF-7 cells were incubated with IL-6Ab or IL-6RAb for 72 hours. MCF-7 cells were also cultured in media containing the rabbit IgG and mouse IgG1, the isotype-matching antibody controls. Cell proliferation was determined by thymidine incorporation. High concentrations of rabbit IgG and mouse IgG1 control produced non-specific inhibitory effects on MCF-7 cells. We found that the IL-6Ab inhibited the growth of MCF-7 cells in a dose-dependent manner, and the addition of recombinant human IL-6 (500 ng/ml) abrogated the inhibitory effect. However, the IL-6RAb had no significant effect on the growth of MCF-7 cells. Additionally, the IL-6RAb showed non-specific inhibition, which was the same as that of the mouse IgG1 control (Figure 2).

Effects of antisense ODNs on target mRNA. Figure 3 shows PCR products of total RNA from MCF-7 cells treated with antisense and sense ODNs specific for IL-6 and IL-6R. cDNA was synthesized by the reverse transcription reaction from mRNA templates. IL-6 and IL-6R cDNA were amplified by PCR with specific primers. To ensure that reverse-transcription efficiencies were comparable between test groups, the level of GAPDH cDNA (a stable housekeeping gene) was measured using PCR for each reverse-transcription reaction. The 628 bp IL-6 band, 251 bp IL-6R band and 981 bp GAPDH band were seen on the agarose gels (Figures 3A and 3B). The mRNA levels of IL-6 and IL-6R were defined as the ratio of IL-6 or IL-6R to GAPDH, and were inhibited by antisense IL-6 and IL-6R ODNs, respectively, in a dose-dependent manner (Figures 3 and 4). Although sense IL-6 and IL-6R ODNs slightly reduced mRNA of IL-6 and IL-6R, respectively, the inhibitory effects of sense ODNs were significantly smaller than those of the corresponding antisense ODNs (both p<0.05, paired t-test, ODNs at 20 μM). The random sequence (RS) ODNs had neither inhibitory nor stimulatory effects on the expression of IL-6 and IL-6R (data not shown).

Inhibition of IL-6 and IL-6R protein secretion by ODNs. MCF-7 cells were treated for 72 h and the concentrations of IL-6 and sIL-6R in the culture media were measured by ELISA. Table I shows that IL-6 and IL-6R sense ODNs at a concentration of 20 μM did not significantly influence the production of IL-6 and siIL-6R, respectively (both p>0.05, paired t-test), but IL-6 and IL-6R anti-sense ODNs significantly suppressed the secretion of IL-6 and siIL-6R, respectively (p<0.05 and p<0.01, respectively, paired t-test).
The RS ODN had no effect on the production of IL-6 and IL-6R in MCF-7 cells (data not shown).

Effects of IL-6 and IL-6R anti-sense ODNs on the proliferation of MCF-7 cells. MCF-7 cells were incubated in media containing ODNs for 72 h and the proliferation was measured by $^{3}$H-thymidine incorporation. We found IL-6 and IL-6R anti-sense ODNs inhibited the growth of MCF-7 cells in a dose-dependent manner (Figure 5). The IC$_{50}$ of IL-6RAS ODN was significantly lower than that for the other ODNs. The IC$_{50}$s of IL-6S and IL-6RS ODNs were significantly higher than those of the corresponding anti-sense ODNs (Table II). In addition, we found recombinant human IL-6 was able to partially abrogate the cytotoxicity of IL-6AS but not IL-6RAS ODN (Figure 6).

**Discussion**

Depending on the target cells, IL-6 induces various and sometimes contrasting biological responses. IL-6 promotes cell proliferation of prostate carcinoma (24), colon carcinoma (25), leukemia (26), melanoma (27), and renal cell carcinoma (28), whereas it has been shown that it inhibits cell proliferation in human colon carcinoma (29), melanoma cell lines (30-31), and M1 leukemia cells (32). In human breast carcinoma, IL-6 has been shown to have contradictory effects on cell proliferation. Badache and Hynes reported that IL-6 inhibited the proliferation of T47D breast cancer cells through STAT3 activation (23). Danforth and Sgagias also showed that IL-1 alpha and IL-6 additively inhibited the growth of MCF-7 breast cancer cells (33). On the other hand, Conze et al.
reported that IL-6 induced multidrug resistance in MCF-7 cells and had no significant effect on cell proliferation (34). Honma’s group also found that IL-6 alone did not significantly effect cell proliferation of MCF-7 cells, but significantly increased the estrone sulfate-induced proliferation when IL-6 and estrone sulfate were simultaneously added to the culture media. They hypothesized that IL-6 regulated proliferation of breast cancer cells through estrogen production by steroid-catalyzing enzymes in the tissue (35). In agreement with Conze et al.’s and Honma et al.’s results, we did not find that the addition of human recombinant IL-6 and sIL-6R significantly stimulated or inhibited cell proliferation in MCF-7 breast cancer cells. The inhibition of proliferation by IL-6 in T47D, A375 melanoma and M1 leukemia cells was associated with STAT3 activation (23, 31-32), whereas the proliferation-promoting effect of IL-6 for some cell types was shown to involve SHP-2 and MAPK activation (32, 36). Thus, in some cells, the biological effect of IL-6 depends on the balance between a growth-inhibitory STAT3-dependent pathway and a growth-promoting MAPK/PI3K-dependent pathway (37-38). However, such a simplistic model for IL-6 action may not apply to all cell systems. For example, STAT3 itself can promote cell growth (39-40) and even behave as an oncogene when constitutively active (41). Thus, the molecular mechanism of IL-6 action on MCF-7 cells remains unclear.

