Characterization of an Interleukin-6–Mediated Autocrine Growth Loop in the Human Multiple Myeloma Cell Line, U266

By Gisela Schwab, Clay B. Siegall, Lucien A. Aarden, Leonard M. Neckers, and Richard P. Nordan

It has been reported recently that freshly isolated human myeloma cell cultures proliferate in response to added interleukin-6 (IL-6). Endogenous levels of IL-6 found in the same cultures suggested that an autocrine growth loop may contribute to cell growth. However, the lack of homogenous cell populations in primary myeloma cultures has made it difficult to distinguish between paracrine and autocrine growth mechanisms. To precisely address the autocrine growth issue we have evaluated the growth of the human myeloma cell line, U266. We have found that a neutralizing anti–IL-6 monoclonal antibody can inhibit U266 proliferation. Furthermore, the addition of IL-6 antisense oligonucleotides also inhibits U266 proliferation. These effects are reversed by adding IL-6, suggesting the presence of an autocrine loop. Using bioassays with two different IL-6–dependent cell lines, we were able to detect IL-6 in concentrated U266 supernatants. IL-6 mRNA was detected by polymerase chain reaction amplification of cDNA. Cell cycle parameter analysis shows that IL-6 acts to release a block in G1. Taken together these results present conclusive evidence for IL-6–mediated autocrine growth in the U266 human myeloma cell line. This is a US government work. There are no restrictions on its use.

INTERLEUKIN-6 (IL-6), also known as interferon β2, B-cell stimulatory factor-2, or plasmacytoma/hybridoma growth factor, has multiple biologic activities. It has been shown to induce B-cell maturation, growth and differentiation of T cells, and has multi-colony-stimulating factor activity on hematopoietic progenitor cells. IL-6 has also been shown to induce the differentiation of myeloid cells and nerve cells, and is responsible for the production of acute-phase proteins in hepatocytes. In addition to its activity on normal cells, IL-6 is necessary to sustain the in vitro growth of many murine plasmacytomas and has been shown to enhance the proliferation of freshly explanted human myeloma cells. The hypothesis that IL-6 can also support myeloma cell growth in vivo is supported by the observation that myelomas arise in or are found in environments that contain cells which produce IL-6 in vitro.

Recently, Kawano et al found measurable concentrations of IL-6 in cultures of freshly explanted myeloma cells as well as cell surface expression of the p80 chain of the IL-6 receptor. They also showed that inhibition of the endogenously produced IL-6 with polyclonal antibodies could inhibit the proliferation of some freshly cultured myelomas, and suggested that an autocrine mechanism may account for the myeloma cell proliferation. However, in a similar study, Klein et al were unable to verify the autocrine hypothesis in human myeloma and assigned the in vitro production of IL-6 to contaminating adherent cells from the bone marrow rather than to the myeloma cells themselves. Thus, according to Klein et al, human multiple myeloma growth is regulated in a paracrine rather than an autocrine manner. Although paracrine growth remains a likely mechanism for many myelomas, in this report we present data on the human multiple myeloma cell line, U266, that clearly show the existence of IL-6–driven autocrine growth.

MATERIALS AND METHODS

Reagents. Moloney murine leukemia virus (M-MLV) reverse transcriptase was obtained from Bethesda Research Laboratories (Gaithersburg, MD). Taq DNA polymerase was purchased from Perkin Elmer-Cetus (Norwalk, CT). Recombinant human IL-6 (rIL-6) was obtained from R&D Systems (Minneapolis, MN). CLB.IL6/8 is a murine monoclonal antibody (MoAb) (IgG1, κ) directed against human IL-6. DMS 1, a murine MoAb (IgG1, κ) directed against human IL-2, was purchased from Genzyme (Boston, MA). Plasmid pIL-6 was purchased from British Biotechnologies Limited (Oxford, UK) and carries a synthetic gene for IL-6 inserted between the HindIII and EcoRI sites in the polylinker of pUC18.

