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c-myb stimulates cell growth by regulation of insulin-like growth factor (IGF) and IGF-binding protein-3 in K562 leukemia cells

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ABSTRACT

c-myb plays an important role in the regulation of cell growth and differentiation, and is highly expressed in immature hematopoietic cells. The human chronic myelogenous leukemia cell K562, highly expresses IGF-I, IGF-II, IGF-IR, and IGF-induced cellular proliferation is mediated by IGF-IR. To characterize the impact of c-myb on the IGF–IGFBP-3 axis in leukemia cells, we overexpressed c-myb using an adenovirus gene transfer system in K562 cells. The overexpression of c-myb induced cell proliferation, compared to control, and c-myb induced cell growth was inhibited by anti-IGF-IR antibodies. c-myb overexpression resulted in a significant increase in the expression of IGF-I, IGF-II, and IGF-IR, and a decrease in IGFBP-3 expression. By contrast, disruption of c-myb function by DN-myb overexpression resulted in significant reduction of IGF-I, IGF-II, IGF-IR, and elevation of IGFBP-3 expression. In addition, exogenous IGFBP-3 inhibited the proliferation of K562 cells, and c-myb induced cell growth was blocked by IGFBP-3 overexpression in a dose-dependent manner. The growth-promoting effects of c-myb were mediated through two major intracellular signaling pathways, Akt and Erk. Activation of Akt and Erk by c-myb was completely blocked by IGF-IR and IGFBP-3 antibodies. These findings suggest that c-myb stimulates cell growth, in part, by regulating expression of the components of IGF–IGFBP axis in K562 cells. In addition, disruption of c-myb function by DN-myb may provide a useful strategy for treatment of leukemia.

Introduction

The c-myb proto-oncogene is a nuclear transcription factor that is homologous to the transforming gene product of avian myeloblastosis virus, which plays an important role in the regulation of cell growth and differentiation [1]. c-myb functions in normal and malignant hematopoiesis by regulating the G1/S transition in cycling hematopoietic cells [2], and by serving as a transactivator of important cellular genes such as the Kit receptor [3] and CD34 [4]. Amplification of c-myb in acute myeloid leukemia and c-myb overexpression in the 6q-syndrome have been reported [5], suggesting that c-myb may play a role in leukemogenesis. Consequently, disrupting c-myb function might provide a possible strategy for controlling leukemia cell growth. In a previous study, we demonstrated that disruption of c-myb by DN-myb resulted in induction of apoptosis in K562 cells [6].

IGF-I and IGF-II are recognized as potent mitogens in many tumor types, and act in an autocrine–paracrine manner [7]. Their mitogenic effects are mediated by IGF-I receptor and thereby, activating the receptor tyrosine kinase [8]. The biological activity of the IGFs is modulated by IGFBP family. Among the six IGFBPs, the IGFBP-3 is the major circulating IGFBP, binding to >75% of serum IGFs in a complex with an acid-labile subunit [9]. It has been shown that IGFBP-3 both potentiates IGF activity [10], as well as functions as a cell growth inhibitor and/or promoter of apoptosis [11]. Several studies have shown that IGFBP-3 mediates transforming growth factor-β [12] and retinoic acid [13]. IGF-I stimulates the growth of myeloid and lymphoid progenitors [14,15] and enhances B-cell differentiation [16]. The human chronic myelogenous leukemia cell line, K562, highly expresses IGF-I, IGF-II, IGF-IR, and IGF-induced cellular proliferation mediated by IGF-IR [17]. It has been reported that the expression of IGFBP-5 is stimulated by c-myb at the transcription level in neuroblastoma cells [18].

Abbreviations: DN-myb, dominant negative myb; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; IGF-IR, insulin-like growth factor-I receptor; Erk, extracellular signal regulated kinase; MAPK, mitogen-activated protein kinase; PI3 kinase, phosphatidylinositol-3 kinase.

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In this study, we investigated whether c-myb regulates cell proliferation by modulation of the IGF axis in leukemia cells. We report here that overexpression of c-myb increased K562 cell growth by up-regulation of IGF-I, IGF-II, and IGF-IR expression, and down-regulation of IGFBP-3 expression.

