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Activation of NF-κB and upregulation of intracellular anti-apoptotic proteins via the IGF-1/Akt signaling in human multiple myeloma cells: therapeutic implications

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Interleukin-6 (IL-6) and insulin-like growth factor-1 (IGF-1) promote the proliferation of multiple myeloma (MM) cells and protect them against dexamethasone (Dex)-induced apoptosis. We have previously shown that Apo2 ligand/TNF-Related apoptosis inducing ligand (Apo2L/TRAIL) induces apoptosis of MM cells, including cells either sensitive or resistant to Dex and cytotoxic drugs, and overcomes the growth and survival effect of IL-6; conversely, NF-κB transcriptional activity attenuates their Apo2L/TRAIL-sensitivity. In the current study, we demonstrate that IGF-1 stimulates sustained activation of NF-κB and Akt; induces phosphorylation of the FKHRL-1 Forkhead transcription factor; upregulates a series of intracellular anti-apoptotic proteins including FLIP, survivin, cIAP-2, A1/Bfl-1, and XIAP; and decreases Apo2L/TRAIL-sensitivity of MM cells. In contrast, IL-6 does not cause sustained NF-κB activation, induces less pronounced Akt activation and FKHRL-1 phosphorylation than IGF-1, and increases the expression of only survivin. Forced overexpression of constitutively active Akt in MM-1S cells reduced their sensitivity to Apo2L/TRAIL and to doxorubicin (Doxo). In contrast, the Akt inhibitor IL-6-Hydroxymethyl-*chiro*-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate induced cell death of both Dex- and Doxo-sensitive and -resistant cells; opposed the protective effect of constitutive Akt activity against Apo2L/TRAIL; and abrogated the NF-κB activation, increase of anti-apoptotic proteins and protection against Apo2L/TRAIL induced by IGF-1. These findings therefore define an important role of the Akt pathway in modulating tumor cell responsiveness to Apo2L/TRAIL, delineate molecular mechanisms for the survival effects of IGF-1, and characterize differential pathophysiologic sequelae of IGF-1 vs IL-6 on MM cells. Importantly, they provide the basis for future clinical trials in MM combining conventional or novel agents with strategies designed to neutralize IGF-1.
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Introduction

Multiple myeloma (MM) remains incurable, despite the use of high-dose chemotherapy with autologous hematopoietic stem cell transplantation. Apo2 ligand/TNF-Related Apoptosis Inducing Ligand (Apo2L/TRAIL) is a TNF-α-like protein which induces apoptosis against a wide range of neoplastic cells, but spares normal tissues (Ashkenazi *et al*., 1999; Pitti *et al*., 1996; Wiley *et al*., 1995). We recently reported that Apo2L/TRAIL induces apoptosis of dexamethasone (Dex)- and cytotoxic drug-resistant MM cells and has potent *in vivo* anti-MM activity, while sparing normal hematopoietic cells (Mitsiades *et al*., 2001b). Importantly, interleukin-6 (IL-6), a major growth and survival factor for MM cells, which abrogates Dex-induced apoptosis (Chauhan *et al*., 2000), failed to protect MM cells from Apo2L/TRAIL-induced apoptosis (Mitsiades *et al*., 2001b). Our studies also showed that the transcriptional activity of NF-κB is a significant determinant of Apo2L/TRAIL-sensitivity; for example, agents which downregulate NF-κB transcriptional activity augment Apo2L/TRAIL-induced cell death in TRAIL-sensitive MM cells, and can even overcome the Apo2L/TRAIL-resistant phenotype in certain MM cells (Mitsiades *et al*., 2001b).

Insulin-like growth factor-1 (IGF-1) is a growth and survival factor for a diverse range of malignant cells (LeRoith *et al*., 1995), including MM cells. In MM cells, IGF-1 activates the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol-3′-kinase (PI-3K)/Akt signaling cascades (Ge and Rudikoff, 2000), which mediate IL-6-induced MM cell growth and survival. IGF-1 also protects MM cells against

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serum starvation- or Dex-induced apoptosis (Ge and Rudikoff, 2000; Georgii-Hemming *et al*., 1996; Jelinek *et al*., 1997; Ogawa *et al*., 2000; Xu *et al*., 1997), but the spectrum and mechanisms of its anti-apoptotic effects are not fully characterized. NF-κB activation mediates resistance of MM cells to multiple apoptotic stimuli (Feinman *et al*., 1999), including Apo2L/TRAIL (Mitsiades *et al*., 2001b). However, the precise effect of IGF-1 on NF-κB activity has not been characterized in detail, neither in hematologic malignancies nor in solid tumors, where there appear to be tissue-specific differential effects of IGF-1 on NF-κB activity (Heck *et al*., 1999; Pons and Torres-Aleman, 2000; Remacle-Bonnet *et al*., 2000).

In the present study, we show that IGF-1 activates NF-κB in MM cells. We also demonstrate differential effects of IGF-1 vs IL-6 on Akt and NF-κB activation, phosphorylation of the pro-apoptotic transcription factor FKHRL-1, and expression of several anti-apoptotic proteins. We also characterize the effect of transfection of a constitutively active Akt construct on MM cell apoptosis. These findings not only define molecular mechanisms for the protective effect of IGF-1 against Apo2L/TRAIL-induced apoptosis, but also suggest that blockade of IGF-1/PI-3K/Akt signaling would enhance anti-MM activity of conventional and novel therapies.

Results

IGF-1 induces activation of NF-κB in MM cells

Following overnight serum-starvation, MM-1S cells were incubated with 200 ng/ml of IGF-1 for 0–60 min. Nuclear extracts of IGF-1-stimulated and control MM-1S cells were analysed for NF-κB DNA binding activity using an ELISA-based assay and by immunoblotting for the p50 NF-κB subunit. As shown in Figure 1a, IGF-1 induced a rapid nuclear translocation of p50 and upregulation of NF-κB DNA binding activity, which was sustained for at least 60 min. Figure 1b also indicates significant upregulation ($P=0.018$) of NF-κB activity after a 3-h incubation with IGF-1 (200 ng/ml). To study the regulation of IGF-1-induced NF-κB activation, MM-1S cells were pre-incubated with the PI-3K inhibitor LY294002 and/or the MAPK inhibitor PD98059. The PI-3K inhibitor LY294002 both suppressed the constitutive NF-κB DNA binding activity and abrogated its upregulation by IGF-I (Figure 1b). In contrast, the MAPK inhibitor PD98059 had no significant effect on NF-κB activity.

