Interleukin-10 Is a Growth Factor for Human Myeloma Cells by Induction of an Oncostatin M Autocrine Loop

By Zong-Jiang Gu, Valérie Costes, Zhao Yang Lu, Xue-Guang Zhang, Vincent Pitard, Jean-François Moreau, Régis Bataille, John Wijdenes, Jean-François Rossi, and Bernard Klein

We have a previously reported that interleukin-10 (IL-10) is a potent but IL-6-unrelated growth factor for freshly explanted myeloma cells (Lu et al, Blood 85:2521, 1995). We have also shown that exogenous IL-10 supported the growth of XG-1 and XG-2 human myeloma cell lines (HMCL) through an IL-6-independent mechanism. (Lu et al, Blood 85:2521, 1995). Because the IL-10 receptor does not involve the gp130 IL-6 transducer, we have attempted to elucidate the mechanisms of IL-10 action on myeloma cells. Our results indicate that the myeloma cell growth factor activity of IL-10 was abrogated by an antibody to the gp130 IL-6 transducer, indicating that it was mediated through one of the gp130-activating cytokines. We found that myeloma cells from XG-1 and XG-2 HMCL and from 5 of 6 patients’ tumor samples produced oncostatin M (OM) constitutively but failed to produce IL-6, IL-11 and leukemia-inhibitory factor (LIF). The autocrine OM was inactive in the absence of IL-10 due to lack of a functional OM receptor on myeloma cells. IL-10, by inducing the receptor for LIF (LIFR), produced a functional autocrine OM loop in XG-1 and XG-2 cells and in primary myeloma cells from 2 patients. We also found that some myeloma cell lines (XG-4, XG-6, and XG-7) were fresh myeloma cells from 3 of 6 patients produced an autocrine IL-10 and that these cells constitutively expressed LIFR. One HMCL (XG-7) produced IL-10, OM, and IL-6 as an expressed LIFR. The XG-7 cells used OM and IL-6 as autocrine growth factors. We have previously shown that IL-10 could induce IL-11 receptor in myeloma cells and confer on them sensitivity to IL-11 (Lu et al, FEBS Lett 377:515, 1996). Taken together, these results show that IL-10 is a key cytokine for inducing the expression of LIFR and IL-11R and possibly another uncharacterized OM coreceptor on myeloma cells and that OM and IL-10 might be produced by myeloma cells. They also emphasize that all myeloma cell growth factors reported to date involve an activation of the gp130 IL-6 transducer.

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Fig 1. Proliferative response of XG cells to various gp130 transducer-activating cytokines. Ten thousand XG myeloma cells were cultured for 5 days in 150 μL RPMI 1640 cell culture medium and 10% FCS together with various gp130 transducer-activating cytokines (1 ng/mL IL-6, 10 ng/mL IL-11, 10 ng/mL LIF and 10 ng/mL OM) and 10 μg/mL of the neutralizing B-R3 anti-gp130 MoAb or a control murine IgG1. Results are the means ± SD of tritiated thymidine incorporation determined in six separate culture wells.

Purification and cultures of freshly explanted myeloma cells. Mononuclear cells from tumoral samples (MC) were separated on Ficoll Hypaque. Myeloma cells were purified by using the antimyeloma cell B-B4 monoclonal antibody (MoAb). We have shown that the B-B4 MoAb recognizes syndecan-1 and that syndecan-1 is present on myeloma cells only in tumoral samples. In addition, this antigen is cleaved by chymopapain without affecting cell viability using a procedure previously described for CD34 purification (B. Klein, unpublished results). To purify myeloma cells, MC from tumoral samples were suspended in Hanks‘ buffer containing 1% of human serum albumin (Hanks/HSA) and 0.5% human Ig at a concentration of 2 X 10⁷ cells/mL and incubated for 30 minutes at 4°C. The B-B4 MoAb was then added (1 μg/10⁶ cells) for 30 minutes at 4°C under gentle agitation. Unbound antibodies were removed by washing (2 times at 400g for 10 minutes in cold), and cells were resuspended in Hanks/HSA at a concentration of 2 X 10⁷ cells/mL. Magnetic beads coated with sheep antimouse IgG (Dynal M450; Dynal, Oslo, Norway) were added at a ratio of 4 beads to 1 target cell and incubated for 30 minutes at 4°C under gentle agitation. The beads and rosetted cells were then captured with a magnet (MPC1; Dynal) and washed three times with the initial volume of Hanks/HSA to remove nonrosetted cells. Rossetted cells were resuspended in Hanks/HSA at an expected concentration of 10⁶ cells/mL. Cells were released from the beads using chymopapain (Boots Pharmaceuticals, Nottingham, UK) at 200 picokatal/mL for 20 minutes at room temperature under gentle agitation. The beads were captured with the MPC1 magnet and the procedure was repeated twice to ensure maximal bead capture. Myeloma cells were then resuspended in RPMI 1640 medium and 5% of fetal calf serum (FCS).