Materials and methods

Materials. Cell culture reagents, fetal bovine serum (FBS), and RPMI-1640 were purchased from Gibco–BRL Life Technologies (Gaithersburg, MD). Recombinant human IGF-I and IGFBP-3 peptides were obtained from Bachem (Toranced, CA) and Abcam (Cambridge, UK). Monoclonal anti-c-myb and anti-IGF-IR antibodies were purchased from Oncogene (Boston, MA) and R&D systems (Minneapolis, MN). Polyclonal anti-IGFBP-3 antibody and all secondary antibodies were purchased from and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal antibodies to total Akt, phospho-Akt, and phospho-Erk were purchased from Cell Signaling Technologies (Beverly, MA).

Cell lines and cell culture. The K562 cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate. All cell cultures were incubated at 37 °C in a 5% CO_2 incubator.

RNA extraction and semi-quantitative RT-PCR. Cells were treated with TRI reagent® (Molecular Research Center, Cincinnati, OH) and total RNA was extracted according to the manufacturer's instructions. RT and agarose gel electrophoresis were performed as previously described [6]. Specific primer sets were designed from published cDNA sequences: sense IGF-I: 5′-CAC AGG GTA TGG CTC-3′, anti-sense IGF-I: 5′-CTT CTG GGT CTT GGG-3′; sense IGF-II: 5′-CGA TGC TGG TGC TTC TCA-3′; anti-sense IGF-II: 5′-GGG GTC TTG GGT GGG TAG-3′; sense IGF-I receptor: 5′-TTG CCC GAA GGT CTG TGA-3′; anti-sense IGF-I receptor: 5′-CCC GTT GTT CCT GGT GTT-3′; sense IGFBP-3: 5′-CTC TCC CAG GCT ACA CCA-3′; anti-sense IGFBP-3, 5′-GAA GTC TGG GTG CTG TGC-3′.

Preparation and administration of adenovirus vectors. The E1/E3-deleted replication-deficient recombinant adenovirus was made using the AdEasy system (Quantum Biotechnologies, Montreal, Canada) described by He et al. [19]. Kpn–XhoI restriction fragments from pcDNA3/*myb* and pcDNA3/DN-*myb* were ligated into Kpn–XhoI-digested pShuttle-CMV [6]. To create AdIGFBP-3, NotI–XbaI restriction fragments from pcDNA3/wild-type IGFBP-3 cDNA were ligated into NotI–XbaI-digested pShuttle-CMV9 [20]. In addition, for Ad/*LacZ*, a SalI–NotI restriction fragment from pcDNA3.1/*LacZ* (Invitrogen, San Diego, CA) was ligated into SalI–NotI-digested pShuttle-CMV. Recombination into the pAdEasy viral backbone was accomplished in bacteria (*Escherichia coli*, strain BJ5183, which is recombination deficient) according to the manufacturer's instructions. The recombination was verified and the adenoviral recombinant DNA was transferred to a regular *E. coli* strain (DH5α). Recombinant pAdEasy plasmids containing CMV-cDNA inserts were purified over Qiagen columns (Valencia, CA) and 5 μg of PacI-digested DNA was used to transfect QBI-293A cells by the calcium phosphate method (Promega, Madison, WI). The cells were seeded at 2×10^6 cells per 150-mm culture dishes 24 h prior to transfection. Lysis of the transfected cells, indicating adenoviral growth, occurred by 4 days. Following amplification, the lysates containing clonal recombinant adenovirus, from 150 mm culture dishes, were purified by CsCl gradient centrifugation. The recovered virus was aliquoted and stored at −20 °C in 5 mM Tris (pH 8.0) buffer containing 50 mM NaCl, 0.05% bovine serum albumin, and 25% glycerol. The virus was titrated by serial dilution infection of QBI-293A cells, and the plaques were counted under an overlay of 0.3% agarose, 10% FBS, and 1% DMEM. For adenovirus infection, subconfluent cells were infected at a known multiplicity of infection (MOI) with adenovirus in culture medium supplemented with 2% FBS. After 1 h at 37 °C with gentle frequent shaking, the cells were washed twice with phosphate-buffered saline and fresh complete medium was added.

