

The Phosphatidylinositol 3-Kinase/AKT Kinase Pathway in Multiple Myeloma Plasma Cells: Roles in Cytokine-dependent Survival and Proliferative Responses¹

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ABSTRACT

Interleukin 6 (IL-6) and insulin-like growth factor I (IGF-I) induce proliferative and antiapoptotic responses in multiple myeloma (MM) plasma cells. Because these cytokines may activate the phosphatidylinositol 3-kinase (PI 3-K)/AKT kinase pathway in other cell types, we investigated the role of PI 3-K/AKT in MM cell responses. IGF-I effectively activated PI 3-K in 8226 and OCI-My5 MM cells, but IL-6 was ineffective. However, IL-6 successfully activated PI 3-K in AF-10 MM cells and IL-6-dependent MH.60 plasmacytoma/hybridoma cells. IGF-I also successfully activated PI 3-K in four of four MM patient specimens, and IL-6 activated PI 3-K in three of four specimens. Inhibition of PI 3-K activity with wortmannin or Ly294002 blocked the antiapoptotic effect of IGF-I and the proliferative effect of IL-6 in the myeloma cell lines. Furthermore, a dominant negative PI 3-K construct, expressed in AF-10 cells by adenoviral infection, also significantly inhibited the IL-6 proliferative response in MM cells. In correlation with activation of PI 3-K, IGF-I also effectively activated the AKT kinase in 8226 and OCI-My5 cells, and IL-6 activated AKT in AF-10 and MH.60 cells. However, although incapable of activating PI 3-K in 8226 and OCI-My5 cells, IL-6 successfully activated AKT in these MM lines, suggesting PI 3-K-independent mechanisms of AKT activation. The prevention of a myeloma cell proliferative response resulting from inhibition of PI 3-K activity was not associated with an inhibition of IL-6-dependent extracellular signal-regulated kinase (ERK) activation. These results support a role for the PI 3-K/AKT pathway in cytokine-dependent responses in myeloma cells, which is independent of any activation of the ERK pathway.

INTRODUCTION

The growth factors IL-6³ and IGF-I induce proliferation and protect viability of MM plasma cells *in vitro* (1–7). Furthermore, the bone marrow microenvironment, the specific locale where myeloma expansion occurs, contains high concentrations of these cytokines (8, 9). Thus, these responses to IL-6 and IGF-I theoretically promote tumor expansion *in vivo*. It, therefore, follows that identification of specific signal pathways that mediate the proliferative and survival pathways of these growth factors may have potential therapeutic relevance.

Several studies have investigated the role of the ERK, jun kinase, and STAT pathways in the proliferative and antiapoptotic responses of myeloma cells to cytokines (10–14). However, an additional signaling cascade that deserves attention is the PI 3-K/AKT kinase pathway. This pathway is important in the antiapoptotic survival-promoting effect of IGF-I in other cell types (15–17). Furthermore, a

recent study (18) indicates that IL-6 can activate PI 3-K and AKT in hepatocytes. Activation of PI 3-K occurs via binding of SH2 domains of the p85 regulatory subunit of PI 3-K to either cytoplasmic domains of receptors or receptor-associated adapter proteins (19, 20). This binding recruits PI 3-K to the membrane and activates the p110 catalytic subunit. PI 3-K phosphorylates PIs on the D-3 position of the inositol ring, and these phospholipids then elicit a diverse set of cellular responses (reviewed in Ref. 21). One target molecule of PI 3-K and the phospholipid second messengers is the serine-threonine kinase c-AKT (also known as protein kinase B), which is the cellular homologue of the *v-akt* oncogene. The phospholipid products of PI 3-K bind to the amino-terminal pleckstrin homology domain of AKT. This interaction results in membrane translocation of AKT, which brings it in proximity to PDKs 1 and 2. Subsequent phosphorylation of AKT on serine and threonine residues by PDK 1 and PDK 2 results in efficient activation of AKT kinase activity (22–24).

Because the PI 3-K/AKT pathway is involved in IGF-I-induced responses and may be potentially activated by IL-6, we, thus, initiated the current study, assessing the activation of the PI 3-K/AKT kinase pathway in cytokine-stimulated myeloma cells and its role in survival and proliferative responses.

MATERIALS AND METHODS

Cell Lines. The myeloma cell lines 8226, OCI-My5, and AF-10 (a subclone of U266; Ref. 12) were kind gifts from Drs. J. Epstein (University of Arkansas), H. Messner (University of Toronto), and James Berenson (UCLA), respectively. The MH.60 plasmacytoma/hybridoma line was a gift from Dr. Oto Martinez-Maza (UCLA). The AF-10 and 8226 cell lines were maintained in RPMI media supplemented with 10% FCS, L-glutamine, nonessential amino acids, pyruvate, and antibiotics. The OCI-My5 cell line was maintained in Iscove's modified Dulbecco's medium containing the same supplements. The MH.60 cell line was maintained in RPMI media supplemented with 10% FCS, 50 μ M 2-mercaptoethanol, antibiotics, and 1 ng/ml recombinant IL-6.

Isolation of Primary Myeloma Cells. Bone marrow cells from four patients with active myeloma were first separated by Ficoll-Hypaque density centrifugation. Resulting mononuclear cells were then further separated on an immunoabsorption column (25) using biotinylated anti-CD38 antibody to isolate high CD38-expressing cells. The separated cells consisted of >96% plasma cells.

Reagents. Recombinant IGF-I and IL-6 were purchased from R&D Systems (Minneapolis, MN). Anti-p85 (PI 3-K) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Ly was obtained from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). Phospho-specific and total anti-AKT and anti-ERK antibodies were obtained from New England Biolabs. All radioisotopes were from Amersham Corp. (Arlington Heights, IL). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

PI 3-K Assay. The assay was performed as described previously (17). Briefly, protein was extracted from cells by lysing them in buffer containing 1% Triton X-100, 150 mM Tris-HCl (pH 7.4), 1 mM EDTA (pH 8), 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM Na3VO4. PI 3-K activity was immunoprecipitated by anti-p85/protein A-agarose. The immunoprecipitates were washed three times with lysis buffer, three times with 0.5 M NaCl and 25 mM Tris-HCl (pH 7.5), and two times with 25 mM Tris-HCl (pH 7.5). The PI 3-K reaction was run in a reaction mixture containing 10 mM Tris (pH 7.5), 100 mM NaCl, 20 mM MgCl₂, 0.2 mM EGTA,

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³ The abbreviations used are: IL-6, interleukin 6; PI 3-K, phosphatidylinositol 3-kinase; IGF-I, insulin-like growth factor I; MM, multiple myeloma; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; Ly, Ly294002; wort, wortmannin; dex, dexamethasone; PI(3,4)P, phosphoinositol 3,4 diphosphate; PDK, 3-phosphoinositide-dependent kinase; MAP, mitogen-activated protein; DN dominant negative; ERK, extracellular signal-regulated kinase.