Purified myeloma cells were cultured at 10⁶ cells/mL in RPMI 1640 supplemented with 2 mmol/L of L-glutamine and 5% of FCS. In some culture groups, recombinant IL-10 (rIL-10; 50 ng/mL) with or without an anti-gp130 IL-6 transducer MoAb (B-R3, 10 μg/mL) or an anti-IL-6 MoAb (B-E8 MoAb, 10 μg/mL) or an anti-OM neutralizing antibody (AB-295-NA, 10 μg/mL) were added at the beginning of the culture. Control cultures were made with 10 μg/mL of a mouse-purified IgG1 MoAb and 10 μg/mL of purified goat IgG recognizing no human antigens. After 5 days of culturing, cells were counted and harvested. The percentage of myeloma cells in S-phase (LI) was evaluated using a double immunofluorescence procedure.* Plasma cells were first labeled using anti-κ or anti-λ Ig light-chain antibodies and then with an anti-S-phase MoAb. At least 5,000 myeloma cells were examined to determine these percentages. This ensured that the lowest LI could be determined with a 30% precision and a P value < .05.

Cell lines. XG-1, XG-2, XG-4, XG-6, and XG-7 cells used in this study possessed cytoplasmic Ig, expressed plasma-cell antigens (Ag; CD38 and B-B4), and lacked the usual B-cell Ag (CD19 and CD20). They were not infected with Epstein-Barr virus. Their detailed characteristics have been reported elsewhere. The U937, 5637, MG 63, and SAOS2 cell lines (purchased from the American Type Culture Collection, Rockville, MD) were routinely grown in culture medium (RPMI 1640 medium and 5% FCS). XG cells were grown with 1 ng/mL of IL-6. All cell lines were free of mycoplasma, as assayed by an enzyme-linked immunosorbent assay (ELISA) kit (Boehringer Mannheim, Mannheim, Germany).
Cell proliferation assay. To investigate the effects of cytokines and antibodies to cytokines on the proliferation of human myeloma cell lines, XG cells were washed once with culture medium, incubated for 5 hours at 37°C in culture medium alone, and washed again twice to remove recombinant bound IL-6. The cells were then cultured in 96-well flat-bottomed microplates for 5 days with either culture medium alone or various cytokines or antibodies to cytokines or gp130 transducer. Tritiated thymidine (2.5 μCi, 25 Ci/mmol; CEA, Saclay, France) was added for the last 8 hours of culture, and tritiated-thymidine incorporation was determined as reported elsewhere.22

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Table 1. Cytokine Production by Human Myeloma Cell Lines

<table>
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<tr>
<th>Cytokine Concentration (pg/mL)</th>
<th>XG-1</th>
<th>XG-2</th>
<th>XG-4</th>
<th>XG-6</th>
<th>XG-1 + IL-10</th>
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<td>0 pg/mL</td>
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<td>18</td>
<td>&lt;5</td>
<td>&lt;15</td>
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Myeloma cells in exponential growth phase were harvested, washed once, incubated at 37°C for 5 hours in culture medium, and washed again twice to remove recombinant bound IL-6. Cells were then cultured for 2 days at a concentration of 5 × 10⁵ cells/mL in RPMI 1640 culture medium supplemented with 5% FCS. Six culture wells were prepared for each culture group. Culture supernatants were harvested and stored at -20°C until needed. Cytokine concentrations were assayed as indicated in the Materials and Methods. Results are the mean cytokine concentrations determined in six culture replicates.
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200 ng/mL of recombinant OM and 10 ng/mL of control murine MoAb (IL-10 + anti-OM + OM). For each culture group, XG cells were diluted every 4 days at a cell concentration of 5 \times 10^6 cells/mL in fresh culture medium containing the initial cytokine and antibody concentrations. Results are the means ± SD of the cumulative numbers of cells generated in sextuplicate culture wells.

To investigate the effects of a neutralizing antibody to IL-10 on their cytokine responsiveness, XG-4 and XG-6 cells were cultured for 15 days with IL-6 (1 ng/mL) and 10 μg/mL of purified goat antibodies to human OM and 200 ng/mL of control murine MoAb (IL-10 + anti-OM + OM). Ten micrograms of this antibody neutralized 5 ng of IL-10. Control cultures were performed with IL-6 (1 ng/mL) and 10 μg/mL of purified goat IgG recognizing no human antigens (control). After 15 days of culture, cells were washed and cultured for 5 days with various concentrations of the different gp130 transducer-activating cytokines with 10 μg/mL of anti-IL-10 antibodies or control goat IgG. Proliferation was assayed as described above.

Phosphorothionate-modified antisense and sense oligonucleotides corresponding to nucleotides +31 to +50 of IL-10 coding region were also used to inhibit IL-10 production, as previously described. XG-4 and XG-6 cells (10^5 cells/culture well) were cultured with 20 μL/mL of antisense or sense oligonucleotides to IL-10 in the presence of graded concentrations of OM in RPMI640 culture medium and 5% heat-inactivated FCS. After 5 days of culture, cell proliferation was assayed as above. The IL-10 concentration in the culture supernatants was assayed with an ELISA.