Western blot analysis. Western blots were performed as previously described [6]. In brief, conditioned media or cell lysates were size-fractionated by 12% SDS–PAGE under non-reducing conditions and electroblotted onto nitrocellulose filters (Hybond; Amersham Biotech, Piscataway, NJ). Filters were blocked with 3% non-fat milk/TBS-T for 1 h at room temperature, and then incubated with primary antibody for 2 h. Protein signals were detected by enhanced chemiluminescence (NEN, Boston, MA).

MTT assay. Cell proliferation was evaluated by MTT assay as previously described [6]. In brief, cells that had been infected with c-myb, DN-myb, IGFBP-3 or treated with IGF-I, IGFBP-3, and anti-IGF-IR antibodies were plated in 10% FBS-RPMI at a density of 2×10^4 cells/well. After the indicated period of culture, 1 ml of MTT solution was added for 4 h. Then, the plates were centrifuged, and DMSO was added. Absorbance at 540 nm was measured by spectrophotometry.

Densitometric and statistical analyses. To quantify the relative induction of the IGF axis, densitometric measurement was performed using a GS-700 Imaging Densitometer with Multi-Analyst software (Bio-Rad, Hercules, CA). Results are expressed as means ± SD. Statistical analysis of the data was performed using a Student's *t* test and *P* values of <0.05 were considered significant.

Results

c-myb stimulated cell growth in leukemia cells

To characterize the role of c-myb in K562 leukemia cells, the cells were infected with adenovirus containing c-myb (Ad/myb) or DN-myb (Ad/DN-myb). The level of c-myb protein was significantly increased in the Ad/myb infected cells by 2 days after infection, compared to the controls (data not shown). By contrast, the c-myb protein in the Ad/DN-myb infected cells was significantly decreased. Next, we examined the effect of c-myb on the growth of K562 cells. As shown in Fig. 1A, K562 cells showed a significant increase of growth after infection with c-myb virus compared to lacZ infected cells at a MOI of 100 with a maximum of a 1.6-fold increase in the growth rate by 4 days after infection ($p < 0.05$). The growth of DN-myb infected cells was significantly reduced in a dose-dependent manner over a 4-day period ($p < 0.05$).

IGF-I receptor antibody blocked c-myb induced cell growth

To determine the effects of IGF-I on the growth of K562 cells, cells were cultured under serum free conditions plus 10 or 50 ng/ml of IGF-I. The cell growth was significantly increased in a dose-dependent manner over a 2-day period (Fig. 1B, $p < 0.05$). Next, we examined whether anti-IGF-IR antibody would block the IGF-I induced cell growth in K562 cells. As shown in Fig. 1B, antibodies to IGF-IR significantly inhibited IGF-I induced cell proliferation. Furthermore, anti-IGF-IR antibodies had a significant inhibitory effect on c-myb induced cell proliferation at 2 days after infection (Fig. 1C).

c-myb effects on IGF–IGFBP-3 axis expression

Since IGF axis serves as a potent mitogen in cancer cells, we examined the effects of c-myb on the expression of IGF-I, IGF-II, IGF-IR, and IGFBP-3 in K562 cells. As seen in Fig. 2A, overexpression of c-myb in K562 cells (at a MOI of 100) resulted in an increase