20 μg of phosphatidyl-4-monophosphate as substrate, 10 μM ATP, 10 μCi of (γ - ^{32}P)ATP, and inhibitors as described above. After proceeding for 5–30 min, the reaction was terminated and lipids were extracted in chloroform:methanol:HCl (100:200:2). The organic phase was collected, dried, and then redissolved in chloroform:methanol (1:1) and spotted on TLC plates. The plates were developed with chloroform:methanol:H₂O:NH₄OH (43:38:7:5), dried, and exposed to film. The location of PI(3, 4)P was determined by comparison with standards in iodine-stained TLC plates. PI 3-K activity was semiquantified by densitometric analysis of the autoradiogram of TLC plates.

Western Blot Analysis. Protein was extracted by lysing cells in lysis buffer [1% Triton X-100, 0.5% NP40, 10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EGTA, 0.2 mM Na₃VO₄, 0.2 mM NaF, and 0.2 mM phenylmethylsulfonyl fluoride]. Twenty-five micrograms of protein from each sample were boiled for 5 min in 1 \times SDS gel loading buffer. Proteins were separated by 12.5% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Membranes were blocked for 1 h in 3% BSA, 5% nonfat dried milk, 10 mM Tris (pH 7.5), 100 mM NaCl, and 0.1% Tween 20. The membranes were washed four times and then incubated with antihuman AKT and ERK antibodies or antibodies specific for phosphorylated AKT or ERK for 1 h. After four more washes, membranes were overlaid with 1 $\mu\text{g}/\text{ml}$ horseradish peroxidase-labeled antimouse IgG and protein bands were detected with an enhanced chemiluminescence system.

AKT Activity Assay. Cells were lysed in 250 μl of buffer containing 50 mM Tris/HCl (pH 7.5), 0.1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM sodium fluoride, 10 mM sodium B-glycerophosphate, 1 mM sodium orthovanadate, 0.1% 2-mercaptoethanol, 1 μM microcystin, 5 $\mu\text{g}/\text{ml}$ leupeptin, and 20 $\mu\text{g}/\text{ml}$ aprotinin. AKT was immunoprecipitated by incubating lysates (approximately 150 μg of protein) for 90 min at 4°C, with 1 μg of anti-AKT antibody. Immunoprecipitates were washed twice with lysis buffer. AKT activity was assayed by incubating immunoprecipitates with histone H2B as a substrate in the presence of (γ - ^{32}P)ATP. The samples were separated by 12.5% SDS/PAGE. After separation, the gel was dried and exposed to film.

Treatment of Cells. OCI-My5, 8226, or AF-10 MM cells were stimulated with either recombinant IGF-I or IL-6 to test activation of PI 3-K, AKT kinase, or ERK MAP kinase. Before challenging MH.60 cells, they were depleted of exogenous IL-6 for at least 18 h. When the effects of wort or Ly were tested, these inhibitors were added 1 h before the addition of IGF-I or IL-6. To induce apoptosis, dex was added at a concentration of 10⁻⁶ M. Controls contained identical concentrations of alcohol (always <0.1%). After 72 h of dex exposure, MM cells were harvested and the degree of death *versus* viability was determined by trypan blue exclusion.

Detection of Apoptosis. As described previously (6), percent apoptotic nuclei was determined by DAPI staining. Cells were first fixed with 3.7% formaldehyde in PBS at room temperature for 10 min and then washed with PBS. Fixed cells were stained with 1 $\mu\text{g}/\text{ml}$ DAPI in PBS at room temperature for 15 min. After washing three times, cells were resuspended in glycerol:PBS (10:1) and were mounted onto glass slides and covered with a coverslip. The slide was examined under $\times 400$ magnification using a fluorescent microscope with a 340/380-nm excitation filter and LP 430-nm barrier filter. At least 300 nuclei were examined per group.

Proliferation Assay. Briefly, 1 $\times 10^5$ MM cells in 200 μl of RPMI media without FCS were cultured for 48 h in 96-well culture plates in the presence or absence of recombinant IL-6. Cells were then labeled with 1 $\mu\text{Ci}/\text{well}$ of (^3H)dThd during the last 6 h of culture, harvested onto filters, and counted in a scintillation counter. Proliferation data are presented as cpm as well as by a proliferation index, calculated as tritium uptake in the experimental group divided by the control group (media alone, no IL-6).

Adenovirus Transduction. The AdLacZ adenoviral vector was a gift from Dr. Raj Batra (West Los Angeles Veteran's Administration Hospital). The adenoviral vector expressing the p85 dominant negative (DN) construct (26) and its corresponding empty vector control were kind gifts from Drs. Prem Sharma and Jerrold Olefsky (UC San Diego). This recombinant adenovirus contains the cDNA encoding the p85N-SH2 domain of p85. It was generated by homologous recombination in 293 cells using two plasmids, pACCMV-pLpA, containing the p85N-SH2 DNA, and pJM17. AF-10 myeloma cells were transduced with adenovirus at an MOI of 100 for 2 h. Adenovirus was then washed away and cells were resuspended in fresh media for 48 h before assays. The fresh media contained low serum concentrations (1% FCS) to minimize proliferation. Expression of p85 DN was confirmed by Western blot

for its *myc* tag, as described previously (26), using clone 9E10 anti-*myc* antibodies (Upstate Biotechnology, Inc.). For X-Gal staining, MM cells were washed three times in PBS, fixed in 0.5% glutaraldehyde, washed four more times with PBS, and stained overnight with 2 mg/ml X-Gal. Cells were counted by light microscopy, and blue-stained cells were enumerated.

Statistics. The *t* test was used to determine significance of difference between groups.

RESULTS

IL-6 and IGF-I activate PI 3-K Activity in Myeloma Cells.

Initially, we tested activation of PI 3-K by immunoprecipitating the enzyme with anti-p85 antibody and testing its ability to phosphorylate PI 4-phosphate on the D-3 position of the inositol ring. We investigated four target cell types: the 8226, OCI-My5, MH.60, and AF-10 plasma cell lines. All these cells express functional IL-6 receptors, but their responsiveness to IL-6 differs. When exposed to IL-6, the former two lines (8226 and OCI-My5) do not proliferate but are protected from apoptotic death (11). In contrast, MH.60 plasma cells are IL-6 dependent in that their continuous proliferation and survival requires the presence of IL-6. The AF-10 plasma cell line is not IL-6 dependent but significantly proliferates when exposed to the cytokine (12). The 8226 and OCI-My5 cell lines also express IGF-I receptors and are protected against apoptosis when exposed to IGF-I (6).

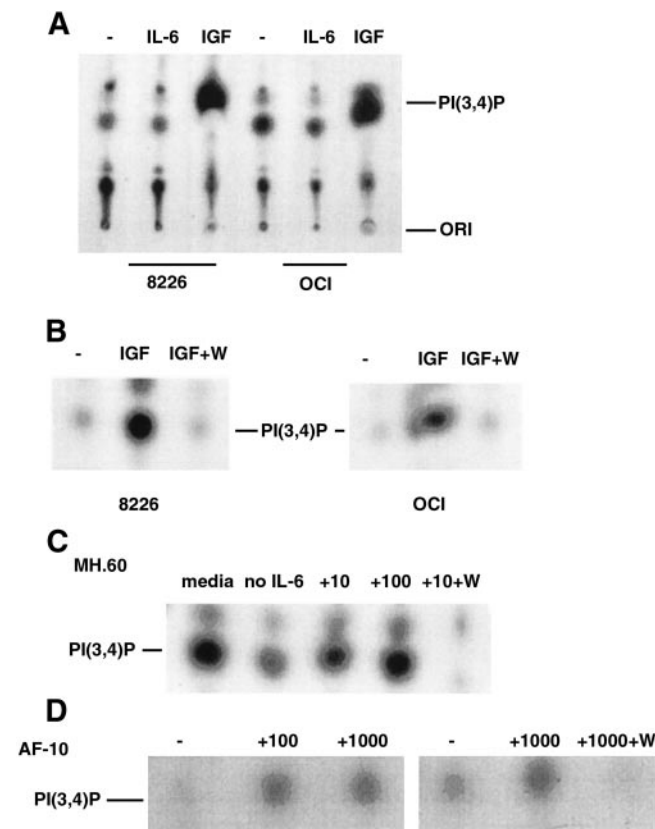


Fig. 1. Activation of PI 3-K in myeloma cells. A, the 8226 or OCI cells were treated without (–) or with IGF-I (400 ng/ml) or IL-6 (1000 units/ml) for 10 min, and then PI 3-K assay was performed. The positions of the reaction product PI(3,4)P and the origin (ORI) are indicated on the right. B, the 8226 or OCI-My5 cells treated with IGF-I alone (400 ng/ml) or IGF-I + wort (IGF+W) for 10 min, and PI 3-K assay was performed. Wort (0.1 μM) was present in a preincubation of 1 h. C, MH.60 cells were continuously cultured in IL-6 (media), depleted of IL-6 for 18 h (no IL-6), or depleted of IL-6 for 18 h, followed by the re-addition of fresh IL-6 at 10 (+10) or 100 (+100) units/ml for 10 min, and PI 3-K assay performed. In one group depleted of IL-6, 10 units/ml of IL-6 were re-added after a 1-h preincubation with wort, 0.1 μM (+10+W). D, left, AF-10 cells treated without (–) or with IL-6 at 100 units/ml (+100) or 1000 units/ml (+1000) for 10 min. Right, AF-10 cells treated without (–) or with IL-6, 1000 units/ml (+1000) or with IL-6 after a 1-h preincubation with wort, 0.1 μM (+1000+W).

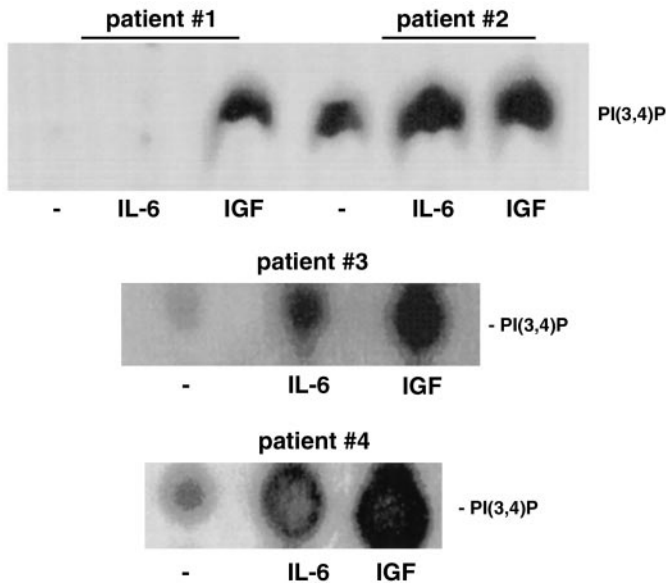


Fig. 2. Activation of PI 3-K in four myeloma patient samples. Isolated CD38+ marrow plasma cells from four patients were treated without (-) or with IL-6 (1000 units/ml) or IGF-I (400 ng/ml) for 10 min.

As shown in a representative experiment (Fig. 1A), IGF-I, but not IL-6, was capable of activating PI 3-K activity in 8226 and OCI-My5 cells. Marked PI 3-K activity was consistently detected between 5 and 15 min of incubation with 400 ng/ml IGF-I (>20-fold increase in each of three separate experiments with both cell lines). Immunoblot analysis demonstrated that the p85 immunoprecipitates contained comparable amounts of PI 3-K enzyme in all lanes (data not shown). In contrast, concentrations of IL-6 up to 1000 units/ml, used in incubations from 5–30 min (three separate experiments at each time point of 5 or 10 min and one at 30 min), were ineffective. The fact that IL-6 protects these cells from apoptosis (11) and activates the AKT kinase within 15 min (see below), confirms the presence of functional IL-6 receptors and the ability of these cells to respond rapidly. IGF-I-dependent PI 3-K activation was inhibited by prior exposure to wort (0.1 μM), a relatively specific PI 3-K inhibitor (Fig. 1B). In other experiments not shown, Ly, a second PI 3-K inhibitor, also inhibited IGF-I-dependent kinase activation in both MM cell lines (when used at 1 or 5 μM).

In the MM cell lines that proliferate when exposed to IL-6 (MH.60 and AF-10), IL-6 was more effective at activating PI 3-K (Fig. 1, C and D). In the MH.60 cell line, which is strictly dependent on continuous exposure to IL-6 for viability and growth (Fig. 1C), target cells were depleted of IL-6 for 18 h (still maintaining >93% viability) and then either incubated in the presence or absence of IL-6 for 15 min. As shown, MH.60 cells continuously cultured in IL-6 demonstrate constitutive activation of PI 3-K (Lane 1, media). A 4-fold decrease in PI 3-K phosphorylation activity (mean of three separate experiments) was detected in cells starved of IL-6 for 18 h (Fig. 1C, Lane 2, no IL-6), and the readdition of the cytokine significantly activated the enzyme [2-fold increase at 10 units/ml (+10) and 4-fold increase at 100 units/ml IL-6 (+100), means of three separate experiments]. Wort, used at 0.1 μM , completely prevented PI 3-K activation in MH.60 cells (+10+W). To study AF-10 cells, we continuously cultured target cells in media + FCS and then simply incubated them in the presence or absence of exogenous IL-6 for 10 min. As shown (Fig. 1D), AF-10 cells demonstrate only modest detectable basal activity of PI 3-K, but IL-6 (at 100 or 1000 units/ml) significantly induced activity (3- and 3.5-fold increase by densitometry, respec-

tively (mean of three experiments). This activity was also inhibited by wort (0.1 μM ; Fig. 1D, right).

Four primary myeloma specimens were also studied. These malignant plasma cells were obtained from patient marrows, and CD38+ plasma cells were isolated. In patient 1, IGF-I (400 ng/ml), but not IL-6 (1000 units/ml), significantly activated PI 3-K activity (Fig. 2). In patient 2, by contrast, there was significant constitutive PI 3-K activation, and both IL-6 and IGF-I were effective in further activation above baselines (7-fold and 6-fold increase by densitometry, respectively). In patients 3 and 4, there was only modest constitutive activation, and, again, IL-6 (5- and 4-fold increase by densitometry in patients 3 and 4) as well as IGF-I (9- and 6-fold increase, respectively) were effective in further activation. Immunoblotting of the anti-p85 immunoprecipitates used in Fig. 2 demonstrated equal loading of PI 3-K protein in all lanes (data not shown).

Inhibition of PI 3-K Activity Abrogates Myeloma/Plasmacytoma Cell Responses to IGF-I and IL-6. To test whether cytokine-induced activation of PI 3-K in myeloma cells has biological significance, we first used PI 3-K inhibitors. Preliminary experiments indicated that concentrations of wort at 1 μM or lower and Ly at 5 μM or lower were not toxic to 8226 or OCI-My5 target cells during a 72-h incubation. In addition, these concentrations of inhibitors did not enhance the toxicity of dex when cells were coexposed to both agents over 72 h. However, nontoxic concentrations of the inhibitors (0.1 μM wort and 5 and 1 μM Ly) prevented the ability of IGF-I to protect myeloma cells against death induced by dex (Fig. 3). This effect was related to the concentration of inhibitors used because Ly used at 0.05 μM in the same experiment (data not shown) had no effect. In additional experiments, we confirmed that these inhibitors were unable to inhibit protection against dex induced by IL-6. For example, IL-6 significantly decreased the lethal effect of dex in 8226 cells from 43% nonviable cells to 19%. In the presence of 0.1 μM wort, 5 μM Ly, and 1 μM Ly, IL-6 was still effective, decreasing the death rate to 18%, 22%, and 24%, respectively. This ineffectiveness of the PI 3-K inhibitors in IL-6-treated 8226 and OCI cells is expected because IL-6 did not activate PI 3-K in these myeloma cells (Fig. 1). These latter data also support the notion that the ability of wort and Ly to abrogate the protective effect of IGF-I is not due to nonspecific effects of these inhibitors but is related to their ability to inhibit PI 3-K. Staining MM

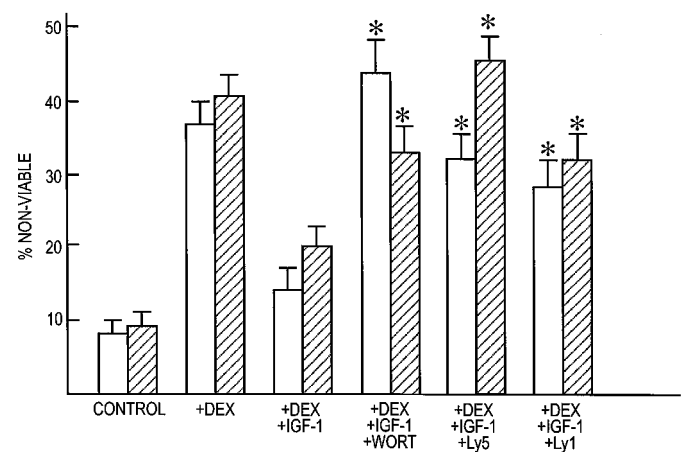


Fig. 3. PI 3-K inhibitors prevent the survival effect of IGF-I. The 8226 (□) or OCI-My5 (▨) cells cultured for 72 h in media (CONTROL); with dex (10^{-6} M; +DEX); with dex + IGF-I (400 ng/ml; +DEX+IGF-I); with dex, IGF-I, and wort at 0.1 μM (+DEX+IGF-I+WORT); with Ly at 5 μM (+DEX+IGF-I+LY5); or Ly at 1 μM (+DEX+IGF-I+LY1). A percentage of nonviable (trypan blue+) cells were then enumerated by counting 300 cells/group. Results are means \pm SEs of three separate experiments. *, significant higher ($P < 0.05$) nonviable percentage compared with +DEX+IGF-I group.

Table 1 Inhibition of IL-6-dependent proliferation by PI 3-K inhibitors in AF-10 cells^a

IL-6	Inhibitor	cpm	Proliferation index ^b
Exp-1			
-		23,970 ± 2,200	
+		44,060 ± 3,100	1.84
+	wort	26,500 ± 1,600 ^c	1.11
+	Ly294002	28,440 ± 1,100 ^c	1.19
	wort	21,740 ± 1,500	
	Ly294002	20,500 ± 1,600	
Exp-2			
-		12,800 ± 1,000	
+		25,100 ± 1,300	1.96
+	wort	14,600 ± 1,400 ^c	1.14
+	Ly294002	15,000 ± 1,600 ^c	1.17
	wort	12,400 ± 2,000	
	Ly294002	12,600 ± 1,200	

^a AF-10 MM cells cultured for 48 h with or without rIL-6 (1000 U/ml) and with or without PI 3-K inhibitors, after which incorporation of tritiated thymidine was assessed and presented as cpm (mean ± SE of quadruplicate samples).

^b Mean cpm of experimental group/mean cpm of control (no IL-6). Wort was used at 0.1 μM and Ly294002 at 5 μM.

^c Significantly lower ($P < 0.05$) than IL-6 group in absence of inhibitors.

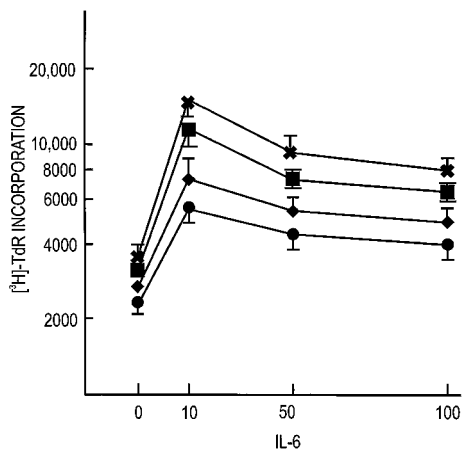


Fig. 4. PI 3-K inhibitors prevent IL-6-induced proliferation of MH.60 plasmacytoma cells. MH.60 cells were assayed for IL-6-dependent proliferation in media (×-×), Ly (0.05 μM; ■-■), wort (0.1 μM; ◆-◆), or Ly (5 μM; ●-●). IL-6 concentrations, 10, 50, and 100 units/ml; data are cpm of thymidine incorporation (mean ± SD of three separate experiments). Proliferation results with wort (0.1 μM) and Ly (5 μM) are significantly ($P < 0.05$) lower than control at all IL-6 concentrations.

cells with the DNA fluorescent dye DAPI also indicated that wort prevented the ability of IGF-I to protect against dex-induced apoptosis. In 8226 cells, exposure to dex (10^{-6} M) resulted in 39 ± 4% apoptosis (by DAPI staining, mean ± SD of three experiments) and IGF-I (400 ng/ml) decreased this to 12 ± 3% apoptosis. However, when wort (0.1 μM) was also present, IGF-I had little effect and dex-induced apoptosis was present at 33 ± 5%.

To address the biological relevance of PI 3-K when it is activated by IL-6 in plasma cells, we also tested the effects of the inhibitors on IL-6-induced proliferation of AF-10 and MH.60 plasma cell lines. We used the thymidine incorporation assay, which measures DNA replication, as a surrogate for proliferation. Exposure of AF-10 cells to IL-6 consistently stimulates an increase in proliferation assayed by thymidine incorporation (Table 1). As shown in Table 1, wort or Ly had no significant effect on the proliferation of AF-10 cells in the absence of IL-6. However, 0.1 μM wort and 5 μM Ly significantly inhibited the IL-6-induced proliferative response of AF-10 cells. The proliferative response was depressed to baseline levels by these inhibitors. Measurement of viability by trypan blue staining demonstrated no difference in cell survival at 12, 24, or 48 h. In similar fashion, the PI 3-K inhibitors prevented the IL-6-induced proliferation of MH.60 plasmacytoma cells (Fig. 4). In this IL-6-dependent cell

line, proliferation is achieved at lower concentrations of IL-6 and is much more remarkable (5–10-fold increase). However, both wort and Ly inhibited the proliferative effect. Significant ($P < 0.05$) inhibition of IL-6-induced proliferation was detected at 0.1 μM wort and 5 μM Ly, whereas 0.05 μM Ly had little effect. Again, trypan blue assessment of viability demonstrated no differences among the groups.

In attempts to further confirm the critical role of PI 3-K in MM cell proliferation, we exploited the ease by which some MM cells are transduced by adenoviral vectors (27, 28). Preliminary experiments consistently demonstrated >80% transfection efficiency in AF-10 cells infected with the AdLacZ reporter adenoviral vector used at an MOI of 100. We then transiently transfected AF-10 MM cells with an adenoviral vector expressing a p85 DN construct (26), which prevents PI 3-K activation. This construct encodes the p85 SH2 domain, and overexpression interferes with binding of endogenous p85 to its SH2-binding targets. P85DN-transfected AF-10 MM cells were then treated with or without IL-6, activation of PI 3-K and its downstream target (the AKT kinase) was monitored, and the proliferative response to IL-6 was assessed. To confirm expression of the p85 DN transgene, we performed Western blot for the myc tag as described previously (26). As shown in a representative experiment (Fig. 5), the DN p85 completely prevented IL-6-dependent activation of PI 3-K (lipid kinase assay) and more modestly, but significantly, inhibited activation of AKT (Western blot using a phospho-specific anti-AKT antibody).

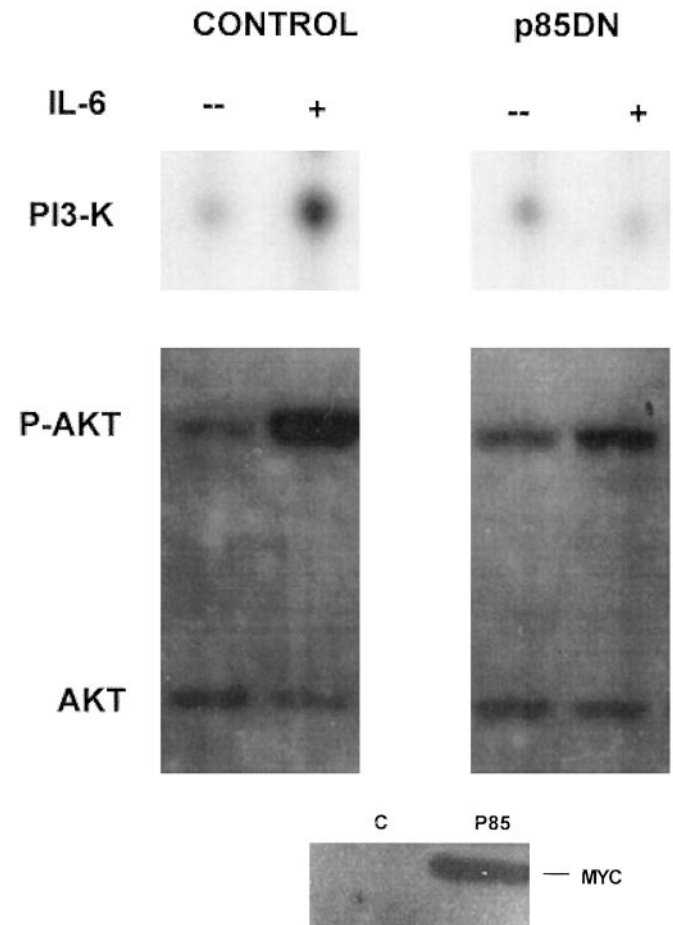


Fig. 5. DN PI 3-K inhibits the IL-6 proliferative response. AF-10 cells were transiently transfected with adenovirus expressing a DN p85 construct (p85DN) or empty vector (CONTROL). Cells were then stimulated with or without IL-6 (1000 units/ml). PI 3-K activation was assessed by lipid kinase assay after 10 min, and AKT kinase activation was assayed by immunoblot for expression of phosphorylated AKT (P-AKT) versus total AKT after 15 min. Expression of p85DN was demonstrated by Western blot for myc-tag (bottom) in p85-transfected cells (p85) as compared with control (C) cells.