Expression of cytokine and cytokine receptor genes by reverse transcription-polymerase chain reaction (RT-PCR). To study the expression of cytokine or cytokine receptor genes, XG cells were cultured in culture medium supplemented with 1 ng/mL of IL-6 and harvested in exponential growth phase (5 \times 10^6 cells/mL). Twenty million XG cells were washed and immediately lysed with thiocyanate guanidium buffer. 25 To study the effect of addition of IL-10 on XG cells, 10 ng/mL of IL-10 was added to cultures of XG cells growing with 1 ng/mL of IL-6, without washing the cells. After 24 hours of culture at 37°C, cells were spun down and immediately lysed with thiocyanate guanidium buffer. SAOS2 and 5637 cells were grown in culture medium and then harvested in exponential growth phase. U937 cells were stimulated for 1 day with 10 ng/mL of phorbol myristate acetate (PMA; Sigma Chemicals, St Louis, MO) and 1 μg/mL of lipopolysacharide (LPS; Sigma Chemicals) before harvesting. Peripheral blood mononuclear cells (PBMC) were isolated from citrated peripheral blood by centrifugation in Ficoll-hypaque solution and cultured for 48 hours at a concentration of 10^5 cells/mL in culture medium supplemented with 1 μg/mL of phytohemagglutinin P (PHA-P; Sigma Chemicals). As for XG cells, 20 million harvested cells were immediately lysed with thiocyanate guanidium buffer.

Total RNA was isolated by the guanidium-ScCl method. cDNA was synthesized from 1 μg of total RNA using a reverse transcription kit (Promega, Madison, WI). Thirty-five PCR cycles (60 seconds at 94°C, 45 seconds at 60°C, and 60 seconds at 72°C), followed by 3 minutes of extension at 72°C, were performed using primers designed to amplify the cDNA of interest. We used primers corresponding to nucleotides 345-364 and 621-640 of IL-10 cDNA, 1160 and 1423-1442 of IL-10 receptor cDNA, 1-22 and 607-628 of IL-6 cDNA, 808-827 and 1346-1365 of LIF cDNA, 6-37 and 269-300 of IL-11 cDNA, 83-102 and 674-693 of OM cDNA, 464-483 and 1297-1316 of IFN (gp190), and 5-24 and 342-361 of β2 microglobulin cDNA. Fifteen microliters of PCR product was run on a 1% agarose gel, and the bands were visualized by ethidium bromide staining. In cases of positivity, the specificity of the vizua-

Fig 3. Long-term growth of myeloma cells with IL-10 and various antibodies to cytokines. XG-1 or XG-2 cells were cultured at a cell concentration of 5 \times 10^6 cells/mL without exogenous cytokine and 10 μg/mL of control murine MoAb and 10 μg/mL of control purified goat antibodies (no cytokine); or 10 μg/mL of B-R3 anti-gp130 murine MoAb and 10 μg/mL of control purified goat antibodies (no cytokine + anti-gp130); or 10 μg/mL of B-E8 anti-IL-6 murine MoAb and 10 μg/mL of control purified goat antibodies (no cytokine + anti-IL-6); or 10 μg/mL of purified goat antibodies to human OM (AB-295-NA) and 10 μg/mL of control murine MoAb (no cytokine + anti-OM); or 10 ng/mL of IL-10 plus 10 μg/mL of control murine MoAb and 10 μg/mL of control purified goat antibodies (IL-10); or 10 ng/mL of IL-10 plus 10 μg/mL of B-R3 anti-gp130 murine MoAb and 10 μg/mL of control purified goat antibodies (IL-10 + anti-gp130); or 10 ng/mL of IL-10 plus 10 μg/mL of purified goat antibodies to human OM (IL-10 + anti-OM); or 10 ng/mL of IL-10 plus 10 μg/mL of control purified goat antibodies to human OM and 10 μg/mL of control murine MoAb (IL-10 + anti-OM + OM). For each culture group, XG cells were diluted every 4 days at a cell concentration of 5 \times 10^6 cells/mL in fresh culture medium containing the initial cytokine and antibody concentrations. Results are the means ± SD of the cumulative numbers of cells generated in sextuplicate culture groups.

anti-OM); or 10 ng/mL of IL-10 plus 10 μg/mL of purified goat antibodies to human OM and 200 ng/mL of recombinant OM and 10 μg/mL of control murine MoAb (IL-10 + anti-OM + OM). For each culture group, XG cells were diluted every 4 days at a cell concentration of 5 \times 10^6 cells/mL in fresh culture medium containing the initial cytokine and antibody concentrations. Results are the means ± SD of the cumulative numbers of cells generated in sextuplicate culture groups.
lized bands was checked by nested PCR using primers 641-660 and 1024-1043 for LIFR cDNA.