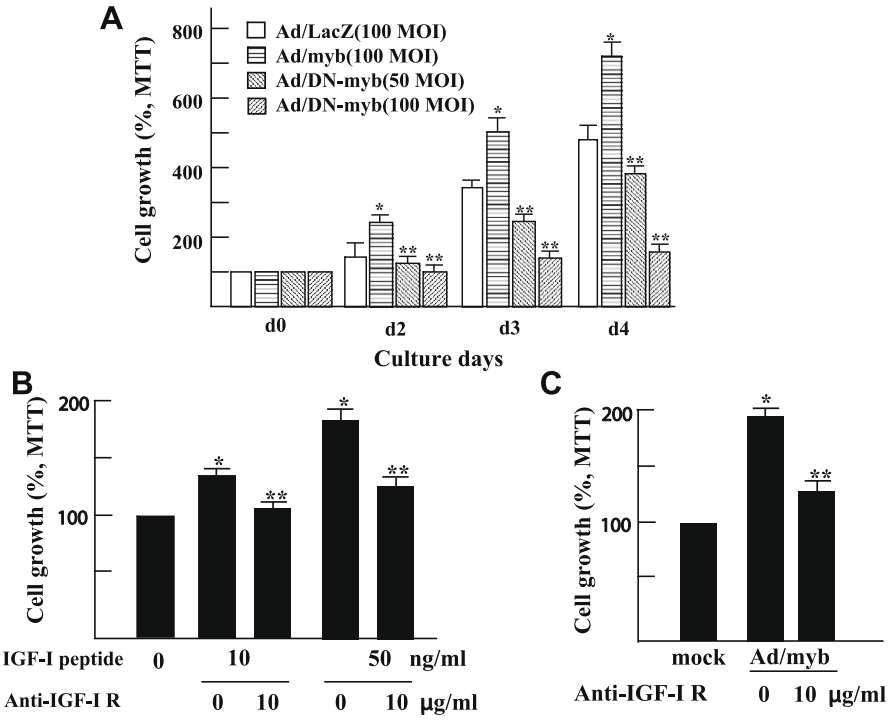

Fig. 1. The effect of c-myb, DN-myb, IGF-I, and IGF-I receptor antibody on cell growth. (A) The cells (2×10^4/well) infected with c-myb (Ad/myb, at a MOI of 100), DN-myb (Ad/DN-myb, at a MOI of 50 or 100) or control vector (Ad/LacZ, at a MOI of 100) were grown over a 4 day period. The number of viable cells was determined by a MTT assay, and is presented as the percent of cell growth. Results are means ± SE of three separate experiments performed in triplicate wells. $^{*}p < 0.05$ compared with LacZ-infected cells. $^{**}p < 0.05$ compared with c-myb-infected cells. (B) Cells were cultured in serum free medium for 24 h and then incubated with IGF-I (10, 50 mg/ml) in the absence (0) or presence of 10 μg/ml of anti-IGF-I receptor antibody for 48 h. $^{*}p < 0.05$ compared with absence of IGF-I treatment. $^{**}p < 0.05$ compared with absence of IGF-I receptor antibody treatment. (C) Cells were mock infected (Mock) or infected with c-myb were grown in the absence or presence of 10 μg/ml of anti-IGF-I receptor antibody for 48 h. $^{*}p < 0.05$ as compared with mock infected cells. $^{**}p < 0.05$ as compared with absence of anti-IGF-I receptor antibody treatment.

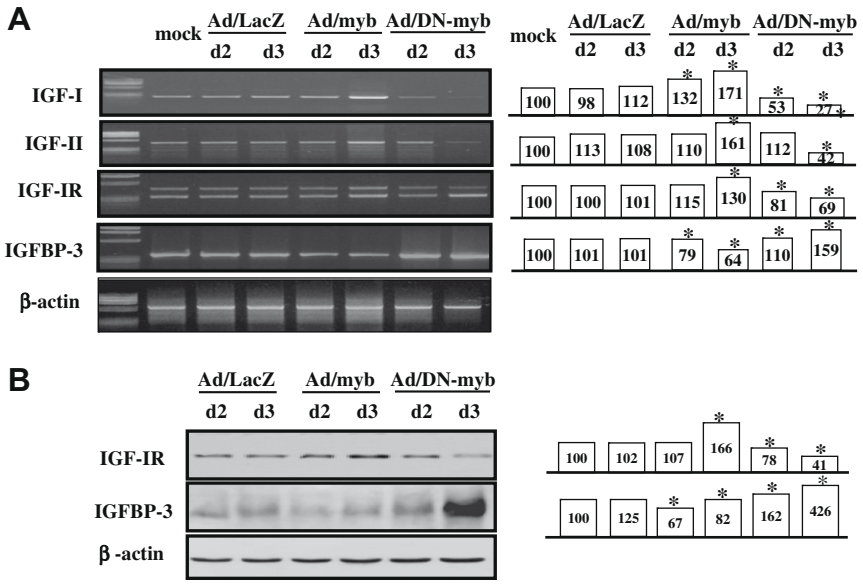

Fig. 2. The effect of c-myb and DN-myb on IGFs and IGFBP-3 expression. Cells (1×10^6 cells/dish) infected with c-myb (Ad/myb, at a MOI of 100), DN-myb (Ad/DN-myb, at a MOI of 50), or control vector (Ad/LacZ, at a MOI of 100) were grown for 2 days (d2), or 3 days (d3). (A) Total RNAs were extracted and analyzed by RT-PCR, as described under Materials and methods. (B) Cell lysates or condition media were collected and analyzed by Western blot analysis with antibodies against the IGF-IR, anti-IGFBP-3, and anti-actin antibodies. Bar graphs are generated from the densitometric analysis of Fig. 3A and B from three separate experiments, and the results represent means ± S.E. Each mRNA or protein level was normalized versus the corresponding actin mRNA or protein level. $^{*}p < 0.05$ compared to the Ad/LacZ-infected cells.