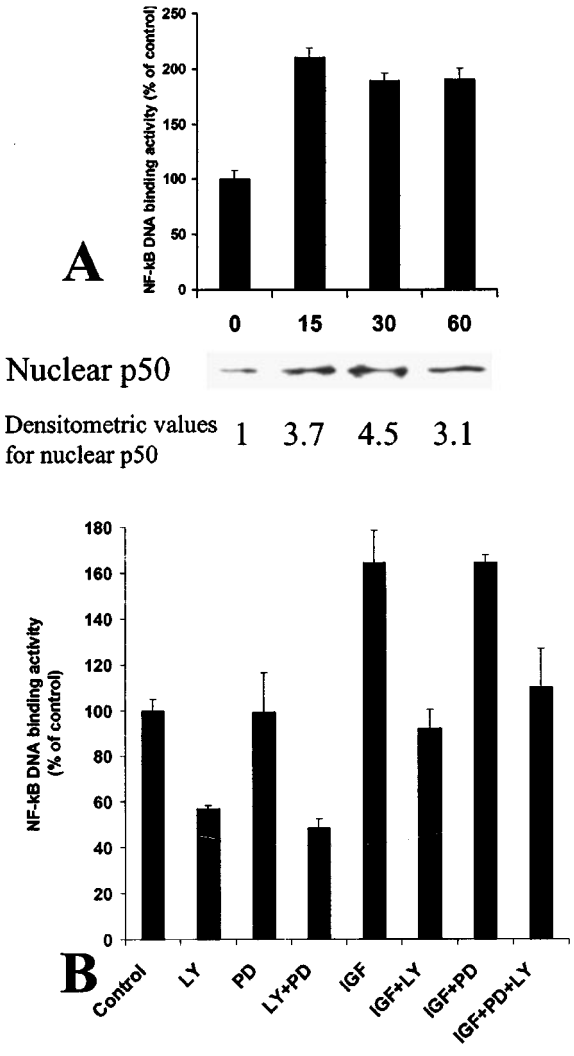

Figure 1 IGF-1 activates NF-κB. MM-1S cells were incubated in the presence or absence of IGF-1 (200 ng/ml), following overnight incubation in serum-free conditions. (**a**) Quantification of NF-κB DNA binding activity shows that IGF-1 induces, at 15 min, a >twofold increase in NF-κB DNA binding activity, which is sustained for at least 60 min. Immunoblotting and densitometric analysis of the p50 subunit of NF-κB in nuclear extracts of MM-1S cells show that IGF-1 also triggers increased nuclear accumulation of p50. (**b**) IGF-1-induced activation of NF-κB is sustained even after 3 h of stimulation. The PI-3K inhibitor LY294002 (20 μM for 1 h) downregulates constitutive NF-κB DNA binding activity and blocks its upregulation by IGF-1. In contrast, the MAPK inhibitor PD98059 (50 μM) had no effect on either constitutive or IGF-1-stimulated NF-κB activity, and did not enhance the inhibitory effect of LY294002

IGF-I reduces the Apo2L/TRAIL sensitivity of MM cells

We have previously demonstrated that NF-κB is an important determinant of resistance of MM cells to Apo2L/TRAIL: inhibition of NF-κB increases the Apo2L/TRAIL-sensitivity of Apo2L/TRAIL-sensitive MM cells, and even overcomes the resistance of certain Apo2L/TRAIL-refractory cell lines (Mitsiades *et al*., 2001a, 2002a). Because IGF-I stimulated NF-κB activity, we next examined the impact of IGF-I on Apo2L/TRAIL-sensitivity of MM cells. IGF-1-induced decreased sensitivity to Apo2L/TRAIL, as shown by decreased apoptotic cells detected with Annexin V-FITC/PI staining and flow cytometric analysis (Figure 2a). Pre-incubation of MM-1S cells with IGF-1 for 2 h triggered a dose-dependent decrease (non-parametric analysis of variance, $P=0.001$) in Apo2L/TRAIL-induced MM cell death. For example, Apo2L/TRAIL (50 ng/ml) induced 45% cell death, whereas the addition of IGF-1 (200 ng/ml) decreased cell death to $\sim$20%

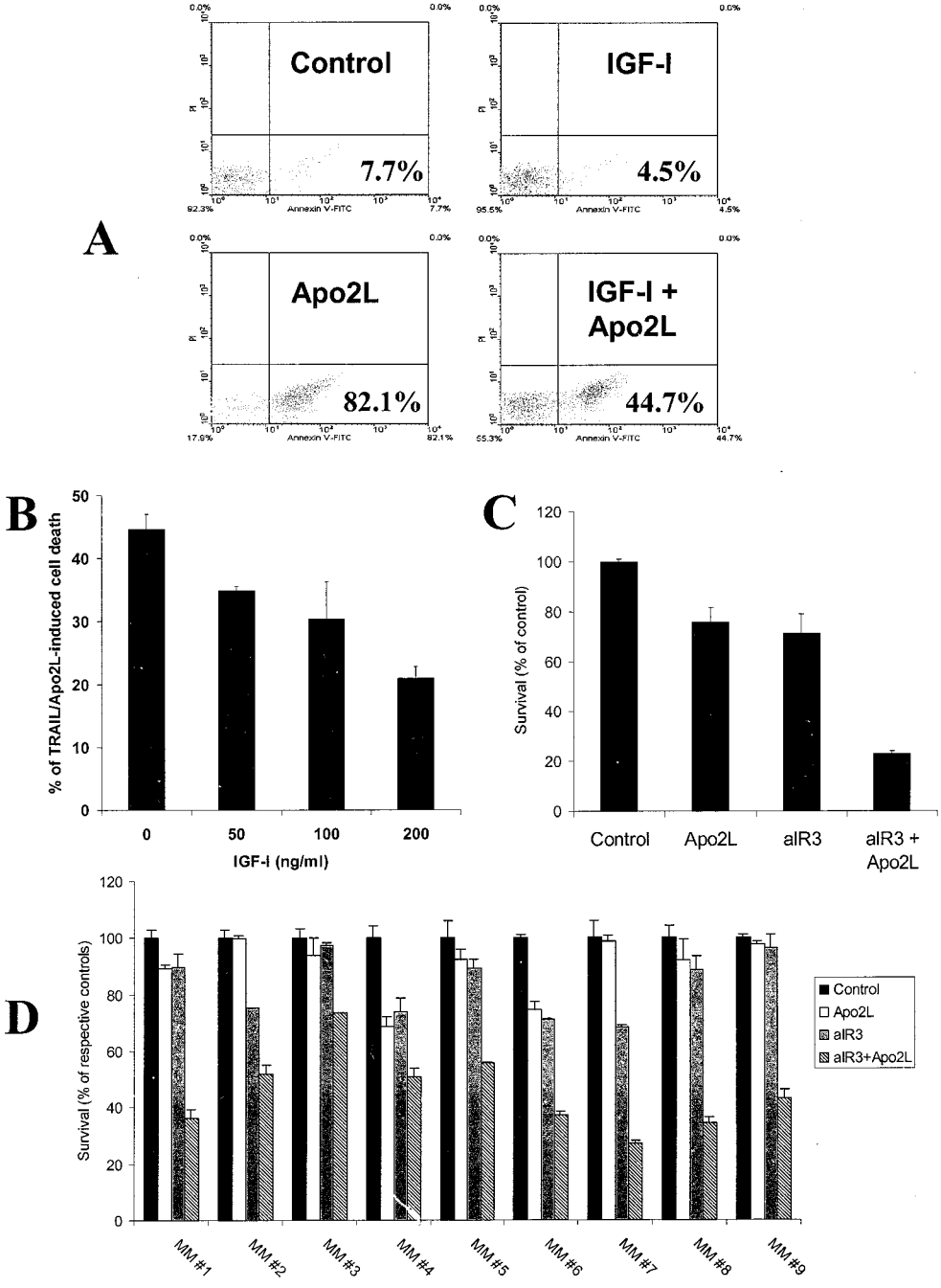

Figure 2 Apo2L/TRAIL-sensitivity of MM cells is reduced by IGF-1 and increased by inhibition of IGF-1 signaling at the level of the IGF-1 receptor. (**a**) Annexin V-FITC/PI staining of MM-1S cells treated with Apo2L/TRAIL (100 ng/ml), with or without pre-incubation (for 1 h) with IGF-1 (200 ng/ml). IGF-1 significantly reduced the percentage of Annexin V^+ PI-apoptotic cells, relative to treatment with Apo2L/TRAIL alone. The dual color flow cytometric histograms display intensity of fluorescence staining with Annexin V-FITC, detected at the FL1 channel (*x* axis), vs PI staining detected at the FL3 channel (*y* axis). The percentage of Annexin V^+/PI^- apoptotic cells is indicated in the respective quadrant. (**b**) Pre-incubation with IGF-1 (0–200 ng/ml for 2 h) reduces Apo2L/TRAIL (50 ng/ml)-induced MM-1S cell death, assessed by MTT colorimetric survival assay. Data shown are representative of three experiments with each condition tested in quadruplicate. (**c**) Inhibition of IGF-1 receptor signaling by the aIR-3 anti-IGF-1R neutralizing Ab increases the sensitivity of MM-1S cells to Apo2L/TRAIL. MM-1S cells cultured in 10% FBS were pre-incubated with the aIR-3 Ab or its isotype control $MsIgG_1$ (1 μg/ml for 1 h, for either antibody) and then exposed to Apo2L/TRAIL (overnight incubation with 100 ng/ml). The significant increase in Apo2L/TRAIL-sensitivity of MM-1S cells in the presence of aIR-3, as shown by MTT assay, indicates that a significant component of the protective effect of serum against Apo2L/TRAIL-induced apoptosis is mediated by IGF-1. (**d**) The aIR-3 neutralizing anti-IGF-1R Ab enhances the Apo2L/TRAIL-sensitivity of tumor cells freshly isolated from 9 MM patients. Each sample was tested in the experimental conditions of Figure 2c by MTT assay

