Interleukin 6 Receptor Superantagonists Are Potent Inducers of Human Multiple Myeloma Cell Death

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ABSTRACT

Interleukin-6 (IL-6) plays a central role in the pathogenesis of multiple myeloma, acting as both a growth and a survival factor for myeloma cells. A series of IL-6 receptor antagonists that are IL-6 variants has recently been obtained, the affinity of which for the ligand-specific receptor chain IL-6Ra has been maintained or even increased, but the signaling of which is impaired by not being able to bind and/or dimerize the signaling chain gp130. Although IL-6 antagonists have been shown to inhibit the growth of IL-6-dependent myeloma, no information has been gathered on their ability to induce myeloma cell death. We show here that IL-6 receptor antagonists are pro-apoptotic factors for the IL-6-dependent human myeloma cell line XG-1. Their capacity to induce cell death is in direct relation to their affinity for IL-6Ra, degree of gp130 binding impairment, and efficiency to inhibit intracellular signaling events. Interestingly, the most potent pro-apoptotic molecule, Sant7, counteracts the protective antitumor effect exercised by the limited amounts of IL-6 produced by XG-1 cells and is thus able to induce cell death at higher rates than just IL-6 deprivation. These findings are particularly relevant for the therapy of multiple myeloma.

INTRODUCTION

IL-6 is a pleiotropic cytokine involved in various physiological processes, such as host defense, bone metabolism, and acute-phase response (1, 2). Overproduction of IL-6 is, however, associated with several pathological conditions, including autoimmune disorders, B-cell malignancies, and postmenopausal osteoporosis (3–5); the pathogenic role of IL-6 in MM in particular is documented by a wealth of data. Briefly, patients with MM show elevated serum levels of IL-6, which are of high prognostic value (6–8). The cytokine itself is a major growth factor for malignant cells, and in vitro cultures of freshly explanted myeloma cells are strongly inhibited by anti-IL-6 antibodies (9). Clinical trials with mAbs to human IL-6 in MM terminal patients have provided evidence that in vivo neutralization of the cytokine is therapeutically effective (10, 11). This is, however, only a transitory and partially effective therapeutic regimen because anti-IL-6 mAbs not only stabilize IL-6 as stable monomeric immune complexes, but also induce IL-6 receptor superantagonists, which are of high prognostic value (6–8). The cytokine itself is a growth inhibitor that they only differ in the amount needed to obtain this effect but not in the maximal degree of inhibition (23). In this paper, we show that IL-6 receptor superantagonists may function not only as growth inhibitory but also as pro-apoptotic agents. We therefore decided to test this possibility on the cell line XG-1 (26). We previously showed that in XG-1, the entire set of IL-6 superantagonists fully inhibits cell growth and that they only differ in the amount needed to obtain this effect but not in the maximal degree of inhibition (23). In this paper, we show that IL-6 receptor superantagonists induce myeloma cell death; however, the degree of apoptosis is not identical for all molecules and inversely correlates with residual binding to gp130. We also analyzed residual activation of intracellular signaling by IL-6 superantagonists and were able to establish a precise relationship between their efficiency as pro-apoptotic molecules and their ability to inhibit the IL-6-dependent activation of STAT transcription factors.

MATERIALS AND METHODS

Cell Cultures. XG-1 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 5 × 10−5 M β-mercaptoethanol in the presence of 2 ng/ml of human wt IL-6. For flow cytometry experiments at low density, after repeated PBS washing, XG-1 cells were grown at 3 × 104 cells/ml in IL-6-depleted conditions, in the presence of 0.2 ng/ml of IL-6 or in the presence of 0.2 ng/ml of IL-6 plus the specific IL-6 antagonist tested. The amount of IL-6 antagonist used caused complete growth inhibition (see Table 1). DFRD was used to compete wt IL-6 at 4 µg/ml; Sant1 at 4 µg/ml; Sant5 at 0.2 µg/ml; Sant7 at 0.2 µg/ml. For flow cytometry experiments at high density, after repeated PBS washing, XG-1 cells were incubated at 106 cells/ml in absence of IL-6, in the presence of IL-6 at 0.2 ng/ml, or in the presence of Sant7 at 1 µg/ml.