of IGF-I and IGF-II mRNA with a maximum of 1.7-fold increase in expression at 3 days after infection, (Fig. 2A, $p < 0.05$). In addition, IGF-IR mRNA and protein expression in the c-myb overexpressed cells increased, with a 1.6-fold increased expression at 3 days after infection (Fig. 2A and B, $p < 0.05$). Overexpression of c-myb in K562 cells resulted in a significant reduction of IGFBP-3 mRNA and protein levels (Fig. 2A and B, $p < 0.05$). By contrast, overexpression of DN-myb induced significant elevation of IGFBP-3 mRNA and protein levels over 3 days after infection that correlated with the decreased expression of c-myb in corresponding cell extracts.

IGFBP-3 inhibited c-myb induced cell growth

To assess whether the reduction of IGFBP-3 expression by c-myb effects cell growth in K562 cells, a cell proliferation assay, in the presence of recombinant IGFBP-3 protein, was carried out. As shown in Fig. 3A, a significant growth reduction was observed ($p < 0.05$) in the IGFBP-3 treated K562 cells; this inhibitory effect of IGFBP-3 was dose and time dependent. Furthermore, c-myb induced cell growth was significantly blocked by IGFBP-3 overexpression compared to the controls in a dose- and time-dependent manner over 4-day period (Fig. 3B, $p < 0.05$).

IGF-I receptor antibody and IGFBP-3 blocked c-myb induced phosphorylation of Akt and Erk

To determine which of the major signaling pathways were involved in c-myb gene function, we examined the phosphorylation states of Akt and Erk in K562 leukemia cells. As shown in Fig. 4A, the phosphorylation of Akt and Erk were significantly increased in the c-myb infected cells over 3 days after infection; these changes were completely blocked by overexpression of DN-myb. Next, we investigated whether Akt and Erk pathway activation by c-myb was mediated by IGF-IR. As shown in Fig. 4B, IGF-IR antibodies markedly reduced phosphorylated Akt and Erk activity in a dose-dependent manner. In addition, overexpression of IGFBP-3 resulted in significant reduction of phosphorylated Akt and Erk induced by c-myb overexpression (Fig. 4C).

Discussion

The results of this study show that overexpression of c-myb increased cell growth and resulted in up-regulation of IGF-I, IGF-II, and IGF-IR in K562 leukemia cells. In addition, the c-myb gene suppressed IGFBP-3 expression. c-myb has been shown to be essential for the proliferation of normal and leukemic hematopoietic cells and for the proliferation and survival of lymphoma cells [1,2]. Although c-myb expression was initially thought to be restricted to the hematopoietic cell system, it has been reported in non-hematopoietic tissues and cancer cells [21]. The presence of a functional IGF-IR is an essential prerequisite for oncogenic transformation, and a number of viral and cellular oncogenes have been shown to activate transcription of the IGF-IR gene and/or stimulate phosphorylation of its β subunit. The role of the c-myb gene in controlling the expression of IGF-I and IGF-IR has been demonstrated in fibroblast [22] and neuroblastoma cells [18]. However, studies on the role of the c-myb gene, in the IGF–IGFBP axis, have not been performed in K562 cells.

The K562 highly expresses IGF-I, IGF-II, IGF-IR, and IGF-induced cell proliferation mediated by IGF-I receptor [17]. The results of this study showed that c-myb stimulated K562 cell growth, and that the c-myb stimulated cell growth was blocked by IGF-IR antibodies. This finding confirmed that c-myb induced cell growth was mediated by IGF-I receptor in K562 cells. Since IGF-I and IGF-II are important mitogens for many cell types, including leukemia cells, we next examined whether c-myb regulated the expression of components of IGF axis, in K562 cells. The findings showed that overexpression of c-myb up-regulated the expression of IGF-I, IGF-II, and IGF-IR. By up-regulation of IGF-I, IGF-II, and IGF-IR, c-myb can increase IGF/IGF-IR survival and mitogenic signaling. In addition, c-myb was found to decrease IGFBP-3 expression. This is the first report of c-myb down-regulating IGFBP-3 in K562 cells.