**Determination of cytokine receptors and cytoplasmic cytokines by fluorescence-activated cell sorting (FACS) analysis.** The expression of cytokine receptors on the XG lines was studied using murine MoAb to LIFR, IL-6R, and gp130 IL-6 transducer and F(ab), fragments of goat antimouse Ig antibodies coupled with fluorescein isothiocyanate (FITC; Immunotech, Marseilles, France). Fluorescence analysis was performed with a FACScan apparatus (Becton Dickinson, San Jose, CA). To detect intracellular cytokines by flow cytometry, MC from cell lines or tumoral samples were fixed for 15 minutes at 4°C in freshly prepared paraformaldehyde (2% in phosphate-buffered saline [PBS]). To detect surface cytokine receptors, MC from tumoral samples were first labeled with anticytokine receptor MoAb or control antibodies before being fixed with paraformaldehyde. After two washes with PBS, cells were permeabilized with 70% ethanol for 10 minutes at room temperature. After two washes with PBS and 1% bovine serum albumin (PBS-BSA1%), samples were incubated in PBS-BSA1% and 10% goat serum (PBS-BSA1%-GS10%) for 30 minutes at room temperature. The mouse anticytokine MoAb (2 μg/10⁶ cells) was added in a total volume of 20 μL per tube with 2 μg of biotinylated goat antimouse Ig light chain antibody and incubated for 30 minutes at 4°C. A mixture of IgG1, IgG2a, and IgG2b murine Igs recognizing no human antigens was used as the negative control. Washing steps with PBS-BSA1%-GS10% were repeated twice. Cells were resuspended in FITC-conjugated F(ab), fragments of goat antimouse Ig (1:50 in 20 μL per tube) and streptavidin-PerCP (2 μg/10⁶ cells) and incubated for 30 minutes at 4°C. After two additional washings, the cells were resuspended in PBS and the fluorescence was analyzed with a FACSScan flow cytometer. Using FSC and SSC parameters, a region was first determined that contained mostly myeloma cells labeled with biotinylated anti-κ or anti-λ light chain antibodies and PerCP streptavidin. Within this region, the percentage of myeloma cells (FL-3) labeled by the anticytokine or anticytokine receptor MoAb (FL-1) was then determined. 10B2 anti-LIFR, M91 anti-IL-6R, B-T2 anti-gp130, B-S10 anti-IL-10, B-E8 anti-IL-6, 11R2 anti-IL-11, 7D2 anti-LIF, and MAB295 anti-OM MoAb were used in these experiments.

**Measurement of cytokines in culture supernatants of myeloma cells.** Myeloma cells in exponential growth phase when cultured with IL-6 were harvested, washed once, incubated at 37°C for 5 hours in culture medium, and washed again twice to remove bound recombinant IL-6. Cells were then cultured for 2 days at a concentration of 5 × 10⁵ cells/mL in culture medium. Six culture wells were prepared for each culture group. Culture supernatants were harvested and stored at −20°C until needed. OM was assayed using an ELISA
purchased from R&D Systems (Minneapolis, MN), IL-10 using an ELISA provided by Diacalone (Besançon, France), LIF using an ELISA provided by Dr. A. Godard (INSERM U211, Nantes, France), and IL-6 using an ELISA provided by Immunotech. IL-1 activity was assayed with a bioassay developed in our laboratory. The sensitivity of these different assays was 1 pg/mL for OM, 1 pg/mL for IL-10, 15 pg/mL for LIF, 1 pg/mL for IL-6, and 5 pg/mL for IL-11. The results are the mean cytokine concentrations determined on six culture replicates.

Reagents. Recombinant IL-6 was provided by Dr Stinchcomb (Synergen, Boulder, CO), OM by Dr Shoyab (Bristol Squibb Myers, Seattle, WA), IL-11 and the 1R2 murine MoAb to IL-11 by Dr Turner (Genetics Institute, Boston, MA), and the B-E8 neutralizing murine MoAb to IL-6 and the B-B4 antimyeloma cell MoAb by Dr Wijdjes (Diacalone). The B-R3 neutralizing and the B-T2 murine MoAb to gp130 transducer were cooperatively obtained by the Diacalone Company and our laboratory. The 10B2 anti-LIFR MoAb and 7D2 anti-LIF MoAb were provided by Drs V. Pitard and J.F. Moreau (CNRS, Bordeaux, France). IL-10; LIF; OM; the AB-217-NA and AB-295-NA purified goat antibodies to IL-10 and OM, respectively; and the MAB-295 murine MoAb to OM were purchased from R&D Systems. PHA, PMA, LPS, BSA, and control goat-puri-
fied IgG were purchased from Sigma Chemicals. Control murine IgG and FITC-labeled F(ab)2 fragments of goat antimouse Ig were purchased from Immunotech. The biotinylated antihuman Ig light chain antibody was from Caltag (San Francisco, CA). Streptavidin conjugated with PerCP was from Beckton Dickinson. Magnetic beads coated with affinity purified sheep antimouse IgG were purchased from Dynal. The phosphotinylated-modified antisense and sense olo-
gonucleotides to IL-10 were purchased from Eurogentec (Seraing, Belgium).