($P<0.05$) (Figure 2b). Cell death was $<2\%$ in control cultures with IGF-1- and Apo2L/TRAIL-free media.

IGF-I is not only produced in the bone microenvironment (BM), but is also present in significant concentrations, similar to those used in our experiments, in the peripheral blood serum of MM patients (Mitsiades, unpublished observations). We therefore next evaluated whether the Apo2L/TRAIL-sensitivity of MM cells cultured in serum can be increased by inhibition of IGF-1 receptor (IGF-1R) signaling. MM-1S cells were pre-incubated for 1 h with 1 μg/ml of the anti-IGF-1R neutralizing monoclonal antibody aIR-3 and then cultured in 10% FBS overnight, either in the presence or absence of Apo2L/TRAIL (100 ng/ml). The survival of these cells, assessed by MTT assay, was compared with that of MM-1S cells pre-incubated with isotype control mouse IgG1 antibody, with or without Apo2L/TRAIL. In the presence of the aIR-3 neutralizing antibody, Apo2L/TRAIL reduced the survival of MM-1S to approximately 25% of control cells (Figure 2c), whereas survival of cells treated with Apo2L/TRAIL alone or aIR-3 mAb alone was approximately 75 and 70% of control cells, respectively. The aIR-3 mAb similarly enhanced Apo2L/TRAIL-sensitivity of tumor cells freshly isolated from 9 MM Patients (Figure 2d). These findings further support the protective role of IGF-1 receptor signaling against Apo2L/TRAIL, and indicate that IGF-1 in serum confers protection against Apo2L/TRAIL-induced apoptosis.

We have previously shown that IL-6, a major survival factor for MM cells (Chauhan *et al*., 2000; Xu *et al*., 1997), does not protect against Apo2L/TRAIL-induced apoptosis (Mitsiades *et al*., 2001b). We therefore next investigated the molecular mechanisms associated with this differential protective effect of IGF-I vs IL-6. We first examined whether IGF-1 or IL-6 modulate the expression of receptors for Apo2L/TRAIL on MM-1S cells. Although our previous studies indicate that the status of the death-inducing DR4, DR5 or the 'decoy' DcR1 and DcR2 receptors alone does not predict for sensitivity or resistance of MM cells to Apo2L/TRAIL, we have nonetheless demonstrated that changes in the expression of Apo2L/TRAIL receptors in individual cell lines or tumor specimens may be associated with modulation of Apo2L/TRAIL-sensitivity (Mitsiades *et al*., 2001b). In this study, incubation of MM-1S cells with IGF-1 or IL-6 for 24 h had no significant effect on the surface expression of DR4, DR5, DcR1 or DcR2 (Figure 3). These data indicate that the protective effect of IGF-1 against Apo2L/TRAIL is not associated with changes in TRAIL receptor levels, suggesting that intracellular molecular events may account for the differential survival effects of IGF-I vs IL-6.

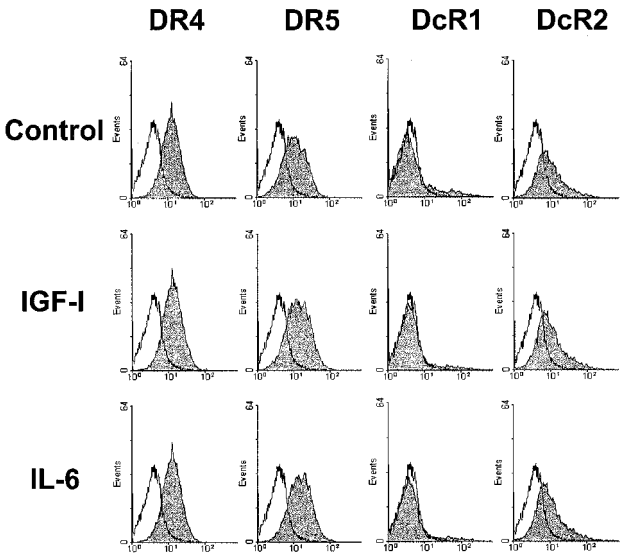

Figure 3 IGF-1 and IL-6 have no effect on surface expression of TRAIL receptors on MM-1S cells. Flow cytometric analyses of MM-1S cells stimulated with IGF-1 (200 ng/ml) or IL-6 (50 ng/ml) for 24 h demonstrates no significant changes in cell surface expression of either DR4, DR5, DcR1 or DcR2 receptors for Apo2L/TRAIL, in comparison to unstimulated control cells. Data shown were obtained after staining with anti-human DR4, DR5, DcR1 and DcR2 monoclonal Abs (Genentech, Inc), which were confirmed using the other anti-TRAIL receptor Abs described in Materials and methods. Shaded curves depict isotype control staining, and unshaded peaks correspond to staining with the respective TRAIL receptor Abs

Differential effects of IGF-1 vs *IL-6 on NF-κB activity, Akt activity, and phosphorylation of Forkhead transcription factor FKHRL-1*

Because of the key role of NF-κB in regulation of Apo2L/TRAIL-sensitivity in MM cells, we next compared the effects of IGF-1 vs IL-6 on NF-κB activation. Using an ELISA-based assay, NF-κB DNA binding activity was measured in nuclear extracts from MM-1S cells stimulated for 3 h with IGF-1 (200 ng/ml) or IL-6 (50 ng/ml). IGF-1, but not IL-6, induced significant and sustained NF-κB activity (Figure 4), correlating with the differential effect of these cytokines on Apo2L/TRAIL-sensitivity. Interestingly, IGF-1-induced NF-κB activation is comparable to that induced by TNF-α, a known stimulator of NF-κB in MM cells (Hideshima *et al*., 2001a). Since NF-κB is a proposed downstream target of Akt in other models (Kane *et al*., 1999), we next examined potential differential effects of IL-6 vs IGF-1 on Akt activation in MM-1S cells. Akt kinase activity was measured by immunoprecipitation of Akt and measurement of *in vitro* phosphorylation of a GSK-3 fusion peptide substrate, quantified by densitometric analysis. Both IGF-1 and IL-6 induced Akt kinase activation; however, there was significantly higher Akt activity in IGF-1- vs IL-6-stimulated cells (Figure 5a). Moreover, IL-6-induced phosphorylation of Akt was modest and comparable to baseline by 1 h, whereas Akt phosphorylation triggered by IGF-1 was sustained for at least 12 h (Figure 5b). These findings suggest that IGF-1 induces more prolonged and sustained activation of Akt than IL-6, consistent with the prolonged activation of NF-κB by IGF-1, but not by IL-6.