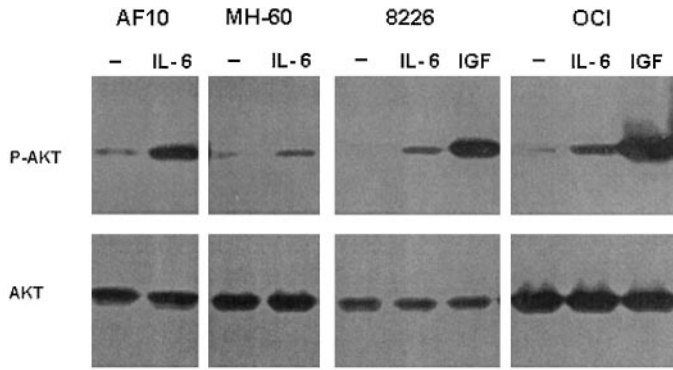


Fig. 6. IL-6 and IGF-I activate AKT kinase in myeloma/plasmacytoma cells. Myeloma/plasmacytoma cells were treated without (–) or with IL-6 (100 units/ml) or IGF-I (400 ng/ml) for 15 min, after which protein was extracted and assayed by immunoblot for expression of phosphorylated AKT (P-AKT) or total AKT.

In three separate experiments, control cells demonstrated an IL-6-dependent 4 \pm 1.7-fold increase in PI 3-K activity over baseline (mean \pm SD by densitometry), whereas p85 DN-containing cells showed no increase. IL-6-dependent AKT kinase activation was 7.5 \pm 2.7-fold over baseline in control cells and 2.5 \pm 1-fold in p85DN-containing cells. Anti-*myc* immunoblotting demonstrated expression of the transgene (Fig. 5, *bottom*). In the experiment shown, the DN p85 also inhibited the proliferative response to IL-6, decreasing the proliferation index from 1.9 to 1.2 when compared with the control group. In two other experiments not shown, a similar inhibition in IL-6-dependent proliferation was detected with a proliferation index of 2.2 and 1.7 in control-transfected cells (empty vector) compared with 1.4 and 1.2 in p85DN-transfected cells. These data confirm the importance of PI 3-K activation in IL-6-dependent proliferation of AF-10 MM cells.

IL-6 and IGF-I Activate the AKT Kinase in Myeloma Cells.

The results described above in Fig. 5 indicated that, in addition to PI 3-K, IL-6 could also activate the AKT kinase. Because AKT kinase activation can mediate antideath responses (15, 29, 30), we more extensively assessed its status in cytokine-activated MM cells. Because AKT kinase is a downstream target of PI 3-K, we predicted that whenever IL-6 or IGF-I activated PI 3-K it should also activate the AKT kinase. Therefore, as exposure of AF-10 or MH.60 cells to IL-6 successfully activated PI 3-K (Fig. 1), it was not surprising that AKT was also activated by IL-6 in these cells [Fig. 6; 2.3-fold and 6.3-fold for MH.60 and AF-10 cells (means of three experiments by densitometry), respectively]. Like-wise, the ability of IGF-I to activate AKT in 8226 and OCI cells (Fig. 6) was consistent with the ability of that cytokine to activate PI3-K in these cells (Fig. 1). In the experiment shown, the increased level of phosphorylated AKT induced by IGF-I in 8226 and OCI-My5 cells was \sim 50- and 15-fold, respectively (by densitometry). However, although IL-6 could not activate PI 3-K in 8226 and OCI-My5 cell lines (Fig. 1), we were surprised to find it successfully activated the AKT kinase in these cells (Fig. 6). Although quite significant, IL-6-induced activation in these cells was less marked than that induced by IGF-I. By densitometry, IL-6 activated AKT 12-fold over control in 8226 cells and 3.5-fold over control in OCI-My5 cells (Fig. 6). The experiment shown was repeated two more times with similar results. Control Western blots with an antibody reactive with total AKT (phosphorylated as well as nonphosphorylated) demonstrated equal loading of the enzyme in each lane (Fig. 6, *bottom*).

To confirm that the more modest induction of phosphorylation of AKT by IL-6 in 8226 and OCI cells resulted in activated AKT kinase activity, we performed an *in vitro* kinase assay. As shown in Fig. 7,

AKT immunoprecipitated from 8226 and OCI MM cells following IL-6 stimulation was significantly activated for its ability to phosphorylate the histone H2B substrate. AKT activation was demonstrated by 15 min of incubation with IL-6, and wort (0.1 μ M) had little effect (15+W). Western blot analysis for AKT (*bottom*) demonstrated equivalent amounts of enzyme in the immunoprecipitates. Similar activation of AKT kinase activity was demonstrated in AF-10 myeloma cells within 15 min of incubation with IL-6 (data not shown).

The absence of IL-6-induced activation of PI 3-K in 8226 and OCI cells (Fig. 1) and the lack of effect of wort on IL-6-induced activation of AKT in these cells (Fig. 7, *top*) suggested that AKT was activated in a PI 3-K-independent fashion. To further address this issue, we compared the sensitivity to wort of IGF-I- versus IL-6-induced AKT activation head to head. We, thus, treated 8226 or OCI cells with either cytokine in the presence or absence of wort (0.1 μ M) and tested phosphorylation of AKT in Western blots. As shown in Fig. 8, wort was much less effective in preventing AKT activation by IL-6 than by IGF-I in 8226 cells. The inhibitor completely prevented the IGF-I-induced increase in AKT activation (by densitometry, a 10-fold decrease in the phosphorylated AKT band) but only modestly decreased IL-6-induced activation (a 2-fold decrease in the phosphorylated AKT band). In three separate experiments in 8226 cells, we consistently demonstrated a difference in the wort sensitivity of IGF-I versus IL-6-induced AKT activation. The mean \pm SD decrease in IGF-dependent AKT activation (by densitometry) induced by wort was 9 \pm 3-fold, whereas the decrease in IL-6-dependent AKT activation was 2.5 \pm 0.8-fold. Similar data were observed with the OCI MM cell line (data not shown). In these latter cells, 0.1 μ M wort completely abrogated the ability of IGF-I to activate AKT (8.5-fold decrease in

