Interleukin-6 Promotes Multiple Myeloma Cell Growth Via Phosphorylation of Retinoblastoma Protein

By Mitsuyoshi Urashima, Atsushi Ogata, Dharminder Chauhan, Maria B. Vidriales, Gerrard Teoh, Yasutaka Hoashi, Robert L. Schlossman, James A. DeCaprio, and Kenneth C. Anderson

Interleukin-6 (IL-6) mediates autocrine and paracrine growth of multiple myeloma (MM) cells and inhibits tumor cell apoptosis. Abnormalities of retinoblastoma protein (pRB) and mutations of RB gene have been reported in up to 70% of MM patients and 80% of MM-derived cell lines. Because dephosphorylated (activated) pRB blocks transition from G1 to S phase of the cell cycle whereas phosphorylated (inactive) pRB releases this growth arrest, we characterized the role of pRB in IL-6-mediated MM cell growth. Both phosphorylated and dephosphorylated pRB were expressed in all serum-starved MM patient cells and MM-derived cell lines, but pRB was predominantly in its phosphorylated form. In MM cells that proliferated in response to IL-6, exogenous IL-6 downregulated dephosphorylated pRB and decreased dephosphorylated pRB-E2F complexes. Importantly, culture of MM cells with RB antisense, but not RB sense, oligonucleotide (ODN) triggered IL-6 secretion and proliferation in MM cells; however, proliferation was only partially inhibited by neutralizing anti-IL-6 monoclonal antibody (MoAb). In contrast to MM cells, normal splenic B cells express dephosphorylated pRB. Although CD40 ligand (CD40L) triggers a shift from dephosphorylated to phosphorylated pRB and proliferation of B cells, the addition of exogenous IL-6 to CD40L-treated B cells does not alter either pRB or proliferation, as observed in MM cells. These results suggest that phosphorylated pRB is constitutively expressed in MM cells and that IL-6 further shifts pRB from its dephosphorylated to its phosphorylated form, thereby promoting MM cell growth via two mechanisms: by decreasing the amount of E2F bound by dephosphorylated pRB due to reduced dephosphorylated pRB, thereby releasing growth arrest; and by upregulating IL-6 secretion by MM cells and related IL-6-mediated autocrine tumor cell growth.

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INTERLEUKIN-6 (IL-6) is a cytokine with pleiotropic activities, including induction of differentiation and regulation of growth in a cell type dependent manner.1 It was originally described as a factor that promoted differentiation of normal B cells to antibody (Ab) producing cells, without triggering B-cell proliferation.2,3 In contrast, IL-6 is known to be a growth factor both for human multiple myeloma (MM) and mouse plasmacytoma cells. Specifically, multiple reports support an autocrine IL-6-mediated growth mechanism in MM, as some MM cells and derived cell lines both produce and respond to IL-6 in vitro.4-10 An IL-6-mediated paracrine growth mechanism has also been postulated, based on these observations: bone marrow stromal cells (BMSCs) are the major source of IL-6 in MM11-13; freshly isolated MM cells cultured without exogenous IL-6 rapidly stop proliferating;14,15 and adhesion of MM cells to BMSCs upregulates IL-6 secretion by BMSCs.16,17 Most recently, IL-6 has also been shown to enhance survival of MM cells, because it can inhibit apoptosis of tumor cells induced by corticosteroids,17 serum starvation,18 and anti-Fas.19 There is also considerable evidence supporting a role for IL-6 in the growth and manifestations of MM in vivo: elevated IL-6 serum levels correlate with poor prognosis and higher tumor cell mass in MM; IL-6 transgenic mice develop histopathologic changes characteristic of MM kidney; IL-6 serves an antiosteoclast activating factor in MM bone disease; and anti-IL-6 monoclonal antibody (MoAb) therapy can transiently reverse disease manifestations in patients with MM.20-24

Despite this central role for IL-6 in MM cell growth, some patient MM cells and derived cell lines that bear IL-6 receptors grow independently of IL-6. Moreover, although overproduction of IL-6 in vivo can occur in MM, it is also observed in other diseases including rheumatoid arthritis, Castleman’s disease, mesangial proliferative glomerulonephritis, and cardiac myxoma.25 Patients with these diseases do not develop MM, suggesting that additional factors are essential for MM pathogenesis. Studies in animals suggest that genetic abnormalities, which result in a pathological response to IL-6, may be required for development of MM.

For example, plasmacytomas can grow in wild type mice, but not in IL-6 knock out littermates,25 confirming the essential role of IL-6. However, C57BL/6 IL-6 transgenic mice develop massive plasmacytosis without plasmacytomas,26 whereas BALB/C IL-6 transgenic mice develop transplantable monoclonal plasmacytomas.27 These animal studies confirm that IL-6 can enhance susceptibility to either plasmacytosis and Ab formation or the development and growth of plasmacytomas, depending on the genetic background.

Mutations of the retinoblastoma (RB) gene contribute to cellular transformation in many types of malignancies. Specifically, normal retinoblastoma protein (pRB) suppresses the transition from G1 to S phase of the cell cycle28,30 by inhibiting E2F-mediated transactivation of a variety of genes involved in initiating DNA synthesis, ie, c-myc, b-myb, cdc2, dihydrofolate reductase, and thymidine kinase.31,32 pRB function is regulated by phosphorylation: hypo or dephosphorylated (activated) pRB binds E2F and induces G1 growth arrest; in contrast, phosphorylated (inactivated) pRB cannot bind E2F, thereby facilitating entry of cells into S phase.30 To date mutations in RB gene or abnormalities in pRB have been noted in up to 70% of MM patients and 80% of MM-derived cell lines.33,34 Moreover, we have recently

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reported that transforming growth factor (TGF β) does not suppress phosphorylation of pRB and proliferation of MM cells, as occurs in normal B cells, consistent with the view that pRB may contribute to MM cell growth. In this regard, it is known that the majority of MM cells and derived cell lines express IL-6 receptors, and yet only a subset are dependent on IL-6 for growth; it is, therefore, possible that abnormalities in cell cycle regulatory proteins, ie, RB, may contribute to MM cell growth and progressive disease. In the present report, we characterized the role of pRB in IL-6-mediated MM cell growth. Although both phosphorylated and dephosphorylated pRB were expressed in all serum-starved MM patient cells and MM-derived cell lines, pRB was predominantly in its phosphorylated form. Exogenous IL-6 downregulated dephosphorylated pRB and decreased dephosphorylated pRB to E2F complexes in MM cells. Culture of MM cells with RB antisense, but not RB sense or oligonucleotides (ODN) triggered IL-6-secretion and proliferation in MM cells; the latter was partially inhibited by neutralizing anti-IL-6 MoAb. These results suggest that both dephosphorylated and phosphorylated pRB are present, and that the former induces growth arrest, even though the latter predominates. However, in IL-6-responsive MM cells, IL-6 further shifts pRB from its dephosphorylated to its phosphorylated form, thereby promoting MM cell growth via two mechanisms: by decreasing binding of dephosphorylated pRB to E2F and releasing growth arrest, and by upregulating IL-6 secretion by MM cells and IL-6-mediated autocrine tumor cell growth.

MATERIALS AND METHODS

MM cells and MM-derived cell lines. Mononuclear cells (MCs) were isolated from three patients with MM by Ficoll-Hypaque (FH) density gradient centrifugation, incubated with HB7 (anti-CD38) MoAb-biotin-streptavidin and 2H4 (anti-CD45RA) MoAb-fluorescein isothiocyanate on ice. MCs (96±2% CD38+ CD45RA-) were isolated using an Epics C Cell Sorter (Coulter Electronics, Hialeah, FL), washed, and resuspended in RPMI-1640 media (Sigma Chemical Co, St Louis, MO) containing 10% fetal bovine serum. ARH-77, IM-9, RPMI-8226, and U-266 human MM-derived cell lines were kindly provided by Drs Kenneth Kirin-Brewrey CO Ltd, Minato-ku, Tokyo, Japan. The XG-1 IL-6-dependent MM cell line was kindly provided by Drs Kenneth Kirin-Brewrey CO Ltd, Minato-ku, Tokyo, Japan. The U-1958-, a lymphoblastic leukemia cell line (ALL), an IL-6 nonresponsive cell line, was obtained from American Type Culture Collection (Rockville, MD). The XG-1 IL-6-dependent MM cell line was kindly provided by Dr Bernard Khoury (Institute for Molecular Genetics, Montpellier, France) and cultured in media with 1 ng/mL of IL-6 (Kirin-Brewrey Co Ltd, Minato-ku, Tokyo, Japan). The U-1958- and OCI-Mys5 MM cell lines were kindly provided by Drs Kenneth Nilsson (Uppsala University, Uppsala, Sweden) and H.A. Messner (Ontario Cancer Institute, Toronto, Ontario, Canada). The JKB acute lymphoblastic leukemia cell line (ALL), an IL-6 nonresponsive cell line, and the CMK acute megakaryocytic leukemia cell line, which grows in an IL-6-mediated autocrine fashion, were used as controls for the MM cells/MM derived cell lines.

B-cell preparation and culture. Normal spleen (n = 5) was obtained from operative specimens of patients not known to have any systemic or malignant diseases. Single cell suspensions from spleen were prepared by extrusion through sterile stainless steel mesh. Splenic MCs were isolated by centrifugation on FH density sedimentation, and adherent cells removed from MCs by double adherence to plastic petri dishes for 1 hour at 37°C. Further enrichment for B cells in spleen was done by rosetting with sheep red blood cells to deplete T cells. B cells were cultured in RPMI-1640 media containing 10% FBS, L-glut, and pen/strep, as previously described. Splenic B cells, which were B1 >90% (+), B2 >85% (+), CD38 negative, PCA-1 negative, gp 80 and gp130 IL-6 receptor (IL-6R) negative (n = 3), were cultured with soluble CD40L (1:4 dilution) for 7 days to trigger their differentiation to 8% B1 (+), B2 negative, 85% CD38 (+), 47% PCA-1 (+), 22% gp80 (+), and 55% gp130 (+), as in previous studies.

Assays of DNA synthesis. DNA synthesis was measured as previously described. Cells (2 × 106/well) were incubated in 96-well plates in the presence of media, IL-6 or CD40L, and DNA synthesis measured using 3H-thymidine (3H-TdR) uptake. Cells were pulsed with 3H-TdR (1 μCi/well) during the last 6 hours of 2-day and last 18 hours of 4-day cultures, harvested onto glass filters with an automatic cell harvester (Cambridge Technology, Cambridge, MA), and counted on a liquid scintillation counter (Packard Tri-Carb 4530, Downers Grove, IL). Stimulation index (SI) was calculated as 3H-TdR uptake of cells with IL-6/3H-TdR uptake of cells with media alone.

Immunoprecipitation and Western immunoblotting. Immunoprecipitation and Western immunoblotting were performed as previously reported.
Table 1. Effects of IL-6 on Cell Proliferation

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<tr>
<th>Concentrations of IL-6 (ng/mL)</th>
<th>XG-1</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>OCI-My5</th>
<th>U-266</th>
<th>U-1958</th>
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<td>0</td>
<td>1.0 ± 0.2</td>
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<td>1.0 ± 0.1</td>
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<td>1</td>
<td>2.0 ± 0.3</td>
<td>2.8 ± 0.2</td>
<td>2.1 ± 0.1</td>
<td>13.4 ± 2.1</td>
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<td>1.5 ± 0.1</td>
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<td>10</td>
<td>3.3 ± 0.3</td>
<td>3.1 ± 0.3</td>
<td>2.9 ± 0.2</td>
<td>22.5 ± 3.1</td>
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<td>100</td>
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<td>3.3 ± 0.2</td>
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<td>1.6 ± 0.1</td>
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</table>

<table>
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<th>RPMI-8226</th>
<th>IM-9</th>
<th>JKB</th>
<th>B Cells</th>
<th>CD40L + B Cells</th>
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<td>10</td>
<td>1.5 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.2 ± 0.0</td>
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<td>100</td>
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<td>1.2 ± 0.1</td>
<td>1.2 ± 0.0</td>
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</table>

Data shown are mean ± SD from three independent experiments.

* Stimulation index (SI) = [3H-Thymidine uptake of cells with IL-6]/[3H-Thymidine uptake of cells in media alone.

30% peroxide (Sigma). The minimal detectable level of IL-6 was 10 pg/mL.

**Reagents.** IL-6 (Kirin-Brewery Co Ltd) was added to cultures of patient MM cells, MM or other cell lines, as well as B cells, and the effects on cell proliferation and phosphorylation state of pRB were assayed. Anti-IL-6 MoAb (Genetics Institute) or unreactive isomatched (IgGl) control MoAb (Coulter) was added to cultures of MM cells with RB sense or antisense ODN. Soluble CD40L was kindly provided by Dr Diane Hollenbaugh (Bristol-Myers Squibb, Seattle, WA). Briefly, CD40L gene was transfected into COS cells and supernatants harvested and characterized as previously described.6 Soluble CD40L was used at a final concentration of 1:4 dilution, previously shown to trigger splenic B-cell proliferation.

**RESULTS**

*Effects of IL-6 on DNA synthesis by MM cells/MM-derived cell lines, B cells, and CD40L-treated B cells.* MM patient cells/MM cell lines were cultured in RPMI-1640 media free of FBS. DNA synthesis was measured using [3H-Thymidine uptake during the last 6 hours of day 2 cultures (Table 1). Proliferation of IL-6-dependent XG-1 cells and of patient MM cells was directly correlated with IL-6 concentration. For XG-1 cells and MM cells from patients 1, 2, and 3, culture with 100 ng/mL of IL-6 increased [3H-Thymidine uptake: S1 5.4 < 0.05, S1 4.6 P < 0.005, S1 3.3 P < 0.005, and 25.4 P < 0.001, respectively. IL-6 (100 ng/mL) also stimulated low level but reproducible proliferation of other MM cell lines: S1 1.6 P < 0.05, S1 1.6 P < 0.01, and S1 1.9 P < 0.01 for U-266, U-1958, and OCI-My5 MM cells, respectively. Significant proliferation of RPMI-8226, IM-9, and ARH-77 MM cells to IL-6 was not observed under any conditions. The CMK megakaryocytic cell line also proliferated in response to IL-6 (100 ng/mL) (S1 1.9 P < 0.01). In contrast, JKB ALL cells did not increase DNA synthesis in response to IL-6. CD40L stimulated proliferation of splenic B cells (S1 3.1 P < 0.05), but IL-6 did not alter [3H-Thymidine uptake in either splenic B cells or CD40L-treated splenic B cells (data not shown).

*Effects of IL-6 on phosphorylation of pRB in MM patient cells/MM cell lines, B cells, and CD40L-treated B cells.* The effect of IL-6 on phosphorylation state of pRB was investigated using immunoprecipitation and Western immunoblotting. Phosphorylation slows electrophoretic mobility; therefore, the upper (115 kD) band corresponds to phosphor-ylated pRB and the lower (105 kD) band to de- or hypophosphorylated pRB. Multiple bands between 105 kD and 115 kD represent several forms of pRB that are phosphorylated at different sites or with different stoichiometry.44 Both phosphorylated and dephosphorylated pRB were observed, at 115 kD and 105 kD respectively, in all MM patient cells and MM-derived cell lines after serum starvation for 48 hours; however, phosphorylated pRB was predominant (Fig 1A). Even after serum starvation for 72 hours, phosphorylated pRB was strongly expressed at 115 kD in MM cell lines (data not shown). Culture with IL-6 (100 ng/mL) led to downregulation of dephosphorylated pRB in MM patient cells and MM cell lines in several patterns. In XG-1, U-266, OCI-My-5, and patient 1 MM cells, as well as CMK leukemia cells, dephosphorylated pRB expression declined in lysates from cells cultured with IL-6 for 30 and 240 minutes. In contrast, dephosphorylated pRB expression persisted at 30 minutes and declined only at 240 minutes in cell lysates of U-1958 and patient 2 MM cells cultured with IL-6. In ARH-77, RPMI-8226, and IM-9 MM cells, there were several pRB bands in addition to phosphorylated pRB at 115 kD; IL-6 increased phosphorylated pRB and decreased dephosphorylated pRB in these MM cell lines. In contrast, pRB in JKB ALL cells was constitutively phosphorylated and unaffected by culture with IL-6.

pRB expression in splenic B cells cultured in media or triggered with CD40L was compared with that in MM cells. pRB in splenic B cells was predominantly in the dephosphorylated form (Fig 1B). In contrast to MM cells (Fig 1A), treatment with IL-6 did not alter pRB phosphorylation state in B cells. CD40L upregulated phosphorylated pRB at 115 kD and downregulated dephosphorylated pRB at 105 kD in splenic B cells (Fig 1B); IL-6 had no effect on pRB phosphorylation in CD40L-treated B cells.

*Effects of IL-6 on binding of pRB to E2F protein in MM patient cells/MM cell lines.* Because dephosphorylated pRB directly binds E2F and the complex of pRB and E2F binds to a variety of gene promoters resulting in growth arrest, we determined whether downregulation of dephosphorylated pRB in MM cells correlates with pRB decreased binding to E2F protein (Fig 2). Lysates of patient 1 MM cells starved of FBS for 48 hours were immunoprecipitated...
with anti-pRB MoAb and immunoblotted with the same MoAb and served as a positive control for phosphorylated pRB (ppRB) and dephosphorylated pRB (dpRB). Dephosphorylated pRB was immunoprecipitated with anti-E2F polyclonal Ab in XG-1, U-266, OCI-My5 and patient 1 MM cells, CMK megakaryocytic cells, and JKB ALL cells after serum starvation for 48 hours. In the presence of IL-6 (100 ng/mL) for 1 hour, binding of dephosphorylated pRB to E2F was downregulated in patient MM cells and MM-derived cell lines, as well as in IL-6 responsive CMK cells, but not in IL-6 nonresponsive JKB ALL cells. E2F expression was not altered in the presence or absence of IL-6 in these cell lines.

Effect of RB antisense and sense ODNs on pRB expression, proliferation, and IL-6 secretion in patient MM cells. The effects of RB antisense and sense ODNs on pRB expres-
sion, proliferation and IL-6 secretion by MM cells from three patients were next examined. Both phosphorylated and dephosphorylated pRB expression decreased in lysates of cells cultured for 4 days in the presence of RB antisense ODN, but not RB sense ODN (Fig 3A). Proliferation, assessed by \(^{3}H\)-Tdr uptake, was significantly increased in the presence of RB antisense ODN (10 \(\mu\)mol/L) relative to cultures in media for patient 1 \((P < .02, n = 4)\), patient 2 \((P < .02, n = 4)\), and patient 3 \((P < .005, n = 3)\) (Fig 3B). In contrast, culture with RB sense ODN did not alter proliferation compared with cultures in media alone. The effects of culture with RB sense and antisense ODNs on IL-6 secretion by the same patient MM cells were assayed using an IL-6 ELISA (Fig 3C). IL-6 secretion by MM cells in media was 0.27 ± 0.04 ng/mL (patient 1), 0.13 ± 0.01 ng/mL (patient 2), and 0.08 ± 0.02 ng/mL (patient 3). When MM cells were cultured with 10 \(\mu\)mol/L of RB antisense ODN, IL-6 secretion increased to 1.21 ± 0.16 ng/mL (patient 1), 0.72 ± 0.12 ng/mL (patient 2), and 1.33 ± 0.15 ng/mL (patient 3), significantly greater than by MM cells cultured in media alone \((P < .01\) for patients 1 and 2, and \(P < .001\) for patient 3) or by MM cells cultured with 10 \(\mu\)mol/L RB sense ODN \((P < .05\) for patients 1 and 2 and \(P < .01\) for patient 3). At concentrations of RB antisense ODN ≤1 \(\mu\)mol/L, no significant differences in either proliferation or IL-6 secretion by MM cells were observed (data not shown).

Effects of anti–IL-6 MoAb on MM cell proliferation triggered by RB antisense ODN. We next examined whether the increased proliferation of MM cells observed in RB antisense cultures was, at least in part, mediated by IL-6. IL-6 responsive patient MM cells were cultured for 4 days with RB antisense or RB sense ODN, in the presence or absence of neutralizing anti–IL-6 MoAb. IL-6 (10 ng/mL) markedly increased proliferation of patient MM cells: SI 32.2 at day 4 in 10% FBS RPMI-1640 media, a response that was significantly blocked (73% reduction, \(P < .001, n = 3\)) by neutralizing anti–IL-6 MoAb (10 \(\mu\)g/mL), but not by control MoAb (Fig 4A). During the first 3 days of culture, there
were no significant differences in proliferation of patient MM cells cultured with RB sense or RB antisense ODNs in the presence or absence of anti-IL-6 MoAb (Fig 4B). However, at day 4 of culture, RB antisense ODN significantly increased MM cell proliferation, compared with cultures in media alone (12.7-fold, \( P < .001, n = 3 \)) or with RB sense ODN (14.1-fold, \( P < .001, n = 3 \)). Neutralizing anti-IL-6 MoAb, but not control MoAb, partially inhibited proliferation triggered by RB antisense ODN (42.2% reduction, \( P < .02, n = 3 \)).

**DISCUSSION**

IL-6 is known to be both an autocrine and paracrine growth factor, as well as an antiapoptotic factor, for MM cells. Recent studies that show that retroviruses containing myc and raf oncogenes induce plasmacytomas in pristane-injected wild type, but not in IL-6 knock out, mice\(^2\) support this central role of IL-6 in the growth and development of plasma cell tumors. However, plasmacytosis without monoclonal plasmacytomas develops in IL-6 transgenic B6 mice,\(^6\) whereas monoclonal transplantable plasmacytomas occur in IL-6 transgenic BALB/c mice, demonstrating the import of genetic background on the biologic sequelae of excess IL-6.\(^2\) Moreover, in man, excess production of IL-6 is associated with a variety of diseases other than plasmacytomas or MM, ie, cardiac myxomas, autoimmune conditions, and Castleman’s disease,\(^1\) further suggesting the importance of other genetic factors.

In human MM, IL-6 triggers proliferation of tumor cells and is, therefore, a growth factor; however, not all MM cells and MM cell lines that bear IL-6 receptors respond to exogenous IL-6, and some can grow independently of IL-6. Recent studies have shed insight into potential differences in IL-6 signaling cascades in MM cells that proliferate and those that do not in response to IL-6. For example, our recent studies suggest that IL-6 triggers activation of the STAT 3 homodimer and STAT1-STAT3 heterodimer cascades without altering DNA synthesis of MM cells, whereas IL-6 triggers MM cell growth via the Ras dependent MAPK cascade.\(^2\) Clearly differences in IL-6 signal transduction cascades may underly the triggering by IL-6 of growth of MM cells versus differentiation of normal B cells, as well as the growth of only a subset of tumor cells.

In the present study, we set out to delineate the importance of the RB cell cycle regulatory protein, as abnormalities of pRB or mutations of RB gene have been noted in up to 70% to 80% of MM patients and derived cell lines.\(^3\) Specifically, mutations of the RB tumor suppressor gene have been frequently observed in patient MM cells as well as the XG-1, RPMI-8226, U-266, and IM-9 MM derived cell lines.\(^3\)
These are monoalleic deletions and pRB, the product of RB gene, can be detected. In contrast, bialleic deletion of RB gene and absence of pRB was reported in U-266 MM cells, which both produce and respond to IL-6 (autocrine growth), but not in parental U-266 cells, which respond but do not produce IL-6 (paracrine growth).\textsuperscript{34,35} Interestingly either partial or complete deletions of chromosome 13, on which RB gene is located, or abnormalities involving 11q, on which bcl-1 is located, are frequent and associated with poor prognosis in MM.\textsuperscript{46} Moreover, cyclin D1, which can trigger phosphorylation of pRB, is located on the 110 kb telomeric of the bcl-1 major translocation cluster region; its overexpression has been reported in various kinds of cancers with 11q abnormalities, such as mantle cell lymphoma.\textsuperscript{77} In the current study, pRB was shown to be expressed both in patient MM cells and MM-derived cell lines, primarily in its phosphorylated form. Although serum starvation for 72 hours both decreased growth and increased expression of dephosphorylated pRB, phosphorylated pRB persisted. This is in contrast to normal splenic B cells in which pRB is dephosphorylated with complete absence of phosphorylated pRB. Complexes of cyclin dependent kinase (CDK) 4, CDK 6, and cyclin D, and of CDK 2 and cyclin E regulate pRB phosphorylation\textsuperscript{50,58}; however, the mechanism whereby MM cells express primarily phosphorylated RB has not yet been delineated.

In our study, pRB was primarily in its phosphorylated form in MM cells/MM-derived cell lines; IL-6 triggered tumor cell proliferation and downregulation of dephosphorylated pRB. In contrast, dephosphorylated pRB was present in B cells; CD40L-induced proliferation and decreased dephosphorylated pRB expression. These observations suggest that dephosphorylated pRB, rather than phosphorylated pRB, affects B and MM cell proliferation. Dephosphorylated pRB binds E2F and the complex induces G1 growth arrest by inhibiting transcription of genes regulating cell replication.\textsuperscript{49}

In our study, dephosphorylated pRB bound E2F protein in MM cells under conditions of serum starvation, whereas complexes of pRB-E2F decreased in the presence of IL-6, further confirming that IL-6 downregulates dephosphorylated pRB. Moreover, in the XG-1 IL-6-dependent MM cell line, phosphorylated pRB expression persisted in the absence of IL-6, although dephosphorylated pRB increased and growth decreased. These data suggest that IL-6 promotes MM cell proliferation by triggering pRB phosphorylation, thereby decreasing complexes of dephosphorylated pRB with E2F and allowing entry into the cell cycle, as expected. In previous studies of mouse M1 leukemia cells, IL-6 has been found to trigger dephosphorylation of pRB, decrease proliferation, and enhance differentiation, further demonstrating that IL-6 can mediate biologic sequelae via its effects on RB phosphorylation state.\textsuperscript{50,51}

Our studies show that RB antisense, but not RB sense, ODN induced both IL-6 secretion and proliferation in patient MM cells. In this setting, the resultant reduced amounts of pRB, including dephosphorylated pRB, facilitate entry into the cell cycle as mentioned above. However, a component of the proliferation noted in RB antisense ODN-treated MM
cells in our study was blocked by neutralizing MoAb to IL-6. These studies, therefore, suggest that inactivation of RB can contribute to autocrine IL-6-mediated growth in MM. Although not directly examined in this study, the increased IL-6 secretion induced by RB antisense ODN treatment of MM cells may be attributed, at least in part, to increased IL-6 transcription as it has been reported that wild type, but not mutated, RB can repress c-fos expression and AP-1 transcription activity in NIH3T3 cells via binding to a cis element (RB control element or RCE) in the c-fos promoter.52 RCE is highly analogous to a region between -126 and -101 of the IL-6 promoter, and pRB binds directly to IL-6 promoter and represses transcription.47,53,54 In addition, McLead et al55 observed nuclear proteins in MM cell lines that were able to bind to NFκB, NF-IL-6, and AP-1 response elements and whose quantity positively correlated with IL-6 production by these cells. These and the current studies suggest that phosphorylated (inactivated) pRB in MM, due to its inability to bind to RCE, cannot inhibit IL-6 transcription; therefore IL-6 transcription and secretion may occur. Ongoing studies are delineating the transcriptional versus posttranscriptional components of the increase in IL-6 secretion observed in the present report. Finally, previous reports that CD40L upregulates IL-6 secretion and phosphorylation of pRB in normal B, as well as MM cells,38,50 are also consistent with a similar mechanism. Altered expression of pRB and related production of IL-6 by tumor cells has also been reported in 30% of patients with acute myeloblastic leukemia and 58% of patients with high grade non-Hodgkin’s lymphoma,57-59 consistent with IL-6-mediated sequelae from abnormalities in pRB in hematologic malignancies other than MM. Future studies in hematologic malignancies of the interactions between growth factors and abnormalities of cell cycle regulating proteins may not only enhance our understanding of tumor cell growth and progression, but also suggest novel therapeutic strategies.

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IL-6 AND RB IN MULTIPLE MYELOMA


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Interleukin-6 promotes multiple myeloma cell growth via phosphorylation of retinoblastoma protein

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