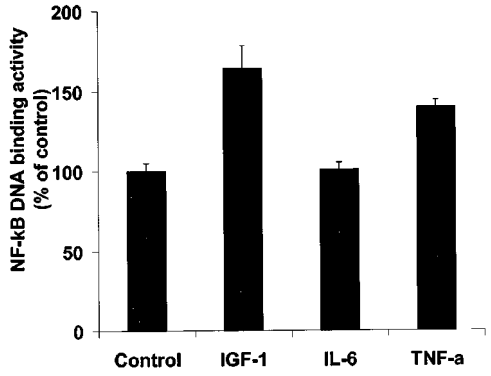

Figure 4 Differential effect of IGF-1 vs IL-6 on NF-κB activation in MM-1S cells. NF-κB DNA binding ELISA of MM-1S cells stimulated for 3 h with IGF-1 (200 ng/ml) or IL-6 (50 ng/ml), following overnight incubation in serum-free conditions. IGF-1 induces sustained significant upregulation of NF-κB activity (comparable to the one induced by TNF-α); in contrast, IL-6 does not induce sustained NF-κB activation

We next examined potential downstream targets of Akt and NF-κB, which could mediate the anti-apoptotic effects of IGF-1. We first studied the Forkhead family of transcription factors (FKHR), since IL-6 induces phosphorylation, cytoplasmic sequestration, and decreased transcriptional activity of these pro-apoptotic factors (Hideshima *et al*., 2001b). Immunoblotting of cytoplasmic extracts of MM-1S cells showed that IGF-1 also induces rapid phosphorylation of the FKHRL-1 transcription factor (Figure 5c), an event which prevents its nuclear translocation and transcriptional activity (Hideshima *et al*., 2001b). Interestingly, IGF-1 led to a more prolonged phosphorylation of FKHRL-1 than IL-6; IGF-1-induced phosphorylation was sustained at 1 h, whereas IL-6-induced phosphorylation was returning to baseline (Figure 5d). These findings are consistent with the more potent and prolonged activation of Akt by IGF-1 than by IL-6, and suggest a possible role of FKHRL-1 in regulation of Apo2L/TRAIL-sensitivity.

Differential effects of IGF-1 vs IL-6 on expression of intracellular anti-apoptotic proteins in MM cells

We next examined the effects of IGF-1 and IL-6 on expression of intracellular anti-apoptotic proteins which are downstream targets of NF-κB in other tissue models, e.g. cIAP-2, cIAP-1 (Chu *et al*., 1997; Wang *et al*., 1998) and A1/Bfl-1 (Wang *et al*., 1999). We have previously demonstrated that the anti-apoptotic proteins FLIP and cIAP-2 are more highly expressed in Apo2L/TRAIL-resistant than Apo2L/TRAIL-sensitive MM cells (Mitsiades *et al*., 2002a), and that inhibition of NF-κB transcriptional activity both increases the sensitivity of TRAIL-sensitive MM cells and overcomes the TRAIL-resistance in certain MM cells (Mitsiades *et al*., 2000). Moreover, we and others have also reported that NF-κB-dependent expression of cIAP-2 inhibits caspase-8 activation and subsequent apoptosis (Mitsiades *et al*., 2002a; Wang *et al*., 1998); conversely, downregulation of NF-κB transcriptional activity is associated with decreased expression of cIAP-2 (Mitsiades *et al*., 2002a). In this study, we first determined whether IGF-1-induced protection against Apo2L/TRAIL is associated with changes in the expression of intracellular anti-apoptotic proteins FLIP, cIAP-1, cIAP-2, XIAP, survivin, Bcl-2, Bcl-X_L, Mcl-1, A1/Bfl-1 or the pro-apoptotic Bax protein. Because caspase-8 is the apical caspase mediating Apo2L/TRAIL-induced apoptosis in MM cells (Mitsiades *et al*., 2002a), we also examined the

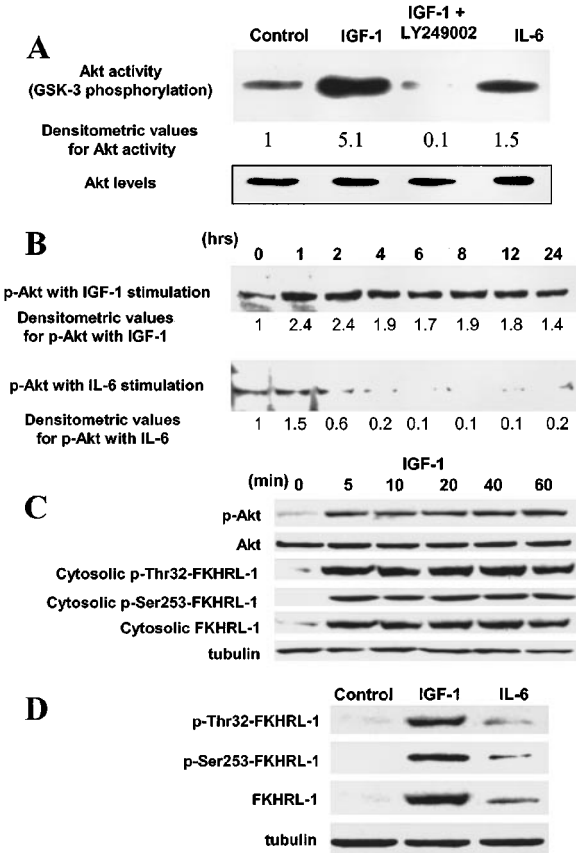

Figure 5 Differential effect of IGF-1 vs IL-6 on Akt kinase activity and Forkhead phosphorylation. (**a**) The kinase activity of Akt was assessed by detection of phosphorylation of a GSK-3 fusion protein substrate and quantified by densitometric analysis. Stimulation of MM-1S cells with IGF-1 (200 ng/ml for 1 h) triggers a significant increase in Akt kinase activity, which is more pronounced than Akt activation induced by IL-6. IGF-1-induced Akt activation is blocked by the PI-3K inhibitor LY249002 (pre-treatment for 1 h). (**b**) Immunoblotting analysis of MM-1S cells stimulated with IGF-1 (200 ng/ml) or IL-6 (50 ng/ml) for 0–24 h shows sustained increase in Akt phosphorylation (quantified by densitometric analysis) induced by IGF-1, but not by IL-6. (**c**) Immunoblotting analysis of cytosolic extracts of MM-1S cells shows that IGF-1 (200 ng/ml) induces significant and rapid phosphorylation of the FKHRL-1 Forkhead transcription factor, which is associated with its cytoplasmic sequestration and neutralization of its pro-apoptotic transcriptional activity. (**d**) Immunoblotting analysis of cytosolic extracts from MM-1S cells stimulated with IGF-1 (200 ng/ml) or IL-6 (50 ng/ml) for 1 h (following overnight incubation in serum-free media) shows more pronounced phosphorylation and cytoplasmic sequestration of FKHRL-1 induced by IGF-1 than by IL-6

effect of IGF-1 on caspase-8 expression. As can be seen in Figure 6, treatment of MM-1S cells with IGF-1 (0–300 ng/ml for 24 h) upregulated the expression of the caspase-8 inhibitor FLIP, the caspase-9 inhibitor XIAP, the caspase-3 inhibitor survivin, as well as the NF-κB-dependent cIAP-2 and A1/Bfl-1 proteins. In contrast minimal, if any, changes were noted in cIAP-1, Bcl-2, Bcl-X_L, Mcl-1, Bax or caspase-8 protein expression. Immunoblotting with anti-tubulin antibody confirmed equal protein loading. In marked contrast to the wide range of anti-apoptotic proteins upregulated by IGF-1, IL-6 stimulation significantly increased the protein expression of only survivin.

We next studied the mechanism whereby IGF-1 induces increases in the levels of the aforementioned anti-apoptotic proteins. As seen in Figure 7a, the PI-3K inhibitor LY294002 abrogated IGF-1-induced upregulation of FLIP, c-IAP2, survivin, XIAP, and A1/Bfl-1, implicating the PI-3K pathway in mediating the effects of IGF-1 on these anti-apoptotic proteins. Furthermore, SN50, a cell permeable peptide inhibitor of NF-κB nuclear translocation and transcriptional activity (Grundker *et al*., 2000; Han *et al*., 2001; Kolenko *et al*., 1999; Lin *et al*., 1995; Qin *et al*., 1998, 1999; Xiao *et al*., 2001; Ye *et al*., 1999), abrogated IGF-1-induced upregulation of survivin, cIAP-2, A1/Bfl-1 and XIAP (Figure 7b), suggesting that PI-3K related modulation of these anti-apoptotic proteins is, at least in part, mediated via NF-κB. Both PD98059, an inhibitor of the MAPK pathway, and LY294002 abrogated the IGF-1-induced upregulation of FLIP (Figure 7a), implicating both the PI-3K and MAPK pathways in mediating IGF-1-induced upregulation of FLIP. Although our prior studies demonstrate that inhibition

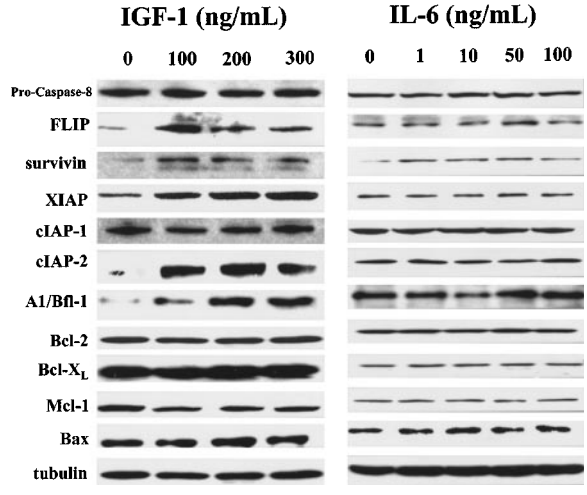

Figure 6 IGF-1- and IL-6-induced changes in the expression of anti- and pro-apoptotic proteins in MM-1S cells. (**a**) Immunoblotting analysis of MM-1S cells stimulated with IGF-1 (0–300 ng/ml for 24 h) demonstrates increased protein levels of caspase inhibitors FLIP, cIAP-2, XIAP, survivin, and the anti-apoptotic Bcl-2 family member A1/Bfl-1. No significant changes were noted in the expression of the other anti- or pro-apoptotic proteins. In contrast to IGF-1, IL-6 upregulates only the expression of survivin. Tubulin detection was used to confirm equal protein loading

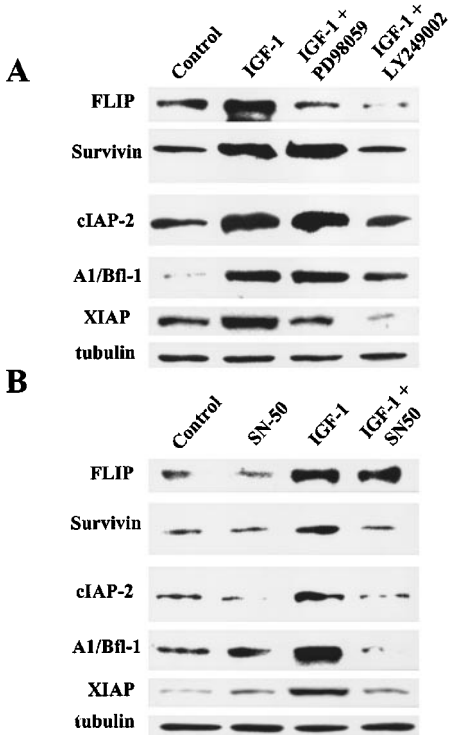

Figure 7 Regulation of IGF-1-induced upregulation of intracellular anti-apoptotic proteins by the PI-3K, MAPK, and NF-κB pathways. (**a**) Pre-incubation of MM-1S cells with the PI-3K inhibitor LY294002 (10 μM for 1 h) abrogated IGF-1-induced upregulation of FLIP, survivin, cIAP-2, A1/Bfl-1 and XIAP. PD98059 (25 μM), an inhibitor of the MAPK pathway, also blocks the upregulation of FLIP, suggesting that the effect of IGF-1 on FLIP regulation involves signaling input via both the PI-3K and MAPK pathways. (**b**) SN50 (10 μM), a cell permeable inhibitor of the nuclear translocation and transcriptional activity of NF-κB, abrogated IGF-1-induced upregulation of survivin, cIAP-2, XIAP and A1/Bfl-1

of NF-κB by SN50 (10 μM) does not significantly affect FLIP expression in MM cells (Mitsiades *et al*., 2002a) and the present study shows no effect of SN50 on FLIP up-regulation by IGF-1 in MM cells, FLIP expression is NF-κB dependent in T cells (Kreuz *et al*., 2001). These data suggest potential lineage- or tissue-specific patterns in regulation of FLIP.

Constitutive Akt activity reduces sensitivity of MM cells to novel and conventional therapies

IGF-1 is a highly pleiotropic cytokine which activates several different signaling pathways, e.g. PI-3K/Akt, MAPK. Our data implicate PI-3K/Akt and NF-κB in mediating the IGF-1-induced upregulation of several anti-apoptotic protein levels and related protection against apoptosis. To confirm the role of Akt in conferring this anti-apoptotic effect, we transfected MM-1S cells with a constitutively active Akt construct. This construct encodes for an Akt form with a myristoylation site targeting the kinase to the cell membrane, which is required for Akt phosphorylation and activation. The stably transfected MM-1S-myrAkt

cells were less sensitive to Apo2L/TRAIL than MM-1S-neo control vector-transfected cells (MM-1S-neo cells) (Figure 8). Interestingly, MM-1S-myrAkt cells also had significantly lower sensitivity to doxorubicin (Doxo) (Figure 9), indicating that Akt activation protects MM cells against both novel and conventional anti-tumor agents.

To further confirm the importance of Akt for the survival or MM cells, we incubated tumor cells with the Akt inhibitor IL-6-Hydroxymethyl-*chiro*-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate, which inhibits the Akt kinase activity with an IC_{50} of 5 μM (in contrast to its much higher IC_{50} for other kinases e.g. PI-3K, $IC_{50} = 83$ μM) (Hu *et al*., 2000). A 48-h incubation with this inhibitor reduced the survival of drug-sensitive (Dex-sensitive MM-1S and Doxo-sensitive RPMI-8226/S), as well as drug-resistant (Dex-resistant MM-1R and Doxo-resistant RPMI-8226/Dox40) MM cell lines (Figure 10a). In contrast, forced overexpression of constitutively active Akt protected MM-1S cells against apoptosis induced by the Akt inhibitor (Figure 10b). Conversely, the Akt inhibitor restored sensitivity of MM-1S-myr-Akt cells to Apo2L/TRAIL (Figure 10c). Furthermore, in MM-1S cells, the Akt inhibitor counteracted IGF-1-induced biologic effects, including protection against Apo2L/TRAIL (Figure 11a), NF-κB activation (Figure 11b) and upregulation of FLIP, survivin, XIAP, A1/Bfl-1 and XIAP (Figure 11c). These findings confirm a role for Akt signaling in regulating the responsiveness of MM cells to pro-apoptotic therapies.