Statistical analysis. Mean values of tritiated thymidine incorporations or cell numbers were compared using a t-test for small samples. The percentages of myeloma cells in the S phase (LI) was determined by examining at least 5,000 cells on four different slides. This ensures that these percentages were determined with a precision of ±2 (LI ± L1/5,000) and a risk of P < .05, because, for each LI considered (LI < .01), 5,000 X LI was always greater than 5.

RESULTS

Production of IL-10 and expression of cytokine receptors discriminated between myeloma cell lines sensitive to IL-6 only and those sensitive to the different gp130 transducer-activating cytokines. As previously shown and illustrated in Fig 1, the proliferation and growth of XG-4 and XG-6 HMCL were supported by various gp130-activating cytokines, unlike the XG-1 and XG-2 HMCL that were only sensitive to IL-6. The proliferative activity of the gp130 transducer-activating cytokines was completely inhibited by a neutralizing antibody to gp130 (Fig 1). In agreement with their sensitivity to IL-11, LIF, and OM, we have previously published that XG-4 and XG-6 cells expressed the IL-11R and LIFR mRNA contrary to XG-1 and XG-2 cells. Expression of LIFR on XG-4 and XG-6 cells, unlike XG-1 and XG-2 cells, was further shown by an MoAb to LIFR and FACS analysis (Fig 2). Another difference between XG-1 and XG-2 HMCL on the one hand and XG-4 and XG-6 HMCL on the other is that the last 2 HMCL expressed IL-10 mRNA and produced IL-10 (Table 1). All cell lines expressed IL-10R (see Fig 5).

XG1 and XG-2 HMCL produced OM, unlike XG-4 and XG-6 HMCL. Exogenous IL-10 did not support the growth of IL-10 producing XG-4 and XG-6 HMCL (results not shown), whereas, in agreement with our previous report, it supported that of XG-1 and XG-2 HMCL (Fig 3). The growth factor activity of IL-10 was unaffected by anti-IL-6 or anti-IL-6R antibodies, but, as shown in Fig 3, it was completely abrogated by a neutralizing MoAb to the gp130 transducer, indicating that it occurred through one of the
gene expression was found in XG-1 cells and XG-1 cells stimulated with IL-10, but not in XG-2 cells (Fig 4). No mRNA for IL-11, LIF, and OM was detected by RT-PCR in XG-4 and XG-6 HMCL (Fig 4). XG-4 cells expressed IL-6 gene, as did XG-1 cells. The production of these cytokines was also searched for by ELISA or bioassay (Table 1). No LIF or IL-11 proteins were detected in the culture supernatants of these four HMCL, in agreement with the lack of LIF and IL-11 mRNA. After extensive washing to remove the bound recombinant IL-6 used for cell growth, no IL-6 was found in the culture supernatant of XG-1, XG-2, XG-4, and XG-6 cells. Interestingly, OM was found in culture supernatants of XG-1 and XG-2 cells, unlike those of XG-4 and XG-6 cells (Table 1).

IL-10 induced LIFR on XG-1 and XG-2 cells and supported the long-term growth through an OM autocrine loop. Because these results suggested that IL-10 could induce responsiveness to the autocrine OM produced by XG-1 and XG-2 cells, we used RT-PCR to search for gene expression of an OM coreceptor (the LIFR) in cells stimulated or not with IL-10. Figure 5 shows that the LIFR gene was not expressed in XG-1 or XG-2 cells cultured with IL-6 and that IL-10 induced the expression of this gene in the two cell lines. The induction of LIFR by IL-10 was further confirmed at the protein level using an MoAb to LIFR. As outlined in gp130 transducer-dependent cytokines. RT-PCR was used to search for the gene expression of the different gp130 transducer-dependent cytokines. LIF and IL-11 genes were expressed in relevant control cells, but not in XG-1 and XG-2 cells (Fig 4), whereas the OM gene was expressed in both XG-1 and XG-2 cells cultured with IL-6 or IL-10. IL-6

![Fig 6. IL-10 induced functional LIFR in myeloma cells. XG-1 and XG-2 cells (10⁴ cells per culture well) were cultured for 5 days with either no cytokine (No cytokine) or 10 ng/mL of LIF (LIF) or 50 ng/mL of IL-10 (IL-10) or 50 ng/mL of IL-10 and 10 µg/mL of AB-295-NA anti-OM antibodies (IL-10 + Anti-OM) or 50 ng/mL of IL-10 and 10 µg/mL of AB-295-NA anti-OM antibodies and 10 ng/mL of LIF (IL-10 + Anti-OM + LIF). Proliferation was assayed using tritiated thymidine incorporation. Results are the means ± SD of the tritiated thymidine incorporations determined in sextuplate culture wells.](image1)