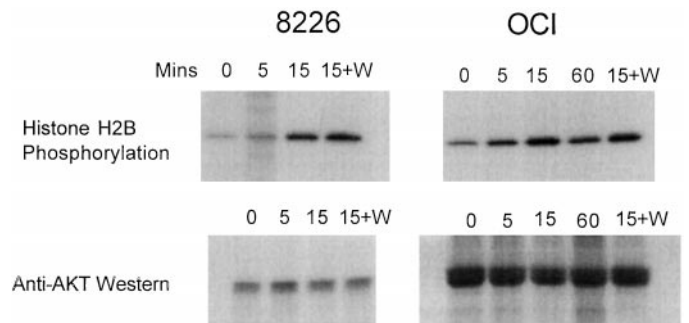


Fig. 7. *In vitro* kinase assay. The 8226 or OCI cells were stimulated for 5, 15, or 60 min with IL-6 (1000 units/ml), and *in vitro* kinase assay was performed against histone H2B as a substrate. In one group, cells were first pretreated with wort (0.1 μ M for 1 h) and then stimulated with IL-6 for 15 min (15+W). *Bottom*, protein in immunoprecipitates was immunoblotted for total AKT.

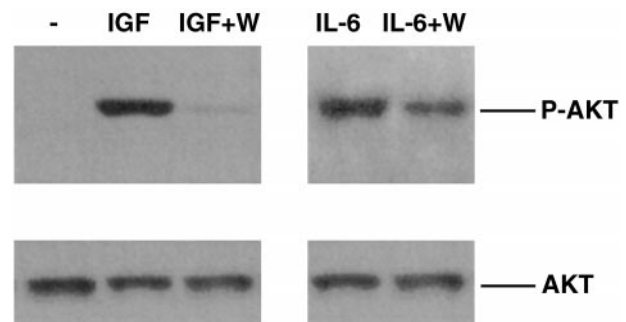


Fig. 8. Differential sensitivity to wort of IL-6 versus IGF-I-induced AKT kinase activation. The 8226 cells treated with IGF-I (400 ng/ml) or IL-6 (100 units/ml) \pm wort (W) were used at 0.1 μ M in a 1-h preincubation. Fifteen min after IGF-I/IL-6 was added, protein was extracted and assayed by immunoblot for expression of phosphorylated AKT (P-AKT) and total AKT.

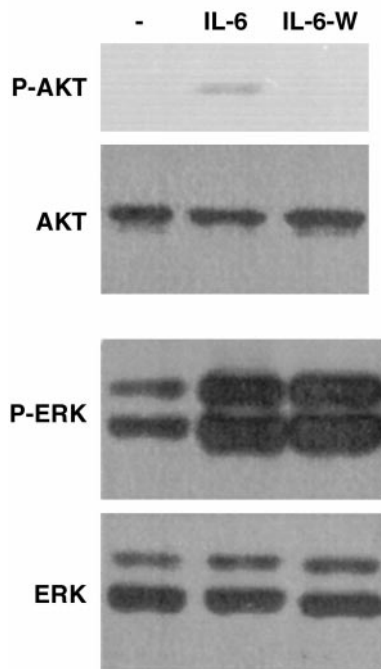


Fig. 9. Wort does not inhibit IL-6-induced ERK activation in AF-10 cells. AF-10 cells treated without (-) or with IL-6 (100 units/ml) +/- a 1-h preincubation with wort (W) were used at 0.1 μ M. Fifteen min later, protein was extracted and assayed by immunoblot for expression of phosphorylated AKT (P-AKT), total AKT, phosphorylated ERK-1 and ERK-2 (P-ERK), or total ERK-1 and ERK-2 (ERK).

phosphorylated AKT band on Western analysis; mean of three experiments), whereas it had only a modest effect on the ability of IL-6 to activate AKT in these cells (2.1-fold decrease in phosphorylation of AKT). These data are consistent with IL-6-dependent mechanisms of AKT activation in 8226 and OCI cells, which are independent of PI 3-K activation. In contrast, the activation of AKT by IL-6 in MH.60 and AF-10 MM cells was completely abrogated by 0.1 μ M wort (data not shown).

Wort Has No Effect on ERK Activation. The ability of wort to prevent IL-6-induced proliferation of AF-10 MM cells indicates a role for PI 3-K in the proliferative response. As described by Ogata *et al.* (13), this proliferative response also depends on IL-6-dependent activation of the ERK-MAP kinase pathway. Because ERK may be activated by a PI 3-K-stimulated cascade in some cell types (31, 32), it was possible that inhibiting PI 3-K activity might prevent IL-6-dependent proliferation via downstream inhibitory effects on ERK activation. To test this possibility, we stimulated AF-10 MM cells with IL-6 in the presence or absence of wort. As shown in Fig. 9, wort completely prevented the IL-6-dependent activation of the downstream AKT kinase. However, both ERK 1 and ERK 2 were both activated by IL-6 in AF-10 cells, and the inhibitory concentration of wort had no effect. Thus, the prevention of IL-6-dependent proliferation by inhibition of PI 3-K activity is not due to adverse effects on ERK activation.

DISCUSSION

The results of this study indicate that both IGF-I and IL-6 are capable of activating PI 3-K in some myeloma/plasmacytoma cells. Activation occurred rapidly, in primary myeloma cells obtained from patients as well as in cell lines and at cytokine concentrations that are effective in inducing biological cellular responses. Activation was sensitive to two PI 3-K inhibitors, wort and Ly.

Experiments using the two PI 3-K inhibitors indicated that kinase

activation is important in both cytokine-dependent proliferative and antiapoptotic responses of MM cells. First, wort and Ly prevented the antiapoptotic effect of IGF-I when MM cells were challenged with dex. Identical concentrations of these inhibitors could not prevent the antiapoptotic effect of IL-6 in these same cells, ruling out a nonspecific adverse effect on MM cells. Our preliminary data also indicate that wort prevents antiapoptotic responses of IGF-I to serum starvation (data not shown). Second, the inhibitors significantly curtailed the IL-6 proliferative response of AF-10 and MH.60 plasmacytoma cells. Furthermore, expression of a DN PI 3-K construct by adenoviral infection also significantly inhibited the AF-10 proliferative response to IL-6.

The antiapoptotic effect of PI 3-K is consistent with previous studies (17, 29) where the role of PI 3-K in the survival-promoting effects of cytokines has been documented in different tumor cell models. However, the potential role for PI 3-K in the proliferative effects of IL-6 is novel. PI 3-K activation may directly affect the cell cycle machinery, possibly by enhancing translation of specific mRNA species and promoting the G₁-to-S transit (33, 34) or maintain cell survival as cell cycle transit proceeds. Because a previous study (13) had documented the role of ERK activation in IL-6 proliferative effects in MM cells, and because the ERK MAP kinases are one of several potential downstream target molecules of PI 3-K (31, 32), we tested whether the ability of wort to inhibit IL-6-dependent AF-10 proliferation correlated with an inhibition of ERK activation. The results of Fig. 9, however, clearly demonstrate that, while markedly inhibiting downstream activation of AKT, wort has no effect on ERK activation. Thus, the data indicate that IL-6-induced MM cell proliferation is dependent on at least two independent pathways: the ras-raf-erk cascade and a second PI 3-K-dependent pathway. The downstream effector molecules of this second pathway remain unknown, but potential candidates are the AKT kinase, the p70 ribosomal protein S6 kinase (p70^{s6k}; Refs. 17 and 19), and, even further downstream, BAD (35), BCL-XL (36), and BCL-2 (37, 38). The potential role of p70^{s6k} is particularly intriguing because a prior study by Chauhan *et al.* (39) demonstrated that adverse effects of dex on MM cells were associated with a down-regulation of p70^{s6k} activity and IL-6 prevented this down-regulation.

Following IGF-I binding to its receptor, tyrosine phosphorylation of IRS-1 presents SH2 docking sites for p85 with subsequent activation of the p110 catalytic subunit (16, 40). In contrast, the molecular mechanism by which IL-6 activates PI 3-K in MM cells is unknown. The gp 130 signaling portion of the IL-6 receptor does not associate with the SH2 domain of p85 after cytokine treatment of cells, nor does it contain consensus binding sites for the SH2 domain of p85 (41). However, several possibilities other than direct binding of p85 to phosphorylated motifs on gp 130 come to mind: IL-6-induced ras activation in MM cells (13) could result in kinase activation because ras has been indicted as an upstream PI 3-K activator in other cell types (24, 42). In addition, the lack of SOS activation and subsequent ras activation in 8226 and OCI-My5 MM cells (43) could explain the inability of IL-6 to activate PI 3-K in those MM cell lines. A second possibility is a direct activation by JAK-1. In cardiac myocytes, leukemia inhibitory factor, presumably signaling through gp130, activates PI 3-K activity that can be immunoprecipitated by anti-JAK1 antibody (44). JAK-1 constitutively associated with PI 3-K in these cells, and leukemia inhibitory factor may activate the associated PI 3-K by phosphorylation of JAK-1. Because IL-6, signaling through gp 130, also phosphorylates JAK-1 in MM cells (13), an identical mechanism of PI 3-K activation may result. A third possibility is that IL-6 induces tyrosine phosphorylation of STAT-3 (well documented in MM cells; Refs. 13 and 14) and phosphorylated STAT-3 serves as a docking site for PI 3-K activation by gp 130. A similar mechanism of

PI 3-K activation occurs in IFN-treated cells (45). Finally, IL-6 may also activate PI 3-K by binding of p85 to other tyrosine phosphorylated intermediary adapter proteins. In particular, the adapter protein vav, which is activated by IL-6 (46), can, in turn, activate PI 3-K (47).

The AKT kinase is a well studied viability-promoting effector molecule that is activated downstream of PI 3-K. Thus, it was not surprising that IGF-I-dependent activation of PI 3-K in 8226 and OCI-My5 MM cells as well as IL-6-dependent activation in AF-10 or MH.60 cells was accompanied by significant AKT activation. However, IL-6 activated the AKT kinase in 8226 and OCI-My5 cells without prior PI 3-K activation. This suggests a PI 3-K-independent mechanism of AKT activation induced by IL-6 in these cells. Exposure to wort in these cells completely abrogated IGF-I-induced AKT activation but had much less of an effect on IL-6-induced AKT activation (Figs. 7 and 8), further supporting the notion of PI 3-K-independent pathways of AKT activation. In contrast, the ability of IL-6 to activate AKT in MH.60 and AF-10 cells was completely abrogated by wort. In addition, exposure of hepatoma cells to IL-6 activates AKT in a PI 3-K-dependent manner (18). Thus, the PI 3-K-independent AKT activation we identified is not a universal finding in IL-6-treated cells or in myeloma cells. It remains to be seen whether other IL-6-responsive myeloma cells can show AKT activation that is independent of PI 3-K activation. Other PI 3-K-independent pathways of AKT activation have been described (48), and IL-6 may use them in selected MM cells.

The AKT kinase promotes survival by protecting against apoptosis in other cell types (30, 35). Thus, activated AKT in 8226 or OCI-My5 MM cells may be participating in the protective effects of IL-6 or IGF-I against dex-induced apoptosis. Additional experiments will require disruption of AKT function by gene transfer to answer this question. AKT phosphorylates BAD (35), which results in dissociation of BAD from BCL-2 and/or BCL-XL, thus liberating these latter antiapoptotic proteins and allowing them to bind and inactivate BAX. However, if AKT function proves to be critical in protection against dex it must function independent of BAD, because we⁴, as well as Kitada *et al.* (49), could not detect any BAD protein in these MM cell lines. As mentioned above, however, activation of p70^{s6k} may also play a role. As a downstream target of PI 3-K, it should be significantly activated in these cells by IGF-I. We have not examined its activation by IL-6, but a previous study (39) documented that IL-6 counteracted a dex-induced down-regulation of p70^{s6k} activity in 8226 and OCI-My5 cells.

In summary, the results here indicate that IL-6 and IGF-I activate the PI 3-K/AKT kinase signal pathway in MM cells and that PI 3-K activation is important in antiapoptotic and proliferative responses to these cytokines. It may be interesting to search for constitutive activation of this pathway in primary MM cells that have become IL-6 independent.

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⁴ Unpublished observations.

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The Phosphatidylinositol 3-Kinase/AKT Kinase Pathway in Multiple Myeloma Plasma Cells: Roles in Cytokine-dependent Survival and Proliferative Responses

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