N-Myc Induction Stimulated by Insulin-like Growth Factor I through Mitogen-activated Protein Kinase Signaling Pathway in Human Neuroblastoma Cells

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Abstract

Insulin-like growth factor I (IGF-I) stimulates proliferation, survival, and differentiation in many cell types, including pediatric neuroblastomas. The effect is mediated via the type I IGF-I receptor (IGF-1R), which is essential for growth in these cells. Several lines of evidence indicate that IGF-IR function may be particularly important in the pathogenesis of neuroblastoma. Amplification of the N-myc oncogene or overexpression of N-Myc oncoprotein has been reported to be associated with resistance to therapy and poor prognosis of neuroblastomas. It was therefore of interest to analyze whether IGF-I signaling regulated expression of N-myc in KP-N-RT human neuroblastoma cells as an experimental model that has amplified N-myc. We found that IGF-I induces N-myc mRNA and protein in the KP-N-RT with maximums of four and six times more than the basal level at 2 and 3 h after stimulation, respectively. These effects of IGF-I were blocked by a neutralizing antibody against IGF-IR (α-IR3). Exogenous IGF-I induced phosphorylation and activation of extracellular signal-regulated kinases p44/42 (ERK1 and ERK2), with a maximal level 30 min after the stimulation. The MEK1 inhibitor PD98059 reduced IGF-I-mediated p44/42 MAPKs phosphorylation and produced a parallel reduction of IGF-I-stimulated N-Myc induction. Furthermore, both α-IR3 and PD98059 inhibited G1-S cell cycle progression stimulated by IGF-I. Our results demonstrate that IGF-I induces N-Myc in the KP-N-RT neuroblastoma cell line at the RNA level and establishes a clear correlation between N-Myc induction and activation of p44/42 MAPK signaling.

Introduction

Neuroblastoma is the most common extracranial solid tumor in young children (1). Prognosis of this disseminated embryonal tumor remains poor, despite aggressive multimodal therapy including stem cell transplantation (2). N-myc gene amplification is one of the most important and independent negative prognostic factors in neuroblastoma (3). The degree of N-Myc protein and mRNA expression in neuroblastoma tissues correlates well with differentiation grade and clinical prognosis (4, 5). Direct evidence of N-myc contributing to tumorigenesis has been obtained from transgenic mice overexpressing N-Myc, which have a high incidence of neuroblastoma (6). Furthermore, there has been reported recently a close correlation between the expression of N-myc and the multidrug resistance-associated protein gene (7, 8). Studies of the Myc family proteins have revealed their role in the integrated regulation of both proliferative and apoptotic signal transduction pathways (9). N-Myc protein appears to act as a transcriptional regulator and has been thought to govern the transcription of critical genes conferring mitogenesis and multidrug resistance (8). It has been shown that the conditional up-regulation of N-myc in SH-EP neuroblastoma cells can induce the expression of two c-Myc target genes, prothymosin and ornithine decarboxylase, and accelerates progression into S phase early after mitogenic stimulation of quiescent cells (10).

IGFs are important growth factors in many tumor types, including neuroblastoma (11–13). Several lines of evidence indicate that IGF-IR function may be important in the pathogenesis of the neuroblastoma (14, 15). Inhibition of IGF-IR expression in neuroblastoma cells has been shown to induce the regression of established tumor in mice (16). In addition, overexpression of the IGF-IR in neuroblastoma cells appears to prevent apoptosis and enhance neuroblastoma tumorigenesis (17).

A recent report has provided evidence of a direct linkage from N-myc to IGF-IR expression by showing transcriptional regulation of IGF-IR by N-myc in an N-myc nonamplified neuroblastoma cell line transfected with N-myc cDNA (18). However, there is no report to indicate whether IGF-I has effect on N-Myc expression. We have found recently IGF-I-mediated c-Myc induction at the translational level through the mTOR (19, 20)/PHAS-I (21) pathway in human rhabdomyosarcoma cells (22). This prompted us to examine whether IGF-I induces N-Myc at transcriptional or translational level. Here, we show that IGF-I is an inducer of N-Myc up-regulation in N-myc-amplified KP-N-RT human neuroblastoma cells (23), which is the first evidence to show the linkage from IGF-I to N-Myc regulation at the RNA level. Furthermore, our results also provide evidence that this N-Myc induction is mediated through the MAPK pathway.