![Fig 7. Autocrine OM and IL-6 control the proliferation of the XG-7 myeloma cell line. XG-7 cells (10⁴ cells per culture well) were cultured for 5 days with either control antibodies (control) or 10 µg/mL of B-R3 anti-gp130 MoAb (anti-gp130) or 10 µg/mL of B-E8 anti–IL-6 MoAb (anti–IL-6) or 10 µg/mL of AB-295-NA anti-OM antibodies (anti-OM) or a combination of the last two antibodies (anti–IL-6 + anti-OM). In two culture groups, 200 ng/mL of recombinant IL-6 (Anti–IL-6 + IL-6) or OM (Anti-OM + OM) were added to reverse the inhibitory effect of the antibodies. Two culture groups with 10 µg/mL of neutralizing antibodies to IL-11 (Anti–IL-11) or LIF (Anti-LIF) were also performed. Proliferation was assayed using tritiated thymidine incorporation. Results are the means ± SD of the tritiated thymidine incorporations determined in sextuplate culture wells.](image2)
Fig 8. Detection of cytoplasmic cytokines by FACS analysis. Cells were permeabilized as described above and labeled with an MoAb to IL-6, IL-11, LIF, OM, or IL-10 or a mixture of isotype-matched murine antibodies (control) and FITC-conjugated (Fab')_2 fragments of goat anti-mouse MoAb. The anticytokine MoAbs were selected for their ability to label cytokine-producer cell lines, unlike nonproducer cell lines. Fluorescence was analyzed with a FACScan apparatus.

Fig 2, XG-1 and XG-2 cells cultured with IL-10 were labeled by the anti-LIFR MoAb, unlike cells cultured with IL-6. IL-10 failed to increase the expression of IL-6R and gp130 IL-6 transducer on these cells as assayed by FACS analysis (results not shown). Finally, the growth activity of IL-10 on XG-1 or XG-2 cells was completely inhibited by a neutralizing antibody to OM, and this inhibition was reversed by adding exogenous OM (Fig 3). The fact that neutralizing antibodies to IL-6, LIF, or IL-11 had no effect on the growth activity of IL-10 (results not shown) was in agreement with...
the lack of production of LIF and IL-11 by XG-1 and XG-2 cells and with our previously published results for IL-6. Finally, we have checked that the LIFR induced on myeloma cells was functional. XG-1 and XG-2 cells were not sensitive to LIF (Fig 1). LIF was able to stimulate their proliferation when these cells were cultured with IL-10 and an anti-OM MoAb to block the autocrine OM (Fig 6).

Neutralizing antibodies or antisense oligonucleotides to IL-10 failed to block the sensitivity of XG-4 and XG-6 cells to IL-11, LIF, or OM. Despite 15 days of culture with a potent anti-IL-10 antibody to neutralize the autocrine IL-10 produced by XG-4 and XG-6 cells, the response of these cells to IL-11, LIF, or OM was unaffected (results not shown). Antisense oligonucleotides to IL-10 inhibited the endogenous IL-10 production by XG-4 and XG-6 cells by 70% and 54%, respectively, but failed to inhibit the sensitivity of these cell lines to OM (results not shown).

Autocrine OM and IL-10 production and LIFR expression

Fig 9. Detection of LIFR and of cytoplasmic cytokines in freshly explanted myeloma cells.

MC from patients' tumoral samples were fixed with paraformaldehyde, permeabilized with ethanol, and labeled with anticytokine murine MoAb or control murine antibodies. To detect membrane LIFR, cells were first labeled with anti-LIFR MoAb before being fixed with paraformaldehyde and permeabilized. Myeloma cells were then labeled with biotinylated antihuman κ or λ light chain antibodies. Cells were finally labeled with FITC-conjugated Flab)* fragments of goat antimouse Igs and PerCP streptavidin. A FSC/SSC region containing mostly myeloma cells was first defined (A). For this patient sample, the myeloma cells were labeled with anti-K light chain antibodies (C), unlike anti-A antibodies (B), in agreement with the light-chain isotype of myeloma Ig. Within this region, the percentage of myeloma cells labeled by isotype-matched control MoAb (C), anti-OM (D), anti-IL-10 (E), or anti-LIFR MoAb (F) was determined by double fluorescence (FL-3/FL-1) analysis with a FACS-can apparatus.
by the autonomously growing XG-7 cell line. As outlined in Table 1, the XG-7 HMCL produced OM and IL-10. These cells expressed LIFR and IL-11R genes and were labeled by anti-LIFR MoAb (results not shown). They also produced IL-6. The results outlined in Fig 7 show that XG-7 cells used OM and IL-6 as autocrine growth factors. Indeed, their proliferation was inhibited by an anti-gp130 MoAb and partially by anti-IL-6 or anti-OM MoAb.