For EMSA experiments, the concentration of wt IL-6 was increased to 0.4 ng/ml to obtain a stronger STAT signal. Obviously, in competition experiments monitoring STAT extinction, the concentration of IL-6 antagonists was increased accordingly.
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Table 1 Properties of the IL-6 antagonists analyzed in this study

<table>
<thead>
<tr>
<th>Mutant protein</th>
<th>sIL-6Ra binding activity</th>
<th>gp130 binding activity</th>
<th>gp130 dimerizing activity</th>
<th>Concentration of antagonist causing full XG-1 growth inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFRD</td>
<td>1 ± 0.1</td>
<td>+</td>
<td>-</td>
<td>4 µg/ml</td>
</tr>
<tr>
<td>Sant 1</td>
<td>4.5 ± 0.6</td>
<td>+</td>
<td>-</td>
<td>4 µg/ml</td>
</tr>
<tr>
<td>Sant 5</td>
<td>40 ± 3.4</td>
<td>+</td>
<td>-</td>
<td>0.2 µg/ml</td>
</tr>
<tr>
<td>Sant 7</td>
<td>65 ± 7</td>
<td>-</td>
<td>-</td>
<td>0.2 µg/ml</td>
</tr>
</tbody>
</table>

*S Relative to wt hIL-6.

**By in vitro immunoprecipitation experiments.

EMSAs. XG-1 cells were collected at the indicated time points, rinsed with ice-cold PBS containing 5 mM NaF, centrifuged, and cell pellets were frozen in liquid nitrogen. Total cell extracts were prepared as previously described (19). STAT activation was monitored by binding to the high affinity SIE m67 oligonucleotide (27), which was labeled by filling in the 5' protruding end with Klenow enzyme, using [α-32P]dATP and [α-32P]dCTP (3000Ci/nmol). EMSAs were performed according to Sadowsky and Gilman (28).

Quantification of Apoptotic Bodies. To estimate DNA fragmentation, cells subjected to different treatments were collected by centrifugation at 800 × g for 10 min and fixed with 1:1 PBS and methanol/aceton (4:1, v/v) solution at -20°C. DNA fragmentation of dead cells was evaluated through flow cytometry by staining with propidium iodide (50 mg/ml; Ref. 29) on a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA). Cells were excited at 488 nm using a 15-mW Argon laser, and the fluorescence was monitored at 570 nm. Events were triggered by the FSC signal with an electronic gating for FSC-H/FSC-A/SSC. Ten thousand events were evaluated using the Lysis II Programme (29). The cell cycle was evaluated through flow cytometry using propidium iodide staining (50 mg/ml) on a FACScan flow cytometer (29).

TUNEL. After treatment, cells were fixed on slides in 2% paraformaldehyde for 3 min at room temperature and then extensively washed in PBS. TUNEL was performed as described by Gavrieli et al. (30), with a few modifications. Endogenous peroxidase was inactivated using 2% H2O2 for 15 min at room temperature, and the slides were incubated in a reaction mix containing terminal deoxynucleotidyltransferase buffer, terminal deoxynucleotidyltransferase enzyme (0.5 unit/ml), biotinylated dUTP (0.025 nmol/ml), and CoCl2 (2.5 mM) for 60 min at 37°C in a humidified atmosphere. The reaction was blocked by transferring the slides in TB buffer (300 mM sodium chloride, 30 mM sodium citrate) for 15 min at room temperature. Cells were rinsed in distilled water, covered with a 2% BSA aqueous solution for 10 min, and then immersed in PBS for 5 min. The slides were incubated with peroxidase-conjugated streptavidin (BioGenex) for 8 min and stained using amin-
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Fig. 2. Three-dimensional flow cytometry evaluation of the cell cycle in human MM XG-1 cells; cells were cultured for 4 days in the absence (B) or in the presence (A) of 0.2 ng/ml of IL-6. The axes represent 580-nm fluorescent emission by propidium iodide (X axis), cell volume (Z axis) and number of events (Y axis). The three main peaks indicate apoptotic cells (peak 1), cells in G0-G1 (peak 2), and cells in G2-M (peak 3).

ethylenediaminetetraacetic acid (EDTA) for 20 min at 4°C. Cell pellets were washed with PBS, counterstained in Mayer's hemalum for 10 s, fixed in 4% paraformaldehyde, and stained the cells for fragmented DNA using the TUNEL technique.