Materials and Methods

Cell Culture and Reagents. A human N-myc-amplified neuroblastoma cell line, KP-N-RT (23), was maintained in RPMI 1640 containing 10% fetal bovine serum at 37°C in a 5% CO2 incubator. The rabbit polyclonal antibody to N-Myc was purchased from Santa Cruz Biotechnology. IGF-I and the mouse monoclonal antibody to β-actin were obtained from Sigma Chemical Co. p44/42 MAPK antibody and phospho-specific MAPK antibody were purchased from New England Biolabs, PD98059 (New England Biolabs) in DMSO was added 1 h prior to stimulation with IGF-I. The final DMSO concentration was <0.1% in all cell cultures. IGF-IR blocking antibody (α-IR3) was a kind gift from Dr Steven Jacobs (Glaxo Welcome, Research Triangle Park, NC).

Western Blot Analysis. Cells were plated into 2 ml of medium at a density of 4 × 10^6 cells/35-mm-diameter well in six-well plates. After 2 days of incubation, the medium was removed, and 2 ml of serum-free RPMI 1640 were added to each well. After 24 h, the serum-starved cells were stimulated by IGF-I. Cells were incubated as required and lysed. Lysates were prepared as described previously (24). The cell lysates were separated by 10% SDS-PAGE and transferred to Immobilon-P (Millipore). The membrane was blocked for 30 min in 5% non-fat milk in TBST buffer.
1 h in PBS-Tween 20 (PBS-T) with 5% nonfat dry milk. The blots were then incubated with 2 μg/ml N-Myc antibody for 1 h, β-actin antibody (1:2000) for 1 h, Thr 202 and Tyr 204 phospho-specific p44/42 MAPK antibody (1:500) overnight, or total p44/42 MAPK antibody (1:1000) for 1 h. After being washed in PBS-T, the blots were incubated with horseradish peroxidase-conjugated antirabbit IgG (1:2000; Amersham) or antimus IgG (1:2000; Amersham) for 1 h. Antibody binding was detected by using the enhanced chemiluminescence (ECL) detection system (Amersham).

**In Vitro ERK Assay.** Cells were cultured in 100-mm tissue culture dishes (2.5 × 10⁵ cells/dish in 5 ml of medium). After 2 days, the medium was replaced with serum-free medium, and culture was continued for 24 h. The quiescent cells were then stimulated with 10 ng/ml IGF-I. Cells were incubated for the time course assay. In **In vitro** ERK assay was performed by using the MAPK immunoprecipitation kinase assay kit according to the manufacturer’s instructions (Upstate Biotechnology). Briefly, cells were washed with ice-cold PBS and lysed in lysis buffer. Insoluble material was then removed by centrifugation, and the precleared cell lysate was incubated for 2 h at 4 °C with anti-ERK1/2, agarose conjugate. Immune complexes were washed three times with lysis buffer and twice with assay dilution buffer. ERK1/2 activity was assayed by resuspend the final pellet in 40 μl of kinase buffer containing 2 mg/ml myelin basic protein (MBP). The reaction was carried out for 20 min at 30°C and stopped by the addition of Laemmli sample buffer. The samples were separated by 15% SDS-PAGE, transferred to Immobilon-P, and analyzed with Western immunoblot using monoclonal anti-phospho MBP antibody.

**RNA Extraction and Northern Blot Analysis.** Cells were cultured in 100-mm tissue culture dishes (2.5 × 10⁵ cells/dish in 5 ml of medium). After 2 days, the medium was replaced with serum-free medium, and culture was continued for 24 h. The quiescent cells were then stimulated with 10 ng/ml IGF-I. Cells were incubated for 2 h, or as required for the time course assay, and then homogenized in TRI Reagent (Molecular Research Center) according to the manufacturer’s instructions. Total RNA was electrophoretically fractionated in 1% agar/formaldehyde gels and transferred to Hybond-N membranes (Amersham). The dry blots were cross-linked by exposure to UV light. Probes for human N-myc and β-actin were radiolabeled with [α-³²P]dCTP. Prehybridization was performed for 1 h at 65°C in rapid-hybridization buffer (Amersham), followed by hybridization with radