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Cytokines Modulate Telomerase Activity in a Human Multiple Myeloma Cell Line[1]

Masaharu Akiyama, Teru Hideshima, Toshiaki Hayashi, Yu-Tzu Tai, Constantine S. Mitsiades, Nicholas Mitsiades, Dharminder Chauhan, Paul Richardson, Nikhil C. Munshi, and Kenneth C. Anderson[2]

Jerome Lipper Multiple Myeloma Center, Department of Adult Oncology, Dana-Farber Cancer Institute, and Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT

Telomerase is a ribonucleoprotein DNA polymerase that elongates the telomeres of chromosomes to compensate for losses that occur with each round of DNA replication and maintain chromosomal stability. Interleukin 6 (IL-6) and insulin-like growth factor 1 (IGF-1) are proliferative and survival factors for human multiple myeloma (MM) cells. To date, however, the effects of IGF-1 and IL-6 on telomerase activity and associated sequelae in MM cells have not been characterized. In this study, we evaluated the effects of IGF-1 and IL-6 on telomerase activity in MM cell lines (MM.1S, U266, and RPMI 8226), as well as patient MM cells. We show that these cytokines up-regulate telomerase activity without alteration of human telomerase reverse transcriptase (hTERT) protein expression. We also demonstrate that increased telomerase activity triggered by these cytokines is mediated by phosphatidylinositol 3′-kinase (PI3k)/Akt/nuclear factor κB (NFκB) signaling. We confirm involvement of PI3k/Akt/NFκB signaling because the PI3k inhibitors wortmannin and LY294002 or the inhibitor of NFκB (IκB) kinase inhibitor PS-1145 block constitutive and cytokine-induced up-regulation of telomerase activity. Furthermore, we show that dexamethasone (Dex) reduces telomerase activity through the inhibition of hTERT expression before the induction of apoptosis. Importantly, IGF-1 and IL-6 abrogate Dex-induced down-regulation of telomerase activity and apoptosis. The protective effect of those cytokines against Dex-induced down-regulation of telomerase activity is blocked by both wortmannin and PS-1145, whereas the protection against Dex-induced apoptosis is blocked by wortmannin but not PS-1145. Therefore, our results demonstrate that telomerase activity is related not only to transcriptional regulation of hTERT by NFκB but also to post-transcriptional regulation because of phosphorylation of hTERT by Akt kinase. These studies therefore demonstrate that telomerase activity is associated with cell growth, survival, and drug resistance in MM cells.

INTRODUCTION

MM[3] is characterized by the expansion of monoclonal plasma cells in the BM (1). We have characterized the mechanisms of myeloma growth, survival, and apoptosis in the BM microenvironment (2, 3). IL-6 (4–12) and IGF-1 (13–16), which are both proliferative and survival factors for human MM cells, are produced at high concentrations in the BM microenvironment by osteoblasts, BM stromal cells, and bone endothelial cells. Importantly, we have demonstrated that these cytokines protect MM cells against both conventional (Dex; Refs. 6–12) and novel (thalidomide and its potent analogs IMiDs; Refs. 17, 18) therapy.

Telomeres are specialized nucleoprotein complexes that protect against fusion and degradation of linear chromosomes (19). Moreover, telomeres regulate mitosis through a checkpoint mechanism because a critical shortening of telomeres leads to cessation of irreversible cell division (senescence; Ref. 20). Telomerase is a ribonucleoprotein DNA polymerase that elongates the telomeres of chromosomes to compensate for losses that occur with each round of DNA replication (21). Therefore, unlimited proliferation in tumor cells requires this enzyme to maintain chromosomal stability and to counteract the cellular mitotic clock. Conversely, inhibition of telomerase shortens telomeres and also increases the susceptibility to apoptosis (22–25). Our recent studies indicate that IGF-1 and IL-6 can activate PI3k, Akt, and NFκB in MM cells (12, 26). Moreover, our studies show that PI3k signaling mediates protection conferred by these cytokines against Dex-induced apoptosis in MM cells. Akt protein kinase not only inactivates proapoptotic proteins (BAD protein or caspase 9; Ref. 27) but also enhances telomerase activity through the phosphorylation of hTERT protein (28). We have shown that NFκB activity in MM cells confers growth and drug resistance (29); although there are two putative NFκB binding motifs on the promotor, no binding was observed (30). Therefore, the role of IGF-1 and IL-6 in regulation of telomerase activity in MM cells is presently undefined.

In this study, we evaluate the effects of IGF-1 and IL-6 on telomerase activity in MM.1S, U266, and RPMI 8226 cells, as well as patient MM cells. We show that IGF-1 induces telomerase activity in MM.1S and U266 cells and that IL-6 up-regulates telomerase activity in MM.1S cells without associated alterations in hTERT protein expression. Moreover, IGF-1 and IL-6 can also activate telomerase activity in freshly isolated MM patient samples. PI3k inhibitors wortmannin and LY294002 and IKK inhibitor PS-1145 block constitutive and cytokine-induced up-regulation of telomerase activity in MM.1S cells. Furthermore, Dex, a known apoptotic agent in MM cells, reduces telomerase activity through the transcriptional repression of hTERT in MM.1S cells. Importantly, both IGF-1 and IL-6 abrogate Dex-induced inhibition of telomerase activity and apoptosis. This protective effect of IGF-1 and IL-6 on telomerase activity is inhibited by wortmannin and PS-1145, whereas the protective effect on apoptosis is inhibited by wortmannin but not PS-1145. Therefore, our results demonstrate that regulation of telomerase activity is associated with cytokine-induced growth, survival, and drug resistance in MM cells and provide the rationale for novel therapies based upon targeting telomerase.

MATERIALS AND METHODS

Reagents. Recombinant human IL-6 (Genetics Institute, Cambridge, MA) and IGF-1 (R&D Systems, Minneapolis, MN) were reconstituted with sterile PBS and stored at −20°C. Activated recombinant Akt was purchased from Upstate Biotechnology (Lake Placid, NY). IKK inhibitor PS-1145 (31) was obtained from Millennium Pharmaceuticals (Cambridge, MA).

Cell Lines and Cell Culture. Human MM cell lines U266 and RPMI 8226 were obtained from the American Type Culture Collection (Rockville, MD). MM.1S cells were kindly provided by Dr. Steven Rosen (Northwestern University, Chicago, IL). All cell lines were cultured in RPMI 1640 (Mediatech, Herndon, VA) with 10% fetal bovine serum (Harlan, Indianapolis, IN), con-

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[2] To whom requests for reprints should be addressed, at Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115. Phone: (617) 632-2144; Fax: (617) 632-2140; E-mail: Kenneth_anderson@dfci.harvard.edu.

[3] The abbreviations used are: MM, multiple myeloma; BM, bone marrow; IL-6, interleukin 6; NFκB, nuclear factor κB; IκB, inhibitor of NFκB; IGF-1, insulin-like growth factor 1; TRAP, telomerase repeat amplification protocol; Dex, dexamethasone; IMiD, immunomodulatory drug; PI3k, phosphatidylinositol 3′-kinase; hTERT, human telomerase reverse transcriptase; IKK, IκBα kinase; Ab, antibody.

taining 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc., Grand Island, NY).

Treatment of Cells. Before stimulating MM.1S, U266, or RPMI 8226 cells with either IGF-1 or IL-6, cells were grown in serum-free media for at least 18 h. When the effects of Dex (Sigma Chemical, St. Louis, MO), wortmannin (Wako Pure Chemical Industries Ltd., Osaka, Japan), LY294002 (Calbiochem, San Diego, CA), and PS-1145 were tested, these agents were added 1 h before the addition of IGF-1 or IL-6.

MM Cells from MM Patients. BM specimens were acquired from patients with MM after obtaining informed consent. MM cells purified as previously described (32) were cultured in RPMI 1640 with 1% fetal bovine serum, containing 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Evaluation of Cell Viability. Viable cells were enumerated by trypan blue dye exclusion using a hematocytometer and a phase-contrast microscope. Experiments were performed three times to determine mean ± SD percentage of viable cells.

Cell Proliferation Assay. Cell proliferation was measured by [^{3}H]thymidine (NEN Life Science Products, Boston, MA) incorporation. MM cells (2×10^4 cells/well) were incubated at 37°C in 96-well culture plates with or without IL-6 and IGF-1. [^{3}H]Thymidine (0.5 μCi) was added in each well for the last 8 h of 48-h cultures. Cells were harvested onto glass filters with an automatic cell harvester (Cambridge Technology, Cambridge, MA) and counted using a MicroBeta Trilux counter (Wallac, Gaithersburg, MD). Proliferation was defined by the stimulation index: [^{3}H]thymidine uptake of sample in media with IGF-1 or IL-6/[^{3}H]thymidine uptake of control sample in media alone.

Telomerase Assay. The telomerase assay was performed using a $TRAP_{EZE}$ Telomerase Detection kit (Oncor, Gaithersburg, MD). Each extract of cells was diluted 1:40 so that an aliquot of 2.0 μl corresponded to 250 cells for cell lines and 5000 cells for patient samples. After incubation for 20 min at 30°C, PCR amplification was performed with 30 cycles at 94°C for 30 s, at 58°C for 30 s, and at 72°C for 60 s. To determine whether telomerase activity is regulated by Akt kinase activity, we used the recombinant Akt kinase (Upstate). The lysate of MM.1S cells, corresponding to 5000 cells, was incubated with or without recombinant activated Akt in a reaction buffer [20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, and 1.3 mM $CaCl_2$) at 30°C for 30 min. Then, 1 μl of the pretreatment reaction mixture, corresponding to 250 cells, was used for telomerase assay. The PCR products were analyzed by electrophoresis on 12% polyacrylamide nondenaturating gels and stained with Sybr Green I (Molecular Probes, Eugene, OR). The gels were photographed using a digital camera and an UV transilluminator (Alpha Innotech Corporation, San Leandro, CA). Telomerase activity was assessed by determining the ratio of the entire telomerase ladder to that of the internal control using NIH image analysis software.

RNA Isolation and Reverse Transcriptase-PCR. Total RNA was isolated using an Isogen RNA extraction kit (Nippongene, Toyama, Japan). The first-strand cDNA was synthesized using M-MLV reverse transcriptase (Life Technologies, Inc.) as described previously (33). Briefly, 6 μg of total RNA were transcribed using a random hexamer primer (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) in a reaction mixture with a total volume of 30 μl. PCR was done using 1.5 μl of first-strand cDNA. The reaction sequence was at 94°C for 30 s, at 60°C for 30 s, and at 72°C for 60 s (30 cycles for hTERT and 21 cycles for β-actin). The amplified products were separated on 2% agarose gel. The primer sequences and amplified product sizes were as follows: hTERT (33), (sense) 5′-TGAACTTGCGGAAGACAGTGG-3′ and (antisense) 5′-ATGCGTGAAACCTGTACGCCT-3′ (301 bp); and β-actin (33), (sense) 5′-GTGGGGCGCCCCAGGCACCA-3′ and (antisense) 5′-CTCCTTAATGTCACGCACGATTTC-3′ (517 bp).

Immunoblotting. Cells were harvested, washed with ice-cold PBS, and lysed with buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP40, 2 mM sodium orthovanadate, and protease inhibitor mixture (Complete; Roche Diagnostics). An equal amount (100 μg) of the samples was separated on SDS-polyacrylamide gel and transferred onto nitrocellulose filters (Bio-Rad, Hercules, CA). The membranes were immunoblotted with Abs against hTERT (Calbiochem), α-tubulin (Sigma), Akt (Cell Signaling, Beverly, MA), and phospho-Akt (Ser^{473}; Cell Signaling). For detection of phosphorylated hTERT, cell lysates were prepared from IGF-1- or IL-6-treated MM.1S cells (2×10^7) using lysis buffer. Lysates were incubated with anti-hTERT Ab (Calbiochem) overnight and then immunoprecipitated for 6 h with protein A-Sepharose (Sepharose CL-4B; Pharmacia, Uppsala, Sweden). Immune complexes were washed, electrophoresed, and analyzed by immunoblotting with anti-phospho-Akt substrate Ab (Cell Signaling). Immunoprecipitates of hTERT in the lysate of MM.1S cells (2×10^7) were also incubated with or without the activated recombinant Akt in protein kinase assay. The reactions were stopped by heating to 95°C for 10 min, and phosphorylated hTERT proteins were similarly analyzed by immunoblotting with anti-phospho-Akt substrate Ab (Cell Signaling) and anti-hTERT Ab (Calbiochem). The immunoblots were detected by ECL chemiluminescence method, according to the manufacturer's recommendations (Amersham Pharmacia Biotech).

Statistical Analysis. A paired *t* test was performed by using Stat View4.5 software (Abacus Concept, Inc., Berkeley, CA). The minimal level of significance was $P < 0.05$.

RESULTS

Effects of IGF-1 and IL-6 on DNA Synthesis and Telomerase in MM Cell Lines. We first studied the effects of IGF-1 and IL-6 on DNA synthesis of MM cell lines (MM.1S, U266, and RPMI 8226) by measuring [^{3}H]thymidine uptake during the last 8 h of 48-h cultures in the presence or absence of IGF-1 or IL-6 at various concentrations. No significant change in DNA synthesis of MM cell lines was noted in response to IGF-1 (data not shown). However, 5–100 ng/ml IL-6 induced proliferation of MM.1S cells (Stimulation index; 2.2–2.3) but not of U266 and RPMI 8226 cells (data not shown).

We next evaluated dose-dependent effects of IGF-1 and IL-6 on telomerase activity in MM cell lines. Culture with IGF-1 (50 and 100 ng/ml) and IL-6 (50 and 100 ng/ml) for 24 h induces 2.3 + 0.2 ($P = 0.006$) and 2.6 + 0.2 ($P = 0.005$) fold increases and 1.4 + 0.1 ($P = 0.007$) and 1.3 + 0.0 ($P = 0.003$) fold increases in telomerase activity, respectively, in MM.1S cells (Fig. 1*A*). In U266 cells, a 1.5 + 0.1 ($P = 0.01$) fold increase in telomerase activity is noted at 12 h in cultures with 100 ng/ml IGF-1. In contrast, no increase in telomerase activity is triggered by IGF-1 or IL-6 in RPMI 8226 cells, which are unresponsive to either cytokine.

We next delineated the time-dependent effects of IGF-1 and IL-6 on telomerase activity. IGF-1 (100 ng/ml) and IL-6 (50 ng/ml) trigger significant increases in telomerase activity in MM.1S cells at 24 h (relative telomerase activity, 2.5 ± 0.7; $P = 0.0009$ and 1.4 ± 0.3; $P = 0.03$, respectively; Fig. 1*B*). Moreover, a significant increase in telomerase activity in U266 cells (relative telomerase activity, 1.5 ± 0.1; $P = 0.01$) is triggered after a 12-h culture with IGF-1 (100 ng/ml). In contrast, no increase in telomerase activity is seen in RPMI 8226 cells cultured with IGF-1 (100 ng/ml) or IL-6 (50 ng/ml).

Having defined these cytokine effects on MM cell lines, we next similarly examined freshly isolated patient MM cells. As seen on Fig. 1*C*, telomerase activity in MM patient 1 decreases by a 24-h culture in media but not in cultures with IL-6 (50 ng/ml): it is increased 1.6-fold in cultures with IGF-1 (100 ng/ml). Moreover, telomerase activity in MM patient 2 increases 3.2-fold in cultures with IGF-1 (100 ng/ml) and 2.9-fold in culture with IL-6 (50 ng/ml).

Mechanisms of IGF-1 and IL-6 Induced Telomerase Activity in MM Cells. We next characterized mechanisms of telomerase activity triggered by these cytokines. We first demonstrated that telomerase activation triggered by IGF-1 (100 ng/ml) and IL-6 (50 ng/ml) is not associated with alterations of hTERT mRNA and hTERT protein in MM.1S cells (Fig. 2).

We next delineated the role of PI3k/Akt-signaling cascade in regulating telomerase activity in MM cells. To specifically confirm that Akt kinase induces telomerase activity through the phosphorylation of hTERT, we performed the TRAP assay and analysis of hTERT phosphorylation with or without pretreatment of the activated recombinant Akt kinase *in vitro*. Pretreatment with 50, 100, and 200 ng

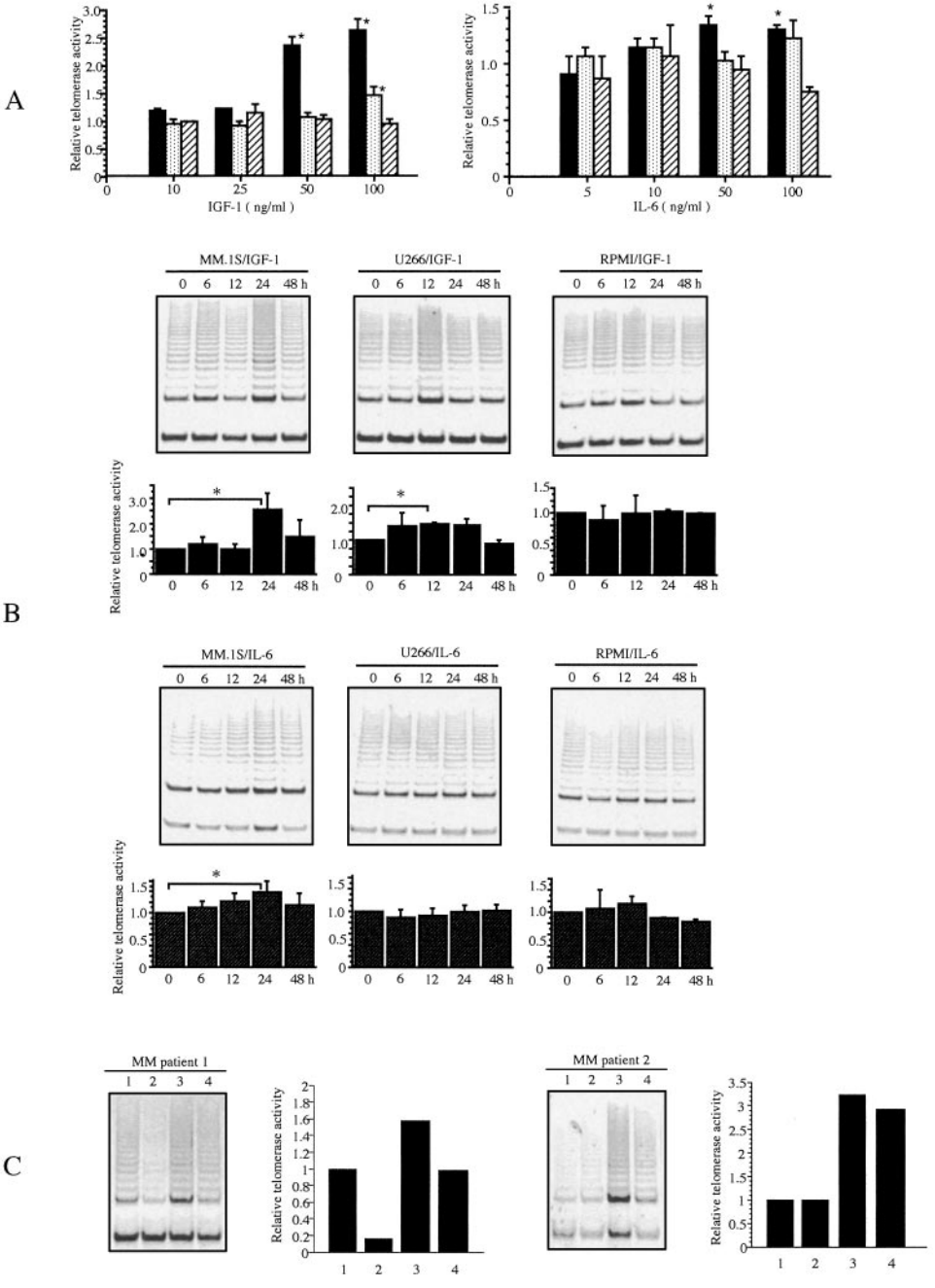

Fig. 1. Effects of IGF-1 and IL-6 on proliferation and telomerase activity in MM cell lines. *A*, MM.1S (■) and RPMI 8226 (▨) cells were cultured with IGF-1 (10–100 ng/ml) and IL-6 (5–100 ng/ml) for 24 h; U266 (▤) cells were cultured with IGF-1 (10–100 ng/ml) for 12 h and IL-6 (5–100 ng/ml) for 24h. Telomerase activity was compared with nontreated cells. *, $P < 0.05$ relative to untreated cells. *B*, these cell lines were cultured with IGF-1 (100 ng/ml) or IL-6 (50 ng/ml) for 0–48 h. Telomerase activity was compared with control (0 h). Values represent the mean ± SD of triplicate cultures. *, $P < 0.05$ relative to control (0 h). *C*, freshly isolated MM cells from two patients were cultured with or without IGF-1 (100ng/ml) or IL-6 (50ng/ml) for 24h: *Lane 1*, MM cells (0 h); *Lane 2*, MM cells in media (24 h); *Lane 3*, MM cells with IGF-1 (24 h); and *Lane 4*, MM cells with IL-6 (24 h).

activated Akt kinase-enhanced telomerase activity by 1.1-, 1.4-, and 2.3-fold, respectively (Fig. 3*A*). Moreover, we observed that the treatment of Akt induced phosphorylation of hTERT protein in a dose-dependent manner: the ratio of phosphorylated hTERT:unphosphorylated hTERT was 0.02, 0.1, 0.93, respectively (Fig. 3*B*).

Having shown that Akt kinase increases telomerase activity through phosphorylation of hTERT, we next determined whether IL-6 and IGF-1 also increase telomerase activity in this fashion. IGF-1 and IL-6 up-regulate telomerase activity in MM.1S cells, whereas both wortmannin (0.2 μM) and LY294002 (10 μM) block constitutive and cytokine-induced telomerase activity without alteration of hTERT mRNA and hTERT protein expression (Fig. 4, *A* and *B*). This decrease in telomerase activity is not related to the loss of cell viability because >70% of cells remain viable at 24 h (Fig. 4*C*). Importantly, both wortmannin (0.2 μM) and LY294002 (10 μM) inhibit Akt phosphorylation, even in the presence of IGF-1 and IL-6 (Fig. 4*D*). Moreover,

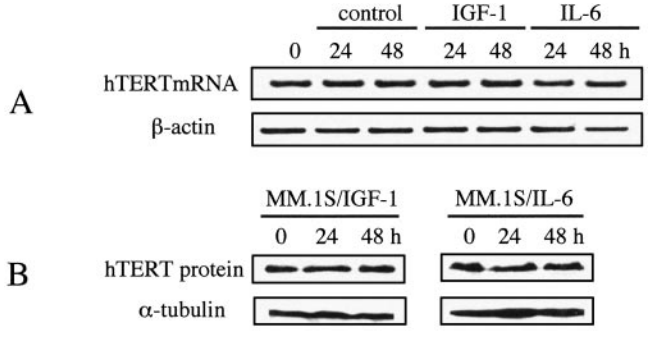

Fig. 2. Effects of IGF-1 and IL-6 on hTERT mRNA and hTERT protein in MM.1S cells. MM.1S cells were cultured without or with IGF-1 (100 ng/ml) or IL-6 (50 ng/ml) for 24 and 48 h. *A*, the expression of hTERT mRNA and β-actin was analyzed by reverse transcriptase-PCR. *B*, the expression of hTERT protein and α-tubulin was analyzed by immunoblotting with anti-hTERT Ab and α-tubulin Ab.

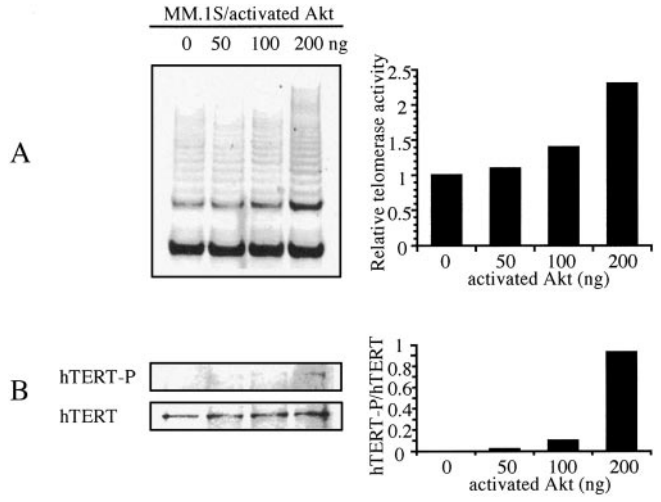

Fig. 3. Akt kinase regulates telomerase activity with hTERT phosphorylation. *A*, to determine whether telomerase activity is regulated by Akt kinase activity, telomerase activity of MM.1S cell lysate after pretreatment with activated Akt kinase (50–200 ng) was assayed as described in "Materials and Methods." *B*, to demonstrate that Akt kinase phosphorylates hTERT protein, a protein kinase assay was performed. Activated recombinant Akt kinase induced dose-dependent increases in hTERT protein phosphorylation.

both wortmannin and LY294002 inhibit IGF-1-induced hTERT phosphorylation (Fig. 4*D*). These results confirm the importance of the PI3k/Akt kinase pathway for telomerase activation.

Because NFκB is a downstream target of Akt signaling (34–37), we examined whether the specific IKK inhibitor PS-1145 abrogated IGF-1- and IL-6-induced telomerase activity in MM.1S cells. PS-1145 in a dose-dependent fashion down-regulates both constitutive and cytokine-induced telomerase activity, with reduction of both hTERT mRNA and protein expression (Fig. 5, *A–C*); there was no significant change in cell viability at 24 h (Fig. 5*D*).

Effects of IGF-1 and IL-6 on Dex-induced Apoptosis and Associated Down-Regulation of Telomerase Activity. Dex induces down-regulation of hTERT mRNA at 6 h and telomerase activity at 12 h (Fig. 6*A*). This decrease in telomerase activity is related to the inhibitory effect of Dex rather than an effect on cell viability because >80% of cells remain viable at 6 h and 12 h (Fig. 6*B*). Both IGF-1 and IL-6 protect against Dex-induced down-regulation of hTERT mRNA and telomerase activity (Fig. 6*A*). As seen in Fig. 6*B*, Dex induces loss of viability at 24 h in MM.1S cells (63.9 $\pm$ 3.7%), whereas both IGF-1 and IL-6 protect against Dex-induced loss of cell viability (87.7 $\pm$ 0.1%; $P = 0.008$ and 86.5 $\pm$ 0.2%; $P = 0.01$, respectively).

To confirm the importance of PI3k/Akt/NFκB signaling, we again specifically blocked PI3k using wortmannin and IKK using PS-1145. Wortmannin (0.2 μM) and PS-1145 (10 μM) abrogate the protective effects of both IGF-1 and IL-6 against both down-regulation of telomerase activity (Fig. 7*A*) and suppression of hTERT mRNA and protein (Fig. 7, *B* and *C*) induced by Dex; both wortmannin and PS-1145 can enhance Dex-induced inhibition of telomerase. However, the protective effect of IGF-1 and IL-6 against Dex is blocked by wortmannin but not by PS-1145 (Fig. 7*D*).

DISCUSSION

IGF-1 and IL-6 play an essential role as proliferative and survival factors for human MM cells (1–16). Telomerase compensates for telomere loss with each round of DNA replication, thereby maintaining chromosomal stability and promoting continued proliferation while avoiding senescence and susceptibility to apoptosis (19–21). In this study, we demonstrate that IGF-1 and IL-6 induce telomerase activity in MM cells without associated alterations of hTERT proteins.