Quantification of IL-6 Produced by Myeloma Cells. To determine the amount of IL-6 produced by XG-1 and XG-2 (26) cells were cultured in medium without exogenously added IL-6 at 10^6 cells/ml, and the supernatants were withdrawn at time 0 and after 4 and 8 days of culture. Supernatants were clarified by spinning for 5 min at top speed in a bench-top centrifuge, and the amount of IL-6 present was determined using a commercially available ELISA kit (R&D Systems, Minneapolis, MN) following the instructions furnished by the manufacturer.

Statistical Analysis. A one-way ANOVA was used for statistical analysis of significance.

RESULTS

Withdrawal of IL-6 from XG-1 Cultures Activates Programmed Cell Death. IL-6 has been shown to protect myeloma cell lines from programmed cell death by modifying the ratio between pro- and anti-apoptotic Bcl proteins (31, 32). To investigate whether apoptosis takes place also in the human XG-1 cell line, which has been shown to be growth-dependent from exogenously added IL-6, we stained the cells for fragmented DNA using the TUNEL technique. Fig. 1 shows that although in the presence of IL-6 (A), only rare cells have a fragmented DNA, the removal of IL-6 (B—D at 3, 4, and 5 days, respectively) significantly enhances the number of cells with fragmented DNA.

To more precisely quantify the extent of apoptotic cell death, XG-1 cells were seeded at the density of 3 X 10^4/ml of culture medium with or without addition of 0.2 ng/ml of IL-6 and flow cytometric evaluation of propidium-iodide-stained hypodiploid cells was performed after 4 days. The results are reported in Fig. 2. In the presence of exogenously added IL-6, most of the cells are actively proliferating, as demonstrated by the amplitude of peak 2 (cells in phase G0-G1) and peak 3 (cells in phase G2-M) and by the low number of cells that show low forward scatter with hypodiploid DNA staining (peak 1). On the contrary, when cells were cultured in absence of IL-6, the number of apoptotic cells rose to 24.9% (peak 1); moreover, most of the cells block their proliferation arresting in G0-G1 phase (peak 2) and do not undergo active mitosis (peak 3).

IL-6 Antagonists Induce Programmed Cell Death with Different Efficacies. We have recently generated a set of IL-6 antagonists that are able to fully block the IL-6-induced proliferation of XG-1 cells as measured by a colorimetric assay (19, 23). The growth arrest is not due to unspecific toxicity of IL-6 superantagonists, and it can be fully reversed by incubating cells with increasing amounts of wt IL-6 (Refs. 19 and 23 and data not shown). The potency of the different IL-6 antagonists nicely correlates with their binding affinity for IL-6Ra and with the residual interaction with gp130 (19, 23). In light of these findings, we decided to analyze the relative potency of a selected subset of IL-6 antagonists to induce apoptosis of XG-1 cells in the presence of IL-6. DFRD carries two substitutions in the A helix, IL-6AntagonistsInducedifferent DFRD and with the residual interaction with gp130 (13) and two in the C helix S118F/V121D (19). This molecule has the same affinity for IL-6Ra as wt IL-6 and has lost the ability to dimerize gp130, but it has maintained the ability to bind a residual gp130 chain at site 3 (17). Sant1 carries three additional amino acid substitutions in the context of the putative D helix that increase affinity for IL-6Ra approximately 5-fold (19); Sant5 carries, with respect to Sant1, substitutions Q7SY/S76K in the putative AB loop, which determine a further increase in the binding to IL-6Ra and have the same affinity for IL-6Ra and have lost the ability to dimerize gp130; finally, Sant7 contains three additional substitutions (L57D/E59F/N60W) with respect to Sant5. This last mutant binds IL-6Ra approximately 10-fold better than wt IL-6 and does not have any residual binding in vitro to gp130 (23). The properties of the IL-6 mutants used in these studies are summarized in Table 1.