Cell culture. U266 (referred to as U266), a human myeloma cell line, and T24, a human bladder carcinoma line, were obtained from the American Type Culture Collection (Rockville, MD). CHP100, a human neuroepithelioma line, was kindly provided by Dr Angelo Rosolen (National Cancer Institute, Bethesda, MD). B9, an IL-6–dependent B-cell hybridoma, has been described previously. 7TD1, another IL-6–dependent B-cell hybridoma cell line, was akind gift from J. Van Snick. All cells were maintained in complete medium that consisted of RPMI 1640, 10% heat-inactivated fetal calf serum (FCS), 50 mmol/L 2-mercaptoethanol, and 50 μg/mL gentamicin. In addition, the IL-6–dependent B9 and 7TD1 cells were maintained in medium supplemented with rIL-6.

IL-6 bioassays. Culture supernatants were evaluated for IL-6 activity using the B9 and 7TD1 hybridoma growth factor assays. Briefly, 2,000 B9 or 7TD1 cells in a final volume of 200 μL of complete medium (no IL-6) were cultured with serial dilutions of the test sample. After 85 hours the B9 assays were pulsed with 0.5 μCi 3H-thymidine per well for 4 hours, harvested over glass fiber filters, and counted in a liquid scintillation spectrophotometer. In the 7TD1 assays the wells were pulsed with 20 μL of 34-S-dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT) at a concentration of 5 mg/mL for 4 hours at 37°C, and then further processed as previously described. Briefly, the cells were pelleted in the wells and resuspended in 200 μL of dimethyl sulfoxide. The samples were then read on an enzyme-linked immunosorbent assay reader at 540-nm and 690-nm wavelengths. The absorbance values in this assay closely correlated with the growth of the 7TD1 cells. In these assays one hybridoma growth factor (HGF) unit represents 1,000 cpm.

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the half maximal growth response of the IL-6-dependent cells and is equivalent to approximately 1 pg/mL of native IL-6.

Cell cycle analysis. Test cells were harvested, washed in phosphate-buffered saline (PBS), and fixed in 50% cold ethanol for 1 hour on ice. After pelleting, the cells were resuspended in RNase A solution (580 U/mL PBS) and incubated for 30 minutes at 37°C. Equal volumes of 0.005% propidium iodide were added. After a 20-minute incubation at room temperature, cell cycle analysis was performed using a FACStar flow cytometer (B-D Immunocytometry Systems, Mountain View, CA) as previously described.19

Oligodeoxynucleotides for in vitro assays. Fifteen-base sequences from three different regions of the IL-6 cDNA2 were chosen for the production of antisense, sense, or random oligodeoxynucleotide (Fig 1). Random oligonucleotide sequences of the same base composition as the antisense oligomers were used as controls. Region I spanned the transcriptional start site. Region II was downstream and immediately adjacent to region I, and region III corresponded to an area in the second exon of the IL-6 gene that had previously been described as an effective antisense sequence in reducing growth and IL-6 production in Kaposi sarcoma cells.20 The oligonucleotides were synthesized by cyanoethyl phosphoramidite methodology using an Applied Biosystems Model 380 B DNA synthesizer (Foster City, CA). An aliquot of each sample was analyzed for homogeneity on a polyacrylamide denaturing gel. The oligomers were ethanol precipitated, washed with 70% ethanol, and subjected to three sequential cycles of sterile water resuspension and hybridization.

cDNA amplification using the polymerase chain reaction (PCR). Poly A selected RNA was prepared using the guanidine isothiocyanate/cesium chloride method,13,21 followed by poly-A-selection on an oligo-dT column. The first strand of cDNA was synthesized using random hexamers and M-MLV reverse transcriptase.24mer oligodeoxynucleotide primers which flanked 639 bases of the mature IL-6 message spanning five exons and four introns in the methodology. cDNA was amplified using Taq polymerasez3 on a Perkin Elmer-Cetus thermocycler for 30 cycles with cycles designed as follows: denaturation at 94°C for 1 minute, annealing at 42°C for 2 minutes, and polymerization at 72°C for 3 minutes with 10 seconds of extension time in each cycle. An aliquot of each sample was analyzed on a 1% agarose gel. Amplified DNA could be detected at the expected size by ethidium bromide staining of the gel. Subsequently, the samples were transferred to nitrocellulose and hybridized with a 32P-labeled IL-6 probe (HindIII-EcoRI insert of pIL-6) prepared by nick translation. The signal was detected by autoradiography.