Autocrine OM production by freshly explanted myeloma cells. Because it is difficult to get enough amount of purified primary myeloma cells, we identified intracytoplasmic cytokines by FACS analysis to study the cytokine production by freshly explanted myeloma cells. We first selected MoAb to IL-6, IL-10, IL-11, LIF, or OM, making it possible to specifically label cell lines producing these cytokines, unlike nonproducer cell lines. Positive and negative labelings obtained with these antibodies are shown in Fig 8. This technique was sensitive enough to label cell lines producing only...
several picograms of a cytokine (Table 1). The expression of IL-6R, gp130, and LIFR was studied with the MoAb described above.

Using this methodology, we detected no production of IL-6, IL-11, and LIF by freshly explanted myeloma cells. Myeloma cells of the six patients expressed IL-6R and gp130. A major finding was that OM was produced by myeloma cells from five of the six patients. For three patients, myeloma cells produced OM but did not produce IL-10 and failed to express LIFR, as was the case with XG-1 and XG-2 HMCL (Fig 9). Myeloma cells from two patients produced OM and IL-10 together and expressed LIFR, as did XG-7 cells (Fig 10). Finally, myeloma cells from one patient had the cytokine profile of XG-4 and XG-6 HMCL, ie, lack of OM production, production of IL-10, and expression of LIFR (Fig 11). For two patients, we obtained enough cells to purify myeloma cells and to study the effect of exogenous IL-10 in in vitro cultures. These myeloma cells produced OM but not IL-10. First, IL-
IL-10 AND OM IN MULTIPLE MYELOMA

Fig 12. Induction of LIFR on freshly explanted myeloma cells by recombinant IL-10. Freshly explanted myeloma cells from patients no. 1 and 2 (Table 2) were purified and cultured for 5 days with 50 ng/mL of IL-10 (IL-10) or with no cytokine at all (control). At the end of the culture period, myeloma cells were labeled with the 10B2 anti-LIFR MoAb or an isotype-matched control murine IgG1 and FITC-conjugated F(ab')2 fragments of goat antimusine IgGs. Myeloma cells were labeled with biotinylated antihuman Ig light chain antibodies and PerCP streptavidin. Fluorescence was analyzed with a FACScan apparatus.

10 induced a detectable expression of LIFR on these myeloma cells (Fig 12). In addition, IL-10 induced a proliferation of myeloma cells that was unaffected by anti-IL-6 antibodies (Table 2). The IL-10–induced proliferation was inhibited by anti-gpl30 or anti-OM antibodies, indicating that it was mediated through an autocrine OM loop (Table 2).

DISCUSSION

We have shown here (1) that myeloma cell lines or patients' freshly explanted myeloma cells frequently produced OM, (2) that IL-10 conferred on myeloma cells the responsiveness to OM by inducing the LIFR that forms with gp130 IL-6 transducer a receptor for OM, and (3) that myeloma

| Table 2. Response of Purified Myeloma Cells to Recombinant IL-10 |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Patient no. 1 (>95%)* | IL-10 | IL-10 + Anti-gp130 | IL-10 + Anti-IL-6 | IL-10 + Anti-OM | IL-10 + Anti-OM + OM |
| 0 | 4.9 ± 0.3 | 1.3 ± 0.1 | 5.1 ± 0.3 | 0.7 ± 0.1 | 5.4 ± 0.3 |
| Patient no. 2 (>95%)* | 0.3 ± 0.1 | 2.4 ± 0.2 | 0 | 2.6 ± 0.2 | 0.2 ± 0.1 | 1.9 ± 0.2 |

Myeloma cells from one patient with MM (no. 1) and one with plasma cell leukemia (no. 2) were purified with the B-84 antimyeloma cell MoAb. Purified myeloma cells were cultured for 5 days without added cytokines (control) or with 50 ng/mL of recombinant IL-10 in the presence of 10 μg/mL of control IgGs or 10 μg/mL of anticytokine antibodies. At the end of the culture period, the percentage of myeloma cells in the S phase (LI) was determined for each culture group with an anti-S-phase MoAb. Five thousand myeloma cells were examined for each group, which made it possible to determine each LI with a standard deviation given by (LI[1 – LI]/5,000)^1/2.

* Percentage of myeloma cells at initiation of culture.
cells might produce IL-10 in association with an expression of LIFR.

These results make it possible to understand our previous data showing that IL-10 is a potent but IL-6-unrelated myeloma cell growth factor.\(^{17}\) In particular, we reported that the growth of 2 HMCL (XG-1 and XG-2) could be supported by IL-10. We have presently shown that IL-10 induced a functional OM autocrine loop in these cell lines. They produced OM that was inactive due to the lack of expression of an OM coreceptor. IL-10, by inducing LIFR, induced a functional OM receptor. Similar results were obtained with freshly explanted myeloma cells from two patients. IL-10 might also induce the second receptor for OM, whose characteristics are not published.\(^{2}\) We found no upregulation of IL-6R and gp130 by IL-10, possibly because these receptors were already maximally expressed. Because we have previously shown that IL-10 induced IL-11R in myeloma cells and conferred upon them sensitivity to IL-11,\(^{16}\) IL-10 appears to be a key cytokine for regulating the expression of the receptors of the gp130 cytokine family.