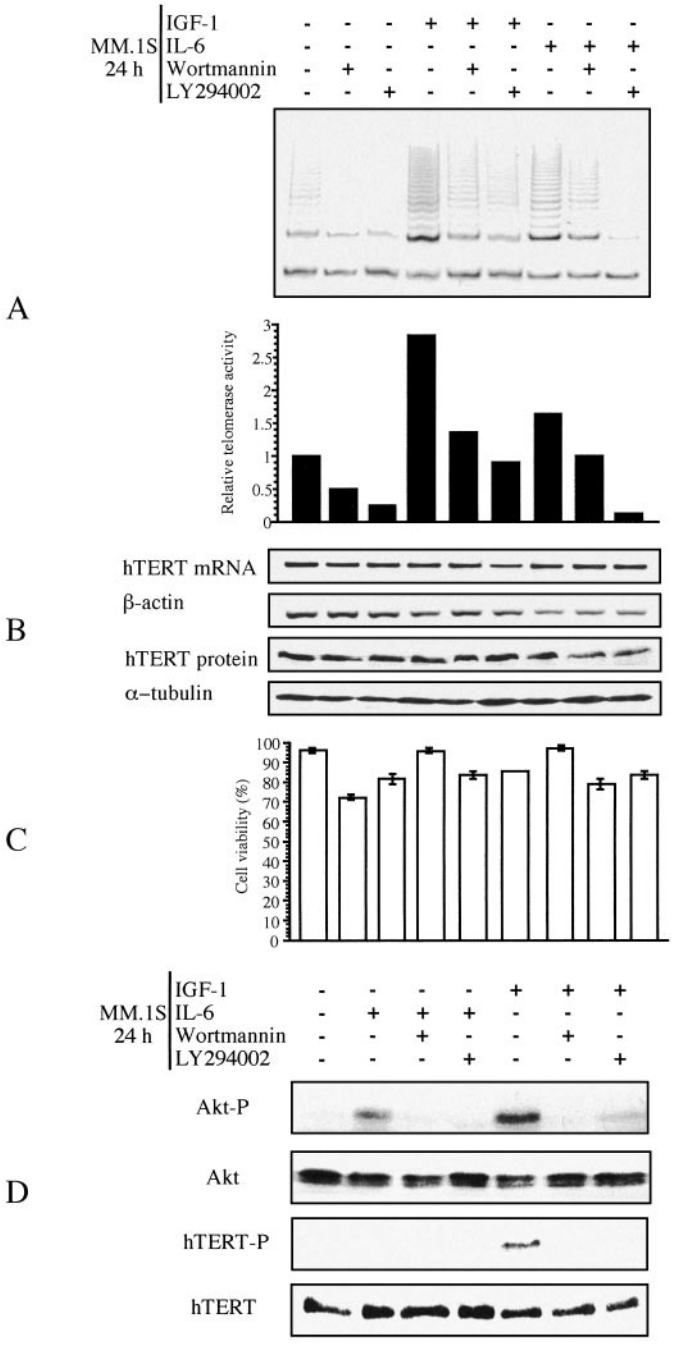

Fig. 4. Cytokine-induced telomerase activity is inhibited by PI3k inhibitors. *A*, MM.1S cells were cultured for 24 h with media (control), wortmannin (0.2 μM), LY294002 (10 μM), IGF-1 (100 ng/ml), wortmannin (0.2 μM) + IGF-1 (100 ng/ml), LY294002 (10 μM) + IGF-1 (100 ng/ml), IL-6 (50 ng/ml), wortmannin (0.2 μM) + IL-6 (50 ng/ml), and LY294002 (10 μM) + IL-6 (50 ng/ml). Telomerase activity was measured using the TRAP assay and compared in treated *versus* untreated controls. *B*, the expression of hTERT mRNA and β-actin was analyzed by reverse transcriptase-PCR. The expression of hTERT protein and α-tubulin was analyzed by immunoblotting with anti-hTERT Ab and α-tubulin Ab. *C*, an evaluation of cell viability was performed by trypan blue dye exclusion using a hematocytometer and a phase-contrast microscope. Experiments were performed three times to determine mean $\pm$ SD percentage of viable cells. *D*, MM.1S cells were cultured for 24 h with media (control), IL-6 (50 ng/ml), wortmannin (0.2 μM) + IL-6 (50 ng/ml), LY294002 (10 μM) + IL-6 (50 ng/ml), IGF-1 (100 ng/ml), wortmannin (0.2 μM) + IGF-1 (100 ng/ml), and LY294002 (10 μM) + IGF-1 (100 ng/ml). Cells were lysed, subjected to SDS-PAGE, transferred to nitrocellulose membrane, and blotted with anti-phospho-Akt Ab. The membrane was stripped and reprobed with anti-Akt Ab. Cell lysates were immunoprecipitated with anti-hTERT Ab. The immunoprecipitates were subjected to SDS-PAGE, transferred to nitrocellulose membrane, and blotted with anti-phospho-Akt substrate Ab (hTERT-p). The membrane was stripped and reprobed with anti-hTERT Ab.

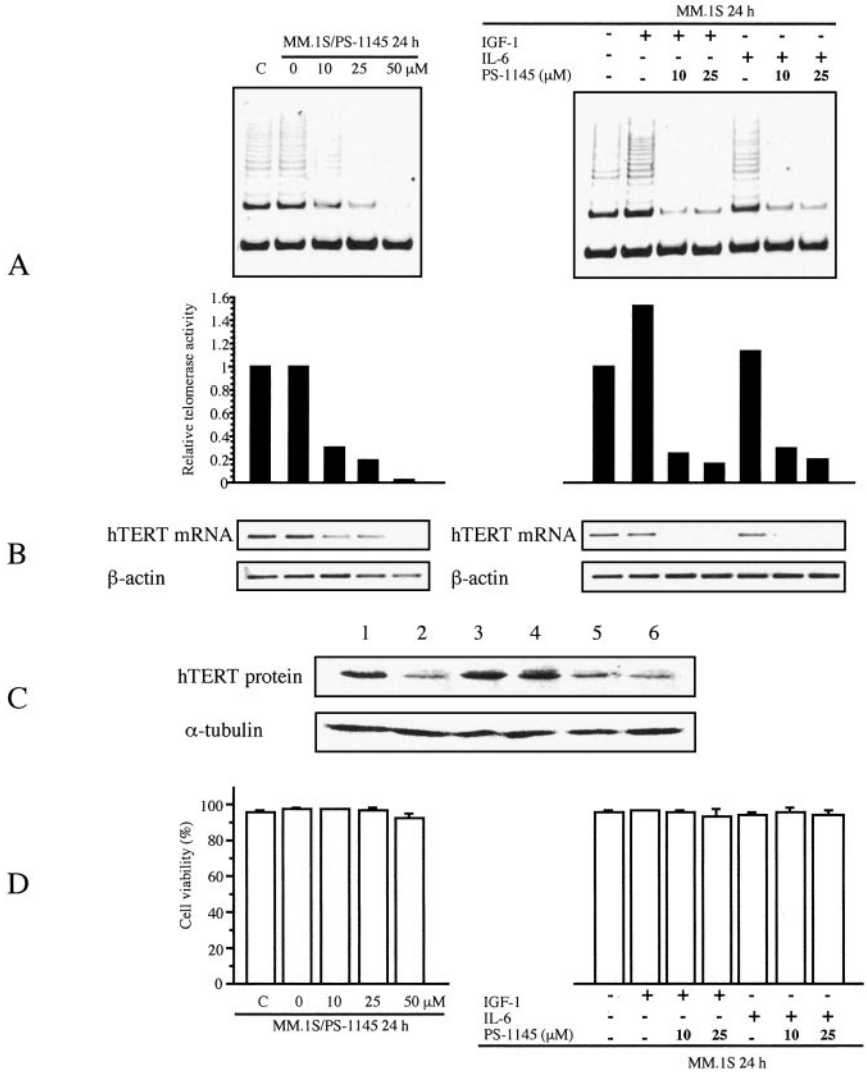

Fig. 5. Cytokine-induced telomerase activity is inhibited by IKK inhibitors. *A*, MM.1S cells were cultured with PS-1145 (0–50 μM) and PS-1145 (10, 25 μM) in the presence of IGF-1 (100 ng/ml) or IL-6 (50 ng/ml) for 24 h. Telomerase activity was compared with untreated cells. *B*, the expression of hTERT mRNA and β-actin was analyzed by reverse transcriptase-PCR. *C*, the expression of hTERT protein and α-tubulin was analyzed by immunoblotting with anti-hTERT Ab and α-tubulin Ab. MM.1S cells were cultured for 24 h with media (*Lane 1*), PS-1145 (10 μM; *Lane 2*), IGF-1 (100 ng/ml; *Lane 3*), IL-6 (50 ng/ml; *Lane 4*), IGF-1 (100 ng/ml) + PS-1145 (10 μM; *Lane 5*), and IL-6 (50 ng/ml) + PS-1145 (10 μM, *Lane 6*). *D*, an evaluation of cell viability was performed by trypan blue dye exclusion using a hematocytometer and a phase-contrast microscope. Experiments were performed three times to determine mean ± SD percentage of viable cells.

We show that the increase in telomerase activity triggered by theses cytokines is mediated via PI3k, Akt, and NFκB signaling.

A recent study shows low telomerase activity despite expression of hTERT protein in normal human T lymphocytes and that stimulation with anti-CD3/CD28 induces telomerase activity via phosphorylation of hTERT protein (38). In contrast, most tumor cell lines have constitutively phosphorylated hTERT protein. To evaluate the effect of IGF-1 and IL-6 on phosphorylation of hTERT protein in MM cells,

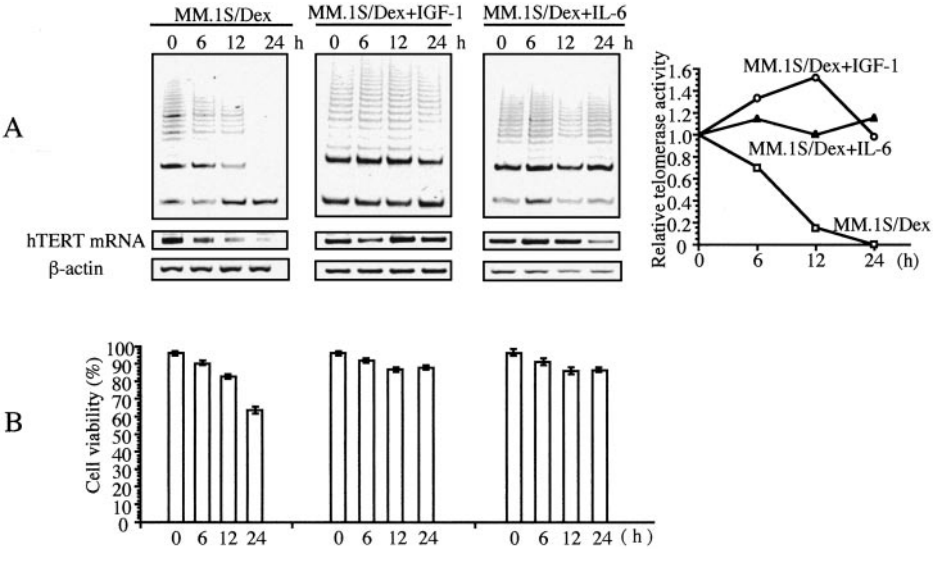

Fig. 6. Effects of IGF-1 and IL-6 on Dex-induced apoptosis and associated down-regulation of telomerase activity. *A*, MM.1S cells were cultured with Dex (1 μM), Dex (1 μM) + IGF-1 (100 ng/ml), and Dex (1 μM) + IL-6 (50 ng/ml) for 0–24 h. Telomerase activity was measured using the TRAP assay and compared in treated *versus* untreated controls. The expression of hTERT mRNA and β-actin was analyzed by reverse transcriptase-PCR. *B*, an evaluation of cell viability was performed by trypan blue dye exclusion using a hematocytometer and a phase-contrast microscope. Experiments were performed three times to determine mean ± SD percentage of viable cells.

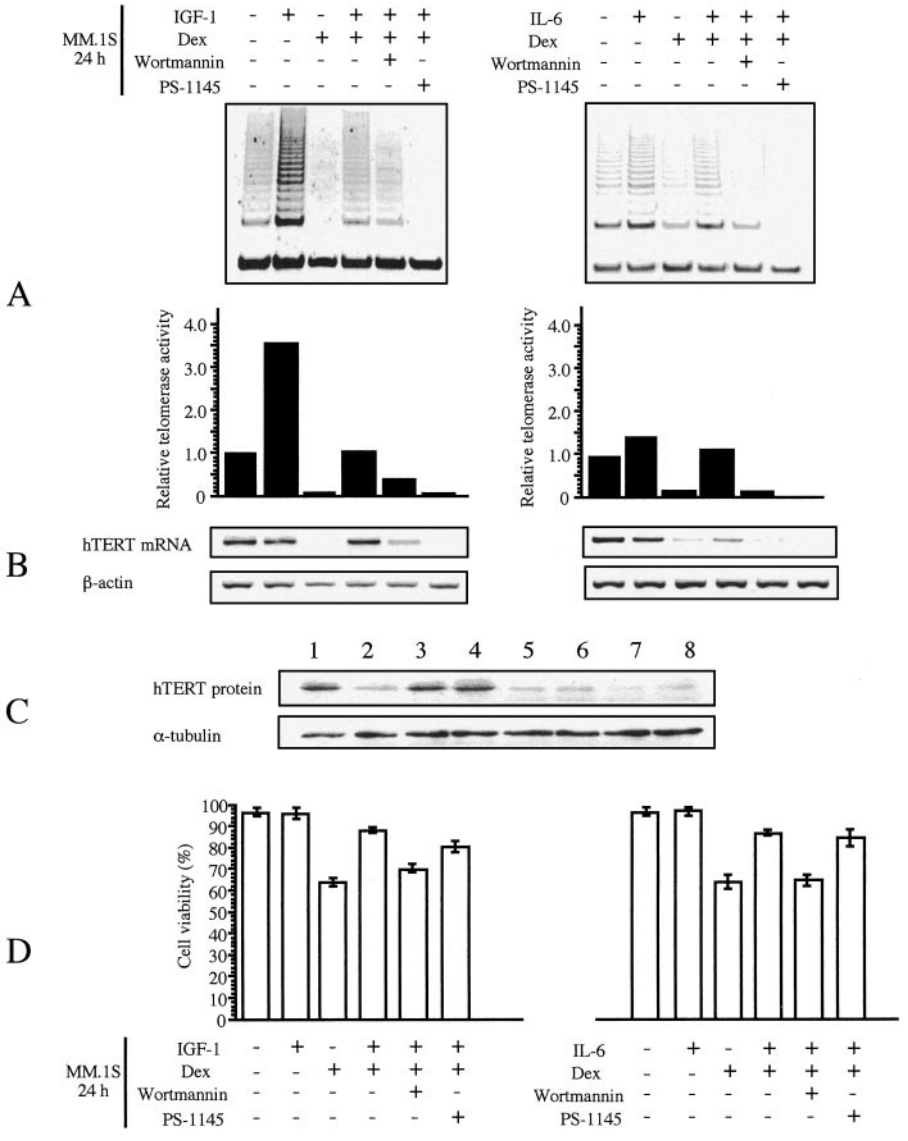

Fig. 7. Effects of IGF-1 and IL-6 on Dex-induced down-regulation of telomerase activity and apoptosis. *A*, MM.1S cells were cultured for 24 h with media, IGF-1 (100 ng/ml), Dex (1 μM), Dex (1 μM) + IGF-1 (100 ng/ml), Dex (1 μM) + IGF-1 (100 ng/ml) + wortmannin (0.2 μM), and Dex (1 μM) + IGF-1 (100 ng/ml) + PS-1145 (10 μM). Telomerase activity was assessed as described above. *B*, the expression of hTERT and β-actin mRNA was analyzed by reverse transcriptase-PCR. *C*, MM.1S cells were cultured for 24 h with media (*Lane 1*), Dex (1 μM; *Lane 2*), Dex (1 μM) + IGF-1 (100 ng/ml; *Lane 3*), Dex (1 μM) + IL-6 (50 ng/ml; *Lane 4*), Dex (1 μM) + IGF-1 (100 ng/ml) + wortmannin (0.2 μM; *Lane 5*), Dex (1 μM) + IGF-1 (100 ng/ml) + PS-1145 (10 μM; *Lane 6*), Dex (1 μM) + IL-6 (50 ng/ml) + wortmannin (0.2 μM; *Lane 7*), and Dex (1 μM) + IL-6 (50 ng/ml) + PS-1145 (10 μM; *Lane 8*). The expression of hTERT protein and α-tubulin was analyzed by immunoblotting with anti-hTERT Ab and α-tubulin Ab. *D*, an evaluation of cell viability was performed by trypan blue dye exclusion using a hematocytometer and a phase-contrast microscope. Experiments were performed three times to determine mean ± SD percentage of viable cells.

we cultured MM.1S cells in serum-free medium for 18 h before addition of either IGF-1 or IL-6 because serum contains cytokines. We found that IGF-1 and IL-6 induce Akt kinase activity, confirming our (12) and other (11) prior studies in MM cells. Furthermore, our study shows that IGF-1 induced Akt kinase activity is associated with phosphorylation of hTERT protein.

Because Akt kinase activity mediates phosphorylation of hTERT protein and up-regulation of telomerase activity (28), we next examined the importance of PI3k/Akt signaling in regulating telomerase activity in MM cells. We demonstrate that the PI3k inhibitors wortmannin and LY294002 down-regulate both constitutive and cytokine-induced telomerase activity through the suppression of both Akt and hTERT phosphorylation without alteration of hTERT protein expression. These results suggest that IGF-1 and IL-6 activate telomerase activity via the PI3k/Akt pathway.

We next examined the importance of NFκB signaling in regulating telomerase activity in MM cells. To confirm the role of NFκB in regulating telomerase activity, we used the specific IKK inhibitor PS-1145, which can abrogate NFκB-mediated binding of MM cells to BM stroma cells and cytokine production in the BM milieu (31). Our study shows that PS-1145 blocks both constitutive and cytokine-induced up-regulation of telomerase activity in MM.1S cells through the inhibition of hTERT transcription without an associated decrease in cell viability. Although the hTERT promotor revealed two putative NFκB binding motifs, no DNA binding was observed (30). Therefore, NFκB may regulate hTERT expression via c-Myc because c-Myc is a downstream target of NFκB that up-regulates hTERT transcription.

We next examined the relationship between telomerase activity and the antiapoptic effects of IGF-1 and IL-6. Our prior studies have shown that IL-6 and IGF-1 confer protection against apoptosis induced by conventional (Dex) and novel (IMiD) therapies via activation of protein tyrosine kinase SHP2 (9), Akt signaling (12), NFκB activation (31), as well as by up-regulating the expression of intracellular inhibitors of apoptosis (17, 18). Others have reported that IGF-1 and IL-6 induced activation of Akt kinase protects against apoptosis via phosphorylation of BAD protein and caspase 3 (27). In this study, we show that Dex induces down-regulation of hTERT mRNA at 6 h and telomerase activity at 12 h before induction of apoptosis. Our recent studies also show that Dex induces down-regulation of NFκB (39) and c-Myc (40) in MM cells. Importantly, we show that both IGF-1 and IL-6 abrogate Dex-induced inhibition of telomerase activity as well as Dex-induced apoptosis, and that these effects of IGF-1 and IL-6 on telomerase activity and hTERT expression are inhibited by both wortmannin and PS-1145, whereas the

cytokine effects on cell viability are blocked by only wortmannin. PS-1145 does not block the protective effects of IGF-1 and IL-6 on Dex-induced apoptosis because PS-1145 may enhance Dex-induced inhibition of IκB kinase activity and growth arrest in G_1 phase (31) but not Dex-induced apoptosis in MM.1S cells with intact p53 (17). These results suggest that IGF-1 and IL-6 induced up-regulation of telomerase activity via PI3k, Akt, and NFκB signaling mediates protection against drug-induced apoptosis. Conversely, the PI3k inhibitors wortmannin and LY294002 can block cytokine-induced telomerase activity and antiapoptotic effects.

The present studies have both biological and clinical relevance. First, it has been reported that activation of telomerase plays an important role in the evolution from monoclonal gammopathy of undetermined significance to MM (41). A recent DNA microarray analysis of a panel of high-risk MM patients revealed genetic profiles similar to MM cell line (42), and similar investigations in patient cells may confirm the relevance of telomerase reactivation in malignant progression of plasma cells *in vivo*. Second, our results demonstrating that cytokine-induced telomerase activity is associated with growth, survival, and drug resistance in MM cells provide additional rationale for therapies based upon targeting telomerase. Novel therapies, including thalidomide and IMiDs (17), proteasome inhibitor PS-341 (39), and arsenic trioxide (43), have demonstrated preclinical and early clinical activity in overcoming resistance to conventional chemotherapy in MM. These agents inhibit NFκB signaling, and ongoing studies are determining whether blockade of downstream telomerase activity contributes to their anti-MM activity.

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