XG-1 cells were grown at low density as above, and antagonists were added at the minimal concentration needed to fully inhibit

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Days of culture</th>
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<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>IL-6</td>
<td>13.1 ± 0.4</td>
</tr>
<tr>
<td>No IL-6</td>
<td>24.5 ± 0.6</td>
</tr>
<tr>
<td>IL-6 + DFRD</td>
<td>17.5 ± 0.4</td>
</tr>
<tr>
<td>IL-6 + Sant1</td>
<td>18.1 ± 0.3</td>
</tr>
<tr>
<td>IL-6 + Sant5</td>
<td>21.8 ± 0.5</td>
</tr>
<tr>
<td>IL-6 + Sant7</td>
<td>27.5 ± 0.6</td>
</tr>
</tbody>
</table>

* Numbers in the columns indicate the percentage of apoptotic bodies in the population at the various days, mean (SE <5%) of triplicate experiments, calculated each on 10,000 gated events.

* The values were compared to IL-6 and no IL-6; all the differences were significant (P < 0.005).
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Time Course of STAT Extinction. The different capabilities of IL-6 antagonists to induce XG-1 apoptosis in conditions that give rise to identical inhibition of cell growth suggests that these molecules may differentially affect intracellular events triggered by the binding of IL-6 to its receptor. To investigate this possibility, we decided to analyze an early event in intracellular signaling by IL-6. It is well known that this cytokine rapidly activates the Jak/STAT signaling pathway, giving rise to the formation of DNA binding-competent STAT complex a few minutes after cell exposure to the cytokine; these complexes are composed for the majority of STAT3 homodimers and for the minority of STAT1 homodimers and STAT3/STAT1 heterodimers (33). XG-1 cells grown in the presence of IL-6 show constitutive activation of STAT (Fig. 4A, lane 1), as detected by EMSA. When cells are deprived of IL-6, there is a rapid extinction of STAT binding until complete disappearance of the signal after 2 h (Fig. 4, lanes 8, 23, and 40).

We have tested the ability of the set of IL-6 antagonists to extinguish STAT activation. The quantities of antagonists used were equivalent to the amounts required to fully inhibit XG-1 growth (23) and used for the apoptosis-induction experiment. All molecules were able to fully abolish STAT binding to DNA in the presence of IL-6 at 2 h (Fig. 4, A–C). However, at later times, STAT binding tended to reappear with different kinetics and intensity, with the exception of Sant7 (Fig. 4, lanes 16, 31, 50, and 51). The reappearance of STAT was inversely related to the ability of the antagonists to induce apoptosis.

XG-1 Cells Grown at High Density Produce IL-6. It has been previously reported that Northern blot analysis on XG-1 myeloma cells does not reveal detectable IL-6 mRNA (34). However, due to the proliferation (Table 1 and Ref. 23). Cells were analyzed 3–7 days after treatment, and the percentage of apoptotic bodies was calculated by three-dimensional flow cytometric evaluation of DNA fragmentation. The results are reported in Table 2 and Fig. 3. All molecules showed a pro-apoptotic effect that was increased in parallel to the increase in affinity for IL-6Ra. It is interesting to note that all the antagonists, even at concentrations that fully inhibit cell proliferation (19, 23), are less efficient in inducing cell death than the simple IL-6 withdrawal, with the sole exception of Sant7, which reproduced IL-6 depletion throughout the entire duration of the experiment.