Another major finding is that IL-10 might be produced by myeloma cell lines or freshly explanted myeloma cells in association with the expression of LIFR. In the present study, we were not able to show that this autocrine IL-10 was responsible for the sensitivity of some myeloma cell lines to the various gp130-activating cytokines.\(^{15}\) This is hardly surprising, because the biologic activity of an autocrine cytokine is difficult to inhibit completely even by using potent neutralizing MoAb or antisense oligonucleotides to IL-10. However, the fact that there was a strict correlation between the ability of myeloma cells from cell lines or patients’ tumor samples to produce IL-10 and to express LIFR and the fact that IL-10 can induce LIFR in IL-10 nonproducer myeloma cells strongly suggest that this autocrine IL-10 was responsible for the expression of LIFR (present study) or IL-11R\(^{16}\) in some myeloma cells. Thus, the ability of myeloma cells to produce IL-10 might be a key marker for identifying patients whose myeloma cells are sensitive to IL-6 only and patients whose myeloma cells are sensitive to the various gp130-activating cytokines. These cytokines might be produced by myeloma cells (OM) or their tumoral environment. We have previously shown that circulating IL-10 is rarely detected in the plasma of patients with MM, except for patients with plasma cell leukemia.\(^{17}\) Thus, the methodology we have described here to identify patients with IL-10-producing myeloma cells might be very useful to find out whether these patients have different disease characteristics. The ability of myeloma cells to produce IL-10 and/or OM might be a part of the malignant process or a characteristic of the normal cell from which the malignant cell originates. In particular, IL-10 is produced by some normal B cells,\(^{37}\) but OM production by normal B cells has not yet been reported. For two of the six patients studied, we found myeloma cells with a possible functional autocrine OM loop, ie, production of OM and IL-10 and expression of LIFR and gp130. However, these cells failed to generate cell lines in vitro. Such a functional OM autocrine loop was shown in the XG-7 HMCL together with an IL-6 autocrine loop.

These results, showing that the myeloma cell growth factor activity of IL-10 is exerted through a gp130-dependent cytokine, emphasize that all myeloma cell growth factors reported so far involve an activation of the gp130 transducer.\(^{28}\) This suggests that abnormalities in gp130-mediated signal transduction might be the major oncogenic event yielding to the continuous proliferation of the myeloma stem cell.

How can we reconcile these results with the previous findings of several groups showing that IL-6 is a major tumoral growth factor in vitro (reviewed in Klein et al\(^{15}\))? As we have previously pointed out,\(^{28}\) the simplest explanation is that the concentration of biologically active IL-6 in culture supernatants of tumor samples or in closed tumoral samples in vivo such as ascites or pleural effusions is about 500- to 5,000-fold higher than that of the other myeloma cell growth factors, which may explain why the myeloma cell proliferation in vivo and in vitro is mainly IL-6-dependent. This major role of IL-6 is probably strengthened by the high concentration of agonist-soluble IL-6R in the plasma of patients with MM.\(^{29}\) This soluble IL-6R binds IL-6 with the same affinity as membrane IL-6R and induces an activation of gp130 transducer.\(^{30}\) However, it is likely that the other gp130-activating cytokines might be functional in vivo, particularly when myeloma cells are proliferating in close contact with stromal cells that might produce these gp130-activating cytokines or IL-10.

We have previously shown that treatment with anti–IL-6 MoAb completely inhibited the in vivo myeloma cell proliferation in some patients.\(^{31,32}\) Failure to respond to treatment was found in patients who produced a too large amount of IL-6 to be neutralized by a single anti–IL-6 MoAb.\(^{33}\) We have developed new strategies that will make it possible to fully neutralize IL-6 activity in vivo.\(^{34}\) Clinical trials with these new antagonists will help to elucidate the relative role of the different gp130-activating cytokines in association with the ability of patients’ myeloma cells to produce IL-10 or not.

Finally, it would also be interesting to investigate whether the property of IL-10 to induce IL-11R\(^{16}\) and LIFR is restricted to myeloma cells or whether it could be extended to B cells or other cells. In particular, recent data indicate that IL-6 and IL-10 are the two main cytokines involved in the generation of normal plasma cells in vitro in the CD40\(^{35,36}\) or T-cell–dependent system.\(^{37,38}\) Our results suggest that IL-10 may induce an autocrine loop of a gp130 transducer–activating cytokine in normal B cells and plasma cells.

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Interleukin-10 is a growth factor for human myeloma cells by induction of an oncostatin M autocrine loop

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