RESULTS

Production of IL-6 by U266 cells. In searching for IL-6-mediated autocrine growth we initially asked if U266 myeloma cells produced biologically active IL-6. Conditioned medium was collected at various times from U266 cells growing at densities ranging from 0.2 to 1.5 × 10⁶ cells/mL and analyzed in the B9/HGF assay. IL-6 was not detected in any of the neat supernatants. However, after a 20-fold concentration, IL-6 could be detected at concentrations of 5 to 20 HGF U/mL in bioassays using either of the IL-6-dependent cell lines, B9 or 7TD1. In the neat supernatants this corresponds to approximately 0.5 to 2 HGF U/mL, which corresponds to 0.5 to 2 μg/mL of IL-6. Specificity of the IL-6-dependent support of B9 or 7TD1 cells was proven by inhibition of the supernatant-mediated growth with CLB.IL6/8, a neutralizing anti-IL-6 MoAb (Table 1).

Inhibition of U266 proliferation with anti–IL-6. To test whether the endogenously produced IL-6 was acting as an autocrine growth factor, the neutralizing MoAb CLB.IL6/8 was added directly to U266 cultures. As measured by 3H-thymidine incorporation, a 70% inhibition of U266 proliferation was obtained with 10 μg/mL CLB.IL6/8 (Fig 2). Partial inhibition of U266 proliferation was observed with antibody concentrations as low as 0.1 μg/mL. This effect was reversible by adding excess IL-6. The addition of CLB.IL6/8 also prevented increases in cell numbers but did not result in cell death, even after 5 days of incubation at 50 μg/mL (data not shown), suggesting that IL-6 is necessary for U266 proliferation but not for the maintenance of viability. In our hands 1 μg/mL of CLB.IL6/8 completely neutralized 20 HGF U of IL-6 in the B9 assay. The monoclonal murine antibody DMS1 that was used as a control did not affect 3H-thymidine uptake of U266 cells.

We also evaluated the effect of CLB.IL6/8 inhibition on U266 cell cycle parameters. As shown in Fig 3, anti–IL-6–treated U266 cells displayed a 50% reduction of cells in the S phase of the cell cycle (P < .001). This was accompanied by an accumulation of cells in G₁ (P < .01), indicating that U266 cells encounter a G₁ block in the absence of IL-6.

Detection of IL-6 message in U266 cells. We attempted to evaluate the level of IL-6 mRNA using Northern blot and RNase protection assays. Although others have reported the presence of IL-6 message in U266 cells,15,24 we were unable to detect IL-6 message in poly A-selected U266 RNA (data not shown) using these methods. PCR amplification of cDNA, a more sensitive assay for the detection of specific mRNAs, was performed. First-strand
Table 1. Endogenous Production of IL-6 by U266

<table>
<thead>
<tr>
<th>Sample</th>
<th>RPMI 1640 + 10% FCS</th>
<th>U266 Medium</th>
<th>RPMI 1640 + 10% FCS</th>
</tr>
</thead>
<tbody>
<tr>
<td>B9 assay Expt 1</td>
<td>2</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>B9 assay Expt 2</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7TD1 assay Expt 1</td>
<td>0.5</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>7TD1 assay Expt 2</td>
<td>0.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7TD1 assay Expt 3</td>
<td>0.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7TD1 assay Expt 4</td>
<td>0.25</td>
<td>0</td>
<td>ND</td>
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</tbody>
</table>

Values are HGF units per milliliter. Production of IL-6 by U266 cells. B9 or 7TD1 cells were cultured in triplicate with serial dilutions of 20-fold concentrated U266 conditioned medium in presence or absence of 10 μg/mL of the monoclonal anti-IL-6 antibody CLB.IL6/8. Twentyfold concentrated RPMI 1640 with 10% FCS served as control. Results were corrected to reflect the IL-6 activity present in the neat supernatant.

Abbreviations: Expt, experiment; ND, not done.

cDNAs were synthesized from 1 μg of poly A-selected RNA using reverse transcriptase. Specific primers corresponding to the 5' and 3' ends of the mature IL-6 message were used in the PCR reaction to amplify the IL-6 cDNA. This reaction produced the 639-bp fragment predicted for the IL-6 cDNA using these primers (Fig 4). The same fragment was generated using T24 bladder carcinoma cells, which have been shown to express IL-6 message, whereas no detectable fragment was generated using CHP100, a neuroepithelioma.

Effect of treatment with IL-6 antisense oligodeoxynucleotides. To establish whether IL-6 antisense oligodeoxynucleotides can be used to inhibit the growth of autocrine myeloma cells, we cultured U266 with an antisense IL-6 oligomer that spanned the transcriptional start site of the IL-6 message (Fig 1, region I). After 48 hours the growth of U266 cells was assayed for 2 days with CLB.IL6/8 (monoclonal anti-IL-6) and then analyzed for DNA content using a flow cytometer. Data represent the mean and standard error of three experiments. (■), Control; (□), anti-IL-6, 1 μg; (△), anti-IL-6, 10 μg.

Fig 3. Effect of anti–IL-6 on U266 cell cycle parameters. U266 cells were cultured for 2 days with CLB.IL6/8 (monoclonal anti-IL-6) and then analyzed for DNA content using a flow cytometer. Data represent the mean and standard error of three experiments. (■), Control; (□), anti-IL-6, 1 μg; (△), anti-IL-6, 10 μg.

Fig 2. Inhibition of U266 proliferation with anti–IL-6 MoAb. U266 cells were cultured at 5 × 10⁴/mL in triplicate with 10,000 HGF U of IL-6 or the indicated concentrations of CLB.IL6/8, a neutralizing monoclonal anti-IL-6 antibody. On days 1 through 5 the cultures were pulsed with [H]thymidine and harvested. Data represent one of four similar experiments. Monoclonal anti–IL-2 antibody of the same isotype (DMS1) served as a control. (■), Control; (□), 10 μg/mL anti–IL-6; (△), 10 μg/mL anti–IL-2; (○), 10 μg/mL anti–IL-6 + IL-6.

Fig 4. Detection of IL-6 mRNA. First-strand cDNA generated from poly A selected RNA was amplified by PCR using 24mer primers corresponding to amino acids 1 through 8 and 206 through 213 of the mature IL-6 protein. PCR products were electrophoresed on a 1% agarose gel, transferred to nitrocellulose, and hybridized with a p32-labeled IL-6 probe. Lane 1, control cDNA; lane 2, CHP100; lane 3, U266; lane 4, T24.
antisense-treated cells was diminished by 40% when compared with sense-treated or control cells ($P < .01$) (Fig 5). Analysis of cell cycle parameters showed that treatment with antisense oligodeoxynucleotides also resulted in a 40% reduction of cells in S phase ($P < .05$), whereas sense treatment had no effect ($P = .63$) (Fig 5). The antisense effect on cell growth and cell cycle parameters could be reversed by adding IL-6 to the cultures. Similar results were obtained in experiments in which the antisense oligomer was added every day for 6 days (data not shown). As with the addition of anti–IL-6, no loss of cell viability was observed. In titration studies of IL-6 antisense oligodeoxynucleotides we saw growth inhibition at concentrations as low as 10 $\mu$mol/L oligomer (data not shown). A 15mer of random configuration, but the same base composition as the antisense oligomer to the transcriptional start region, also had no effect on cell growth or distribution of the cells in the cell cycle. We further evaluated the effect of antisense oligonucleotides directed to other regions of the IL-6 cDNA (Fig 6). An antisense 15mer directed against a region of the second exon of the IL-6 gene (Fig 1, region III) exhibited similar effects on the distribution of cells in S phase and G$_1$ phase to those obtained with antisense oligonucleotide against the transcriptional start site of the IL-6 gene (region I). After treatment with this oligomer, U266 cells in S phase decreased by 45% ($P < .001$) and cells in G$_1$ increased by 45% as compared with untreated U266 cells ($P < .001$). The corresponding random base oligomer did not affect either parameter significantly. An antisense oligonucleotide directed to the region immediately adjacent to and downstream from the transcriptional start site oligomer (Fig 1, region II) did not exert any effect on cell growth or cell cycle distribution of U266 cells.

**DISCUSSION**

The ability of IL-6 to serve as an in vitro growth factor for myelomas has been well documented. Both murine and human IL-6–dependent myeloma cell lines have been established in vitro, and studies with freshly explanted human myeloma cells have shown that IL-6 enhances, in a paracrine manner, the in vitro proliferation of some tumors. The issue of autocrine growth in myeloma is less clear. Using freshly explanted myeloma cells, Kawano et al detected exogenous IL-6 production and mRNA expression and could inhibit the proliferation of some myelomas with anti–IL-6 antibodies. In a similar study Klein et al were unable to confirm the autocrine hypothesis and attributed the endogenous in vitro IL-6 expression to the presence of contaminating cells. The concept of an autocrine mechanism in tumor development is supported by the studies of Freeman et al, who found IL-6 message expressed in some B-cell lymphomas, but because of the presence of contaminating cells were unable to determine if freshly isolated myeloma cells expressed IL-6 message. In our studies we avoid the issue of contaminating cells by analyzing the human myeloma cell line, U266. In this report we demonstrate the existence of an IL-6–mediated autocrine growth loop in U266. Evidence of an autocrine growth loop is provided by the specific inhibition of U266 growth by the monoclonal anti–IL-6 antibody, CLB.IL6/8. When U266 is inhibited with anti–IL-6 the cells accumulate in G$_1$, and the proportion of cells in S phase is reduced by 40% to 50%, indicating that these cells encounter a G$_1$ block in the absence of IL-6. This observation agrees with experiments in which IL-6 has also been shown to relieve a G$_1$ block in IL-6–dependent murine plasmacytomas and B-cell hybridomas. It is interesting that unlike the IL-6–dependent murine plasmacytomas and hybridomas, which die in the absence of IL-6, the inhibition of U266 does not lead to cell death even with a 100-fold excess of antibody. This suggests either that IL-6 is not required to maintain the viability of U266 or that IL-6 can interact with its receptor in regions that cannot be reached by the antibody, perhaps internally. In either case, if the inhibition of IL-6 does not result in the death of autocrine myeloma cells, the potential therapeutic effectiveness of such reagents against in vivo autocrine tumors will be reduced.

The production of IL-6 by U266 was documented using two different highly sensitive IL-6–dependent hybridoma cell lines, and this response was specifically inhibited with the monoclonal anti–IL-6 antibody. The accumulated IL-6 reached levels of about 0.5 pg/mL to 2 pg/mL, and could be detected only after concentration of the U266 conditioned medium. These exceedingly low levels of IL-6 would be unable to support most IL-6 responses and raise questions about the level of sensitivity of the U266 myeloma to IL-6. Most cells that exhibit a response to IL-6, eg, IL-6–dependent murine plasmacytomas, require levels of native IL-6 that are 100-fold higher than levels required by B-cell hybridomas, such as the B9 and 7TD1 cell lines used in this study (half maximum $= 100 \mu$g/L). The low levels of IL-6 found in U266 medium might imply that, like B9 and 7TD1, the stimulation of U266 growth occurs at very low concentrations (1 pg/mL) of IL-6. Alternatively, U266 may be similar in sensitivity to IL-6–dependent murine plasmacytomas ($> 100 \mu$g/L) and may produce a concentration gradient of secreted IL-6 in the immediate vicinity that is high enough to meet this requirement. It can also be argued...
that U266 rapidly internalizes the secreted IL-6, thus depleting it from the medium and complicating the detection of this molecule. Our results do not allow us to distinguish between these possibilities.