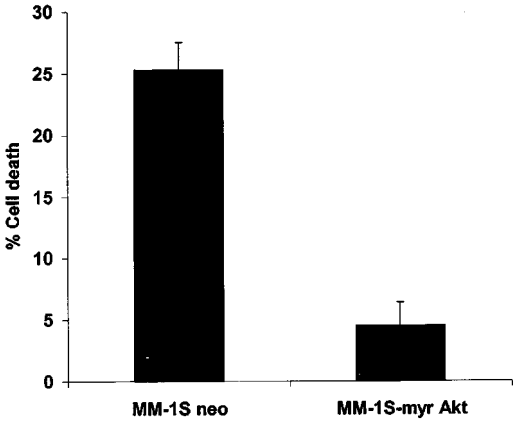

Figure 8 Constitutively active Akt reduces sensitivity of MM cells to Apo2L/TRAIL. MM-1S cells overexpressing constitutively active Akt construct (MM-1S-myr-Akt) incubated overnight with Apo2L/TRAIL (50 ng/ml) demonstrated significantly reduced sensitivity to Apo2L/TRAIL-induced than empty vector transfected MM-1S-neo cells, as evidenced by MTT colorimetric assay

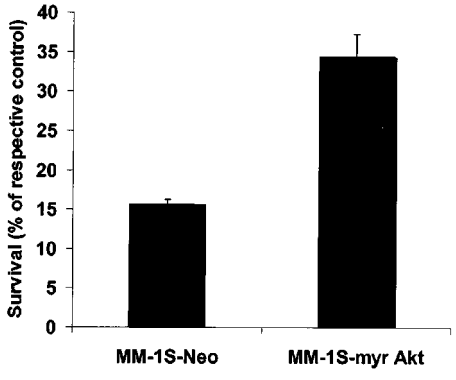

Figure 9 Reduced chemosensitivity of MM-1S cells transfected with constitutively active Akt construct. MM-1S-myr-Akt and MM-1S-neo cells were incubated for 72 h with doxorubicin (250 ng/ml) in quadruplicate samples per condition. MTT colorimetric assay showed significantly higher survival of MM-1S-myr-Akt cells with constitutively active Akt, in comparison to empty vector transfected MM-1S-neo cells

Discussion

IGF-1 and IL-6 are both major growth factors for MM cells and confer protection against Dex-induced apoptosis (Chauhan *et al*., 2000; Xu *et al*., 1997). However, our studies indicate that these cytokines may differ in their ability to protect MM cells from other apoptosis-inducing agents. In this study, we report that IGF-1 reduces the sensitivity of MM cells to Apo2L/TRAIL, in contrast to IL-6 which, as our prior studies show, confers no protection (Mitsiades *et al*., 2001b). These findings suggest that IGF-1 can exert anti-apoptotic effects against a wider range of pro-apoptotic agents, which is important from both a pathophysiologic and clinical standpoint. In upcoming clinical trials, Apo2L/TRAIL and potentially other novel biologically-based therapies may have enhanced anti-myeloma activity when combined with therapeutic strategies designed to abrogate this anti-apoptotic effect of IGF1.

By investigating the molecular pathways implicated in the anti-apoptotic effect of IGF-1, we documented that IGF-1 and IL-6 have differential effects on a series of important mechanisms regulating cell survival. We found that IGF-1 stimulated pronounced and prolonged activation of NF-κB and Akt; induced sustained phosphorylation and cytoplasmic sequestration of FKHRL-1 transcription factor, leading to neutralization of its pro-apoptotic transcriptional activity; and induced increased levels of several anti-apoptotic proteins, such as the caspase-8 inhibitors FLIP and cIAP-2, the caspase-3 inhibitor survivin, the caspase-9 inhibitor XIAP, and the anti-apoptotic mitochondrial protein A1/Bfl-1. In marked contrast, IL-6 did not induce significant NF-κB activity; Akt activation and FKHRL-1 phosphorylation were induced by IL-6, but were less pronounced and not as prolonged as that triggered by IGF-1. Furthermore, IL-6 upregulated only the expression of survivin and had no effect on the other anti-apoptotic proteins whose expression is upregulated by IGF-1. These findings suggest that IGF-1 exerts broader anti-apoptotic effects on MM cells due to its ability to activate multiple mechanisms promoting cell survival and, importantly, to induce protracted stimulation of key anti-apoptotic effectors, such as Akt and NF-κB.

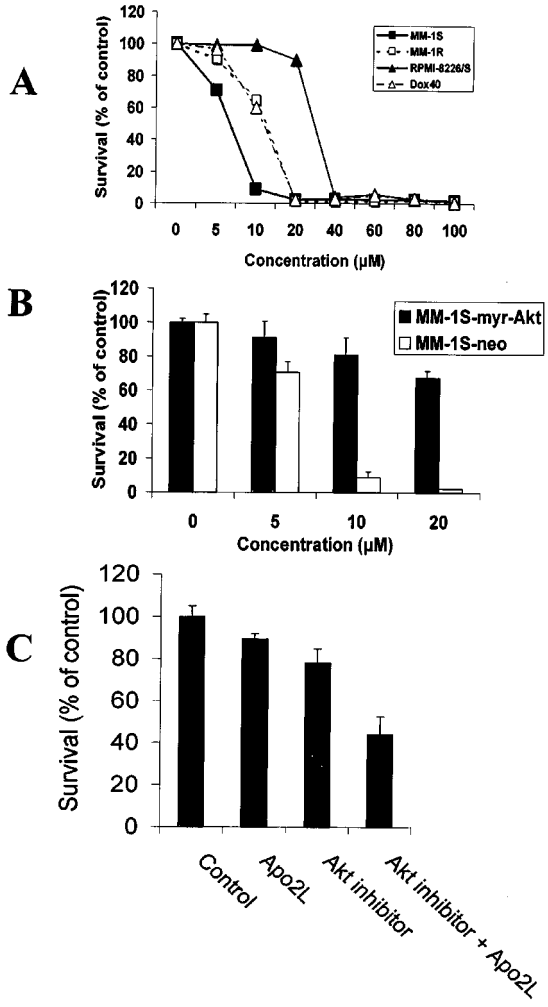

Figure 10 A small molecule inhibitor of Akt modulates the survival of drug-resistant and drug-sensitive MM cells. (**a**) A panel of MM cell lines, including the Dex-sensitive (MM-1S), its Dex-resistant subline MM-1R, the Doxo-sensitive RPMI-8226/S, and its Dox-resistant subline (RPMI-8226/Dox40) were incubated for 48 h with IL-6-Hydroxymethyl-*chiro*-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate, a specific inhibitor of Akt kinase. MTT assays showed that the Akt inhibitor reduced survival of both drug-sensitive and resistant MM cell lines. (**b**) Stable transfection of constitutively active Akt in the MM-1S-myr-Akt cells significantly reduced the impact of the Akt inhibitor on their survival, in comparison to MM-1S-neo cells. (**c**) Pre-incubation of MM-1S-myr-Akt cells with the Akt inhibitor IL-6-Hydroxymethyl-*chiro*-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (20 μM for 6 h) increases their sensitivity to Apo2L/TRAIL (overnight incubation with 50 ng/ml), as determined by MTT assay