IGFBP-3 was originally defined, by the somatomedin hypothesis, as the principal carrier of IGF-I in the circulation and the primary regulator of the amount of free IGF-I available for

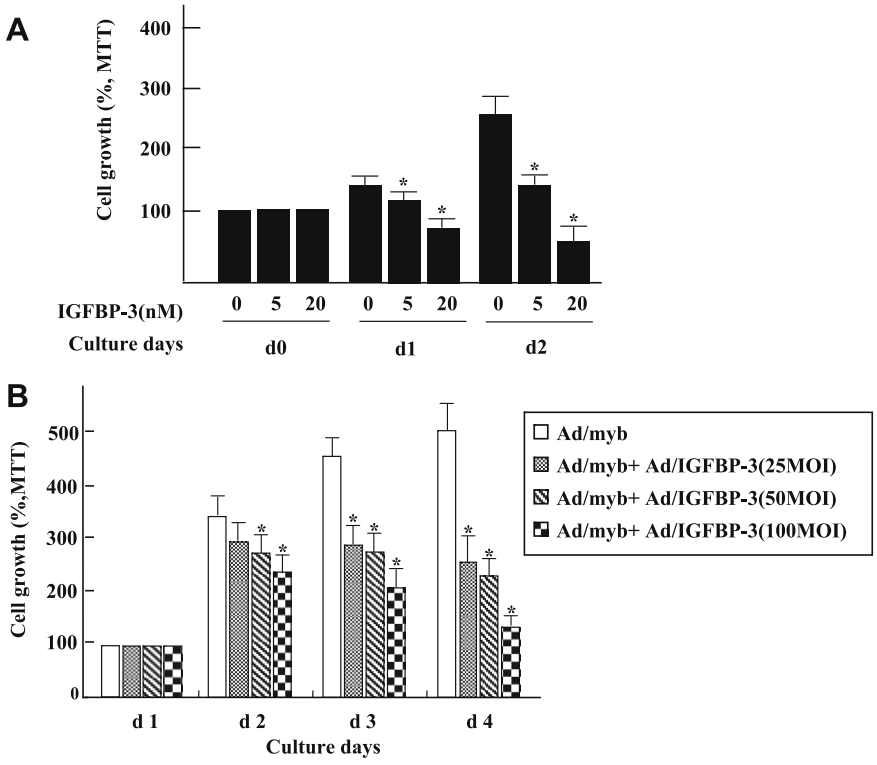

Fig. 3. The effect of exogenous IGFBP-3 and IGFBP-3 overexpression on the cell growth. (A) Cells (2×10^4 cells/well) were grown until 60% confluency and changed to serum free media in the absence (0) or presence of IGFBP-3 (5, 20 nM) over a 2 day period. (B) The cells (2×10^4/well) infected with c-myb (Ad/myb, at a MOI of 100) were grown in the absence or presence of IGFBP-3 infection (Ad/IGFBP-3, at a MOI of 25, 50, or 100) over 4 day period. The number of viable cells at the indicated times were determined by a MTT assay. Results are means ± SE of three separate experiments performed in triplicate wells. *$p < 0.05$ compared to the absence of IGFBP-3 treatment (A) or infection with c-myb(Ad/myb) only (B).