Antisense oligodeoxynucleotides to proto-oncogenes and growth factors have previously been used to inhibit cell growth. In our studies a specific inhibition of U266 cell growth is seen after treatment with an antisense oligomer to the transcriptional start site of the IL-6 cDNA, whereas the sense and random sequence oligomers do not affect growth. Cell cycle analysis of antisense-treated U266 cells yields essentially identical results to treatment with anti-IL-6 antibody, ie, reduction of cells in S phase and accumulation of cells in G1. Recently, an antisense IL-6 15mer against a region in the second exon of the IL-6 gene has been shown to inhibit IL-6 production and growth of Kaposi sarcoma cells. Using this same antisense oligonucleotide on U266 also yielded the same growth inhibition and cell cycle effects obtained with the transcriptional start site sequence. The inhibition of U266 with two distinct IL-6 antisense sequences and their reversal with exogenous IL-6 confirms the specificity of the antisense effect on the IL-6 message. The failure of a third sequence to inhibit is not surprising because the most effective region for antisense inhibition spans the transcriptional start site, whereas inhibition in other regions of the mRNA is unpredictable. IL-6 antisense oligomers presumably exert their effect by annealing with the IL-6 mRNA in the region of complementarity, thus producing a DNA/RNA hybrid. At least two potential mechanisms exist that may mediate the inhibitory effect. In the first, the hybrid becomes a substrate for RNase H and is degraded, allowing the oligomer to hybridize to another mRNA molecule. In the second, if the antisense oligomer is complementary to the initiation codon, the attachment of the ribosome is inhibited and translation is arrested.

Other investigators have conducted studies that address various aspects of IL-6 and U266 growth; however, none have specifically demonstrated an IL-6-mediated autocrine loop. Taga et al initially described the expression of IL-6 receptors on U266 cells, and we have repeated this observation for the subline used in this study. In addition, the expression of IL-6 message in U266 has been reported by Kawano et al; however, Klein et al did not detect IL-6 message in their studies of U266. Likewise, Kawano et al detected a low level of IL-6 activity produced by U266, whereas Klein et al found no IL-6 produced by U266. As suggested by Klein et al, these discrepancies are possibly due to the use of different U266 sublines.

The presence of IL-6 mRNA is a condition sine qua non of IL-6 antisense oligomer action. In our hands Northern blot analysis was not sensitive enough to detect IL-6 mRNA in U266 cells. We find that IL-6 mRNA can be detected in U266 cells, but only after amplification of the reverse transcribed mRNA by PCR. The need to use the PCR technique indicates that the level of IL-6 message is exceedingly low and correlates with the low levels of IL-6 detected in the conditioned medium. Thus, our results indicate that vanishingly low amounts of IL-6 are sufficient for allowing autocrine growth of this U266 subline. Furthermore, these studies show that the failure to detect message in Northern blots should not be taken as evidence for the absence of an autocrine mechanism. Taking all results together, we conclude that in the U266 cell line there is clear evidence of an IL-6-mediated autocrine growth loop in which IL-6 acts to relieve a G1 block in the cell cycle. Monoclonal anti-IL-6 antibody or antisense IL-6 oligodeoxynucleotides interrupt this loop at different stages and may be useful for further understanding the role of this cytokine in human myeloma. Interestingly, in the sera of patients with advanced disease of multiple myeloma increased levels of IL-6 have been described, whereas in early stages IL-6 is usually not detected.
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