![Time course of XG-1 cells apoptosis induction by IL-6 mutants. XG-1 cells were cultured as described in the legend to Table 2. The percentage of apoptotic bodies in the cell population was determined by cytofluorometry, as described in “Materials and Methods.” Fig. 3 shows the increase in the percentage of apoptotic bodies in the cultures treated with IL-6 mutants as a function of time.](image-url)

![Fig. 4. Time course of STAT extinction. XG-1 cells were grown in the presence of 2 ng/ml of IL-6, washed twice with PBS, and resuspended in medium without IL-6 or with 0.4 ng/ml of IL-6 in the absence or in the presence of the various mutants as indicated above the lanes. At the indicated time points, aliquots were withdrawn and subjected to EMSA, as described in “Materials and Methods.”](image-url)
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Fig. 5. Accumulation of IL-6 in the cell culture supernatants. XG-1 or XG-2 cells were cultured at 10^6 cells/ml, cell culture supernatants were withdrawn at the indicated time points, and the IL-6 concentration was measured as described in "Materials and Methods." Data points, average of triplicate measures; bars, SE.

...relatively low sensitivity of this method, it cannot be excluded that very low amounts of cytokine are indeed produced and released, which might contribute to cell survival in the absence of exogenously added IL-6.

...XG-1 cells were cultured at high density in IL-6-depleted conditions for 1 week, samples of conditioned medium were collected at days 0, 4, and 8, and IL-6 production was measured by ELISA. IL-6 was already detected at day 4 (6 pg/ml), but after 8 days, the amount of IL-6 reached 25 pg/ml (Fig. 5), indicating a release and a progressive accumulation of the cytokine in the culture supernatant.

...Like XG-1 cells, XG-2 cells were also directly derived from a terminal MM patient; they show approximately the same cell surface markers as XG-1 (26). IL-6 acts as a growth factor also for XG-2 cells, but whereas IL-6 deprivation determines a complete growth arrest of XG-1 cells, it determines only an increase in the replication time of XG-2 cells (34). As a control for IL-6 production by XG-1 cells, we cultured XG-2 cells in the same conditions, and we did not detect any IL-6 in the culture supernatant (Fig. 5).

...Sant7 is more potent than IL-6 withdrawal in promoting XG-1 cell death. From the experiments described above, we established an inverse correlation between the pro-apoptotic effect of the different IL-6 antagonists and their residual biological activity in terms of STAT activation. Sant7 is the most powerful inducer of apoptosis and is able to completely block intracellular signaling. Because XG-1 cells grown at high density produce detectable amounts of IL-6, which may exert a partially protective autocrine effect against cell death, we decided to investigate the pro-apoptotic effect of Sant7 in these conditions. Table 3 shows that the administration of 1 pg/ml of Sant7 determines a stronger pro-apoptotic effect than IL-6 withdrawal; this starts after 24 h and is sustained during the following days. At day 3, the percentage of apoptotic bodies is 20.2% in absence of IL-6 and increases to 23.4% when Sant7 is added. At day 4 this difference is further enhanced: we detected 24.1% of apoptotic bodies without IL-6 and 29.6% with Sant7 treatment. This suggests that by treating myeloma cells with Sant7, we were able to counteract the action of IL-6 endogenously produced by XG-1 cells and increase the rate of cell death.

**DISCUSSION**

In this paper, we show that in a human myeloma cell line that proliferates in response to IL-6, this cytokine also protects cells from apoptosis and that this effect is reversed by IL-6 superantagonists. The IL-6 variants used in this study have been shown previously to be effective inhibitors on a variety of nonmyeloma IL-6-responsive cell lines and to block IL-6-induced XG-1 cell growth, as measured both by colorimetric and [3H]thymidine incorporation assays (19, 23). However, they are a somewhat heterogeneous class of molecules in two respects: a) their affinity for IL-6Rα ranges from that of the wt to 65-fold enhanced; b) they can be subdivided into two categories based on their residual binding to gpl30. Whereas DFRD, Sant1, and Sant5 efficiently interact with but do not dimerize gpl30, Sant7 is apparently unable to bind the signaling chain at all. These differences are nicely mirrored by their efficiency in promoting apoptosis: only Sant7, which combines high affinity for IL-6Rα with simultaneous inactivation of both gpl30 binding sites, is as potent as IL-6 deprivation, when used at low cell density and in the presence of wt IL-6. Furthermore, Sant7 potentiates the pro-apoptotic effect of IL-6 deprivation when tested at high cell density. On the basis of the available data, we believe that in these latter experimental conditions, the limited amounts of IL-6 produced and released by XG-1 cells, although not sufficient to induce growth, offer partial protection from death. This effect is counteracted by Sant7.