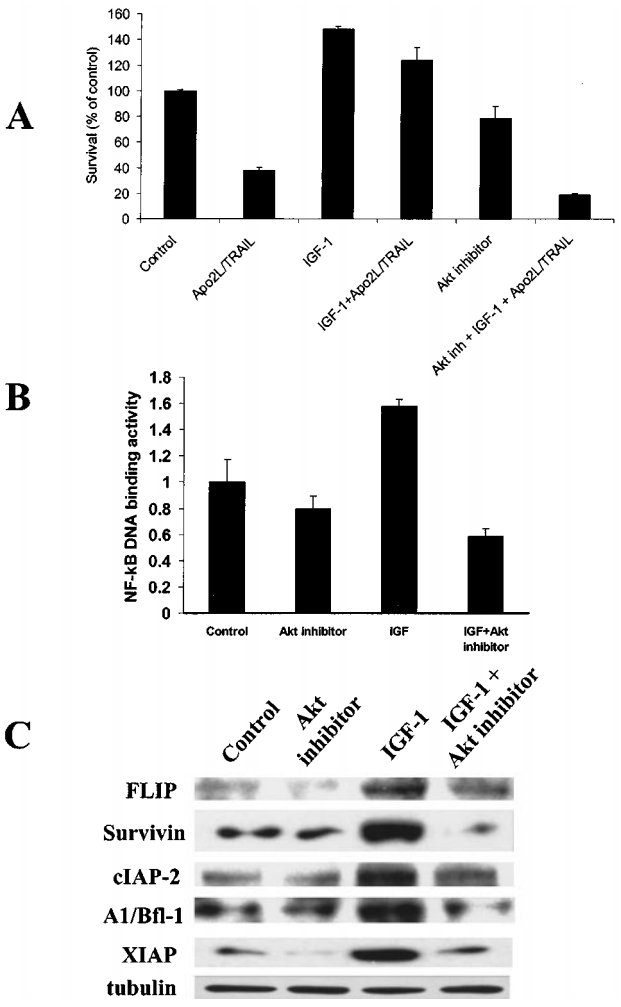

Figure 11 A small molecule inhibitor of Akt counteracts the biologic effects of IGF-1 on MM-1S cells. MM-1S cells were pre-incubated with the Akt inhibitor IL-6-Hydroxymethyl-*chiro*-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (20 μM for 6 h). The Akt inhibitor opposes the IGF-1-induced decrease in sensitivity to Apo2L/TRAIL (overnight incubation with 50 ng/ml), as determined by MTT assay (**a**); blocks the IGF-1-induced upregulation of NF-κB DNA binding activity (**b**); and blocks the IGF-1-induced upregulation of the anti-apoptotic proteins FLIP, survivin, cIAP-2, A1/BFl-1, and XIAP (**c**)

NF-κB is a key regulator of Apo2L/TRAIL-sensitivity of MM cells: NF-κB inhibition increases their sensitivity to this tumoricidal ligand or even reverses the resistance against it, in certain MM cells (Mitsiades *et al*., 2001b, 2002a). We have also recently shown that FLIP and cIAP-2 (which both inhibit caspase-8, the apical caspase for Apo2L/TRAIL-induced cell death) as well as XIAP, are more highly expressed in Apo2L/TRAIL-resistant vs -sensitive MM cells (Mitsiades *et al*., 2002a). Furthermore, FLIP antisense oligodeoxynucleotides sensitize MM cells to Apo2L/TRAIL-induced apoptosis (Mitsiades *et al*., 2002a). These findings, coupled with the present study, suggest that NF-κB activation and upregulation of FLIP, cIAP-2, and XIAP contribute to the protection conferred by IGF-1 against Apo2L/TRAIL.

Although IL-6 does not stimulate NF-κB activation or upregulation of FLIP, cIAP-2 or XIAP, it does induce, similarly to IGF-1, increased protein levels of the caspase-3 inhibitor, survivin. This protein may play a role in the protection that both IGF-1 and IL-6 confer against Dex-induced apoptosis. In a recent study of gene expression profiling, we found higher survivin expression in Dex-resistant MM-1R cells than in parental Dex-sensitive MM-1S MM cells (Chauhan *et al*., 2002). Moreover, Dex induces apoptosis of MM

cells via activation of caspase-9 (Chauhan *et al*., 2000), which is upstream of caspase-3, further supporting the notion that upregulation of survivin may be a common inhibitory mechanism whereby both IL-6 and IGF-1 protect against Dex-induced caspase-3 activation and apoptosis. In contrast, Apo2L/TRAIL-induced apoptosis in MM cells occurs in a caspase-3-independent fashion (Mitsiades *et al*., 2002a), which may explain why upregulation of survivin by IL-6 cannot confer protection against Apo2L/TRAIL. Since caspase-8 is the apical caspase mediating Apo2L/TRAIL-induced apoptosis in MM cells (Mitsiades *et al*., 2002a), upregulation of its inhibitors (e.g. FLIP, cIAP-2) by IGF-1 can protect against Apo2L/TRAIL.

In our studies, the PI-3K/Akt pathway is implicated in the entire spectrum of IGF-1-induced anti-apoptotic mechanisms (e.g. NF-κB, FKHRL-1, caspase inhibitors, A1/Bfl-1). This raises the question as to why IL-6, which also activates the PI-3K/Akt pathway (Hideshima *et al*., 2001b) cannot induce significant NF-κB activation or upregulation of as many caspase inhibitors as does IGF-1. It is possible that these molecular events require pronounced and prolonged activation of Akt, which, as shown in this study, is achieved by IGF-1 but not by IL-6. Another possibility is that IGF-1, but not IL-6, activates additional signaling cascades which cross-talk and amplify Akt signaling. These pathways triggered by IGF-1 are under active study in our laboratory.

To confirm the key role of the PI-3K/Akt pathway in the anti-apoptotic properties of IGF-1, we transfected MM-1S cells with a construct for constitutively active Akt. This forced overexpression significantly reduced the sensitivity of MM cells to Apo2L/TRAIL, as well as doxorubicin, suggesting that Akt confers protection against a spectrum of both conventional (e.g. doxorubicin, dexamethasone) as well as novel pro-apoptotic agents. In our study, the Akt inhibitor IL-6-Hydroxymethyl-*chiro*-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate induced cell death of MM cells, including both Dex- or Doxo-sensitive and -resistant MM cells, further confirming the role of Akt in mediating tumor cell survival. In MM-1S-myr-Akt cells, this Akt inhibitor had significantly reduced anti-MM activity, but could restore sensitivity to Apo2L/TRAIL. In MM-1S cells, the small molecule Akt inhibitor could also counteract IGF-1-induced biologic effects, including its protective effect against Apo2L/TRAIL, the upregulation of NF-κB activity, and the increased protein levels of anti-apoptotic proteins such as FLIP, XIAP, survivin, cIAP-2 and A1/BFl-1. These findings indicate that Akt plays an important role in regulating the responsiveness of MM cells to apoptosis-inducing agents; and conversely, that inhibition of the IGF-1/Akt axis can enhance the anti-tumor activity of novel, e.g. Apo2L/TRAIL, as well as conventional, e.g. Doxo or Dex, agents. Our studies further suggest distinct molecular levels where the IGF-1/Akt axis may be targeted therapeutically, i.e. inhibition of the IGF-1 receptor (e.g. with aIR-3 neutralizing antibody) or Akt itself (e.g. with small molecule inhibitors).

The role of Akt activity in mediating Apo2L/TRAIL-resistance has not been previously addressed in hematologic malignancies, but has been studied in only two solid tumor models with apparently conflicting results: constitutive Akt activity protects from Apo2L/TRAIL in prostate carcinoma (Chen *et al*., 2001; Nesterov *et al*., 2001; Panka *et al*., 2001; Thakkar *et al*., 2001), but not in colon carcinoma (Burns and El-Deiry, 2001), suggesting tissue-specific differences in the anti-apoptotic role of Akt. Therefore, our present study specifically addressed whether Akt mediates survival and drug resistance in myeloma. Importantly, we showed that IGF-1 can potently activate PI-3K/Akt pathway and downstream signaling to modulate sensitivity to conventional or novel anti-MM therapies. The concentrations of IGF-1 used in our studies are present in MM patient sera (Mitsiades, unpublished observation); moreover, IGF-1 is produced by the bone marrow (BM) microenvironment (e.g. osteoblasts and BM stromal cells), suggesting that MM cells in the BM milieu may be exposed to higher levels of IGF-1 than those present in circulation, with more pronounced IGF-1-induced biologic sequelae.