We used IL-6 antibody to neutralize the IL-6 synthesized by MCF-7 cells and found that the antibody inhibited cell growth. This growth-inhibitory effect can be reversed by the addition of exogenous IL-6 to the culture media (Figure 2). These results suggested that IL-6 is required to maintain MCF-7 cell survival and growth. However, in the present study, the IL-6RAb had no inhibitory effect on MCF-7 cell growth. We increased the concentration of IL-6RAb in the culture medium, but high concentrations of IL-6RAb (>300 μg/ml) only led to non-specific inhibition of proliferation (Figure 2). It has previously been reported that monoclonal
antibodies directed to different regions of a protein may have different effects on cell proliferation (42). Therefore, multiple clones of monoclonal antibodies, directed to different epitopes of the IL-6R, need to be tested in MCF-7 human breast cancer cells to determine if the binding of IL-6 to the IL-6R can be disrupted.

To demonstrate the relationship between expression of IL-6 and IL-6R mRNA and MCF-7 cell growth, we used IL-6 and IL-6R anti-sense ODNs to target IL-6 and IL-6R genes in MCF-7 cells, respectively. mRNA levels were measured by RT-PCR and cell growth was evaluated by 3[H]-thymidine incorporation. RT-PCR showed that mRNA levels of IL-6 and the IL-6R in MCF-7 cells were reduced by IL-6 and IL-6R anti-sense ODNs, respectively, in a dose-dependent manner (Figure 4). The secretion of IL-6 and IL-6R protein was also suppressed by the corresponding anti-sense ODNs (Table I). 3[H]-Thymidine incorporation showed that the proliferation of MCF-7 cells was also inhibited by IL-6 and IL-6R anti-sense ODNs in a dose-dependent manner (Figures 5 and 6). The cytotoxicity of IL-6R anti-sense ODN was five times greater than that for IL-6 anti-sense ODN (Table II). Furthermore, addition of IL-6 to the culture media partially abrogated the inhibitory effect on MCF-7 cells of IL-6 antisense ODN but not IL-6R antisense ODNs in MCF-7 cells (72-hour incubation).

Breast cancer patients have often been found to have increased serum IL-6 levels (16, 43-46). This IL-6, produced by the tumor cells, can neutralize the inhibitory effect of IL-6 anti-sense ODN on breast cancer cells. However, IL-6R anti-sense ODN can inhibit breast cancer cells in the presence of exogenous IL-6, and additionally, IL-6R antisense ODN are more cytotoxic than are IL-6 anti-sense ODN. Therefore, IL-6R anti-sense ODN might have greater efficacy in cancer therapy than IL-6 anti-sense ODN.

Cancer therapy through suppression of IL-6 gene expression in cancer cells by curcumin has also been reported (47-50). Curcumin is a dithydroxyphenolic compound isolated from the rhizome of the plant Curcuma longa, and has anticancer, antiviral, antioxidant and anti-inflammatory properties. The underlying mechanisms of these effects are diverse and appear to involve the regulation of various molecular targets, including transcription factors (such as nuclear factor-κB), growth factors (such as vascular endothelial growth factor), inflammatory cytokines (such as tumor necrosis factor, IL-1 and IL-6) and protein kinases (such as MAPKs and AKT) (47). Saydmohammed et al. have reported that treatment of cancer cells with curcumin induces a dose- and time-dependent decrease in IL-6 expression and IL-6-induced phosphorylation of STAT3, which is associated with reduced cell viability and increased cleavage of caspase-3 (48).
et al. have also reported that treatment with curcumin dramatically inhibited the production of pro-inflammatory cytokines and vascular endothelial growth factor of human multiple myeloma U266 cells, and reduced IL-6/iIL-6R-induced STAT3 and ERK phosphorylation. In addition the combination of curcumin and bortezomib synergistically inhibited the growth of multiple myeloma (49).

In summary, the data in the present study demonstrates that IL-6 is a growth factor which maintains MCF-7 cell survival and growth. Neutralization of IL-6 with an IL-6-specific antibody or down-regulation of IL-6 mRNA and protein with IL-6 antisense ODN resulted in growth inhibition of MCF-7 cells. Exogenous IL-6 can abrogate the inhibitory effects of both antibody and antisense ODNs. IL-6R antisense ODN reduced the expression of IL-6R mRNA and protein of MCF-7 cells, and suppressed MCF-7 cell growth. The cytotoxicity of IL-6R antisense ODN was greater than that for IL-6 antisense ODN and was not affected by addition of exogenous IL-6. In conclusion, IL-6 plays an important role in the growth of MCF-7 breast cancer cells, and careful modulation of IL-6 and IL-6R expression of cells by using antibodies and antisense ODNs, especially IL-6R anti-sense ODN, is a potential approach for breast cancer therapy.

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References


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