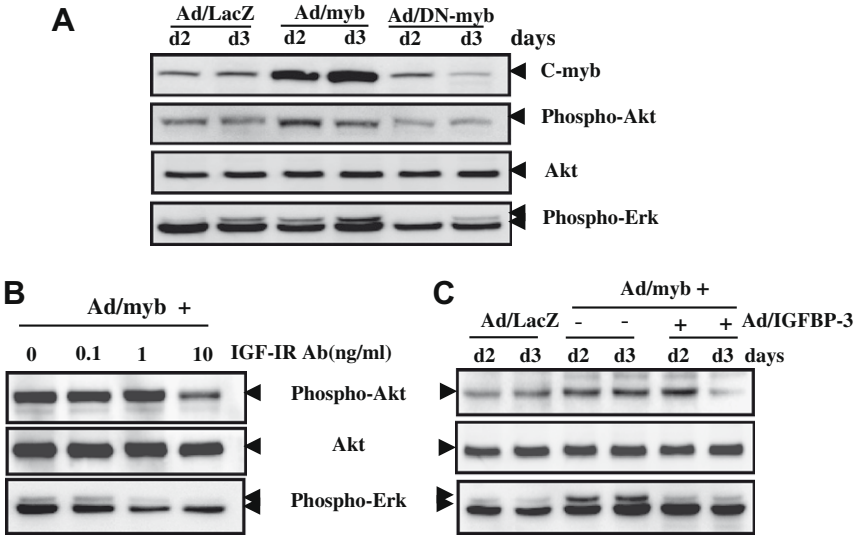

Fig. 4. The effect of c-myb and DN-myb on the Akt and Erk pathways, and effects of IGF-I receptor and IGFBP-3 on c-myb activated Akt and Erk pathways. (A) Cells (1×10^6 cells/dish) infected with Ad/myb (at a MOI of 100), Ad/DN-myb (at a MOI of 50), or Ad/LacZ (at a MOI of 100) were grown for 2 days (d2) or 3 days (d3). (B) Cells infected with Ad/myb were grown for 2 days in the absence (0) or presence (0.1, 1, and 10 ng/ml) of the IGF-I receptor antibody. (C) Cells infected with Ad/myb or Ad/LacZ were grown for 2 days (d2) or 3 days (d3) in the absence (−) or presence (+) of Ad/IGFBP-3 at a MOI of 50. Total proteins from cell lysates were extracted and analyzed by Western blot using c-myb, phospho-Akt (p-Akt), total Akt (Akt), or phospho-Erk antibodies, as described under Materials and methods.

interaction with IGF-I receptor [9]. IGFBP-3 can also function as a direct cell growth inhibitor, independent of its IGF-binding activity [11]. In the present study, exogenous IGFBP-3 inhibited K562 cell growth, and c-myb induced cell growth was blocked by IGFBP-3 overexpression. However, whether the IGFBP-3 inhibitory effect is IGF-dependent or -independent remains to be determined. Up-regulation of the IGFs and IGF-IR, and down-regulation of IGFBP-3 by the c-myb gene, resulted in an increase of IGF/IGF-IR survival in the leukemia cells. Many studies have shown high expression of IGF-II, IGF-IR, and IGFBP-2 in leukemia [23,24]. Petridou et al. [24] reported that reduced levels of IGFBP-3 were associated with a higher risk of childhood leukemia. Therefore, the IGF signaling pathway might be important for the proliferation of leukemia cells.

Since the proliferation of K562 cells, induced by c-myb gene, was found to be mediated by the activation of IGF axis, we examined the two major IGF-IR mediated signaling pathways, PI3-kinase and MAP kinase. PKB/Akt is an important downstream effector of PI3-kinase that mediates many of the growth-promoting effects of PI3-kinase activation [25]. The MAPK cascade involves the sequential phosphorylation of Raf, MEK, and ERK. ERKs are likely to play a distinct role in cell differentiation and/or proliferation depending on the cell lineage and on the extracellular stimuli [26]. c-myb overexpression significantly increased Akt and Erk phosphorylation in leukemia cells. In addition, the phosphorylation of Akt and Erk, induced by c-myb, was blocked by IGF-I antibodies and IGFBP-3 overexpression. Taken together, these data support the hypothesis that c-myb gene stimulates leukemia cell growth by IGF-IR mediated Akt and Erk signaling pathway.

In summary, the results of this study showed that overexpression of c-myb up-regulated IGF-I, IGF-II, and IGF-IR expression, and down-regulated the expression of IGFBP-3. On the basis of this work, it can be speculated that c-myb stimulates, at least in part, cell growth by IGF-I receptor mediated growth stimulating effects including up-regulation of IGF-I and IGF-II, and down-regulation of IGFBP-3 in leukemia cells. Therefore, disruption of c-myb function might provide a powerful alternative treatment strategy for leukemia patients.

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