It is important to compare the apoptotic effect of IL-6 antagonists in relation to their ability to impair signaling. As a measure of signaling activation, we decided to monitor STAT factor binding to DNA both because it is very sensitive and because it has been shown in several other cell types to follow a very rapid kinetics of activation/deactivation after cytokine administration (35). XG-1 cells grown in the presence of IL-6 show a constitutive STAT activation. All IL-6 antagonists cause a rapid decline of DNA binding activity, which is in line with their mode of action. However, only Sant7 is able to maintain this effect for a prolonged period of time (up to 48 h) and inhibit STAT reappearance. This, together with the previous results, leads to the conclusion that a weak signaling activation by IL-6 is still possible in the absence of detectable cell proliferation (as observed for DFRD, Sant1, and Sant5 at 24 h), and this is sufficient to ensure partial protection from death. When signaling is fully impaired, as observed for Sant7, cell death is identical to or even greater than that obtained in the absence of the cytokine.

The reactivation of STAT binding, which takes place at late incubation times and with different kinetics for DFRD, Sant1, and Sant5, cannot be easily explained. We can eliminate the possibility that it is due to protein instability or depletion, because the supernatant of XG-1 cells incubated with the various antagonists for 24 h is still able to induce a complete short-term shut-off of STAT binding when used on fresh cells and to fully inhibit IL-6-induced acute phase gene expression in hepatoma cells (not shown). An alternative interpretation could be that site 2 antagonists (but not site 2 + 3 antagonists, such as Sant7), have the potential, albeit at very low efficiency, to assemble functional receptor complexes and hence to activate signaling. This effect has indeed been observed by us in a different IL-6-dependent myeloma cell line, XG-2, in which DFRD, Sant1, and Sant5, but not Sant7, are still able to induce a modest proliferation at high concentrations (23). What is unclear is why signaling in XG-1 is activated only upon prolonged incubation. Although several hypotheses can be formulated, the most attractive is that of up-regulation and clustering of the receptor. Future efforts will be addressed to investigating this possibility.

Cell death by apoptosis in other myeloma cell lines has been shown to involve changes in bcl-X alternative splicing or in Bcl-2 steady state levels (31). To establish to what extent similar changes take place in XG-1 cells starved of IL-6 or incubated with IL-6 antagonists, a preliminary set of Western and Northern blot analyses were per-
formed. These allowed us to exclude the possibility that the mechanism of death is due to gross modification of Bcl-2, bax, bc1-X, and p53 expression, as no changes were detected in the mRNA or protein steady state levels. It will be, therefore, important in the future to investigate the possible involvement of other bcl-related proteins or the activation of the autocrine suicide Fas-L/Fas circuitry, as observed in T-cell receptor and HIV-1 Tat/gp120-induced apoptosis (36, 37).

The results presented in this paper are of potential value for the therapy of MM. In this disease, the pathogenetic role of IL-6 as a proliferative and anti-apoptotic factor has been demonstrated by a wealth of data (6–8, 10, 11). On the other hand, mAbs against the cytokine have been shown to reach only a limited clinical efficacy, mainly because of a stabilizing effect of the cytokine in vivo, due to the formation of circulating IL-6/IL-6 receptor complexes (11). The IL-6 superantagonists offer an alternative testable possibility, which implies a different pharmacological mode of action. The site 2 + 3 superantagonist Sant7, a “full-proof” inhibitor of signaling and a potent pro-apoptotic agent, which counteracts the action of autocrinally produced IL-6, might be evaluated as an alternative treatment in the therapy of this serious disease.

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