In summary, our data indicate that IGF-1, a protein present in both the bone microenvironment and the peripheral blood, induces a wide range of anti-apoptotic molecular events, including potent and prolonged activation of NF-κB and Akt; phosphorylation of FKHRL-1; and increased protein expression of several anti-apoptotic proteins, such as the caspase-8 inhibitors FLIP and cIAP-2. Importantly, our study suggests that the activity of novel as well as conventional anti-MM therapies may be enhanced when combined with treatment strategies designed to neutralize this protective effect of IGF-1.

Materials and methods

Materials and tissue culture

Reagents were obtained as follows: mouse monoclonal antibodies (Abs) for Bcl-2, Bcl-X_L, Mcl-1, Bax and the polyclonal Ab for A1/Bfl-1 from Santa Cruz Biotechnology (Santa Cruz, CA, USA); human recombinant IGF-I as well as polyclonal antisera against c-IAP-1, cIAP-2 and XIAP from R&D Systems, Inc. (Minneapolis, MN, USA); rabbit polyclonal Abs against FLIP, FKHRL-1, phospho-Thr32-FKHRL-1 and phospho-Ser-253-FKHRL-1 from Upstate Biotechnologies (Lake Placid, NY, USA); rabbit polyclonal Ab for survivin, mouse monoclonal aIR-3 neutralizing Ab against the IGF-1 receptor, and its mouse IgG1 isotype control from Oncogene Research (San Diego, CA, USA); the LY249002 inhibitor of PI-3K and the PD98059 inhibitor of MAPK pathway from Sigma Chemical (St. Louis, MO, USA); the Akt inhibitor IL-6-Hydroxymethyl-*chiro*-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate from Calbiochem (La Jolla, CA, USA); the goat polyclonal Abs for human DR4, DR5, DcR1 and DcR2; and recombinant human Apo2L/TRAIL from Genentech, Inc. (San Francisco, CA, USA); mouse MsIgG1 and MsIgG2b isotype controls, as well as the Annexin V-PI early apoptosis detection kit from Beckman Coulter/Immunotech (Miami, FL, USA); IGF-1, IL-6, TNF-α, goat anti-human DR4, DR5 and DcR2

polyclonal Abs from R&D Systems, Inc. (Minneapolis, MN, USA); anti-human DcR2 rabbit polyclonal Ab from Imgenex (San Diego, CA, USA); rabbit anti-human DcR1 polyclonal Ab from Affinity Bioreagents (Golden, CO, USA); goat anti-mouse IgG (FITC) conjugated $F(ab')_2$ fragment, donkey anti-goat IgG FITC conjugated $F(ab')_2$ fragment, and donkey anti-rabbit IgG FITC conjugated $F(ab')_2$ fragment from Jackson Immunoresearch Laboratories (West Grove, PA, USA); MTT and doxorubicin from Sigma Chemical Co. (St Louis, MO, USA); the inhibitory peptide SN50 and its mutant control peptide SN50M from BIOMOL (Plymouth Meeting, PA, USA). MM-1S and MM-1R cells were kindly provided by Dr Steven Rosen (Northwestern University, Chicago, IL, USA); RPMI-8226/S and RPMI-8226/Dox40 were kindly provided by Dr William Dalton (University of Arizona, Tucson, AZ, USA). All cells were cultured in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY, USA) supplemented with 10% charcoal dextran-treated fetal bovine serum (FBS; Hyclone, Logan, UT, USA) as well as L-glutamine, penicillin, and streptomycin (GIBCO). Tumor cells from MM patients were freshly isolated from bone marrow (BM) aspirates by Ficoll density centrifugation and subsequent purification of MM cells using either flow cytometric cell sorting or immunomagnetic sorting for CD138 (post-sorting samples were $>95\%$ $CD38^+$ $CD138^+$). IGF-1 stimulation studies of MM cells were performed following overnight culture in serum-free medium. The effect of IGF-1 on Apo2L/TRAIL-induced apoptosis was studied by pre-incubation of MM-1S cells (2 h) with IGF-1 (0–200 ng/ml), followed by overnight exposure to 100 ng/ml of Apo2L/TRAIL. Apo2L/TRAIL-induced reduction in cell survival was quantified by both MTT colorimetric assay and by flow cytometric analysis following staining with Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI), as previously described (Mitsiades *et al*., 2001b).

NF-κB DNA binding assay

The DNA binding activity of NF-κB was quantified by enzyme linked immunosorbent assay (ELISA) using the Trans-AM™ NF-κB p65 Transcription Factor Assay Kit (Active Motif North America, Carlsbad, CA, USA), according to the instructions of the manufacturer. The use of this assay for quantification of DNA binding activity of NF-κB (or other transcription factors) has been previously substantiated in both MM and other models (Joussen *et al*., 2002; Mitsiades *et al*., 2002b,c). Briefly, MM-1S cells (following overnight incubation in serum-free medium) were stimulated with IGF-1 (200 ng/ml) for 0–3 h (each condition was performed in triplicate), with or without pre-incubation (1 h) with LY249002 or PD98059. The effect of IGF-1 on NF-κB activity was compared with its modulation by IL-6 (50 ng/ml) or TNF-α (5 ng/ml). Nuclear extracts were prepared as previously described (Powers *et al*., 1996) and incubated in 96-well plates coated with immobilized oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′) containing a consensus (5′-GGGACTTTCC-3′) binding site for the p65 subunit of NF-κB. NF-κB binding to the target oligonucleotide was detected by incubation with primary Ab specific for the activated form of p65 (Active Motif North America), visualized by anti-IgG horseradish peroxidase (HRP)-conjugate and Developing Solution, and quantified by spectrophotometry at 450 nm with a reference wavelength of 655 nm. Background binding, obtained by incubation with a 2-nucleotide mutant oligonucleotide (5′-AGTTGAGGC-CACTTTCCCAGGC-3′), was subtracted from the value obtained for binding to the consensus DNA sequence.

Detection of Akt activity

MM-1S cells were treated with IGF-1 (200 ng/ml) or IL-6 (50 ng/ml) for 1 h, in the presence (1 h pre-incubation) or absence of LY294002 (20 μM). Akt was immunoprecipitated and its enzymatic activity was assessed with the Akt Kinase Assay Kit (Cell Signaling Technology, Beverly, MA, USA) using a GSK-3 fusion protein as substrate, according to the instructions of the manufacturer.

Immunoblotting

Immunoblotting analysis for intracellular anti-apoptotic proteins was performed as previously described (Mitsiades *et al*., 2001b; Poulaki *et al*., 2001). Immunoblotting was performed to detect FKHRL-1 and phospho-FKHRL-1 in the cytoplasmic fraction, and the p50 NF-κB subunit in nuclear extracts of MM-1S cells, which were obtained, as described in previous studies (Poulaki *et al*., 2001). Densitometric analyses of scanned immunoblotting images were performed with the NIH Image Software. Densitometric values were expressed as fold change relative to control.

Transfection of constitutively active Akt construct

MM-1S cells were transfected with a vector carrying an Akt construct containing a myristoylation site, leading to constitutive activation of Akt kinase (Upstate Biotechnologies, Lake Placid, NY, USA), or the empty (neo) vector with the Lipofectamine 2000 (Life Technologies), according to the instructions of the manufacturer. Forty-eight hours later, the cells were incubated in growth medium containing G418 (500 μg/ml, Life Technologies) to select pools of stable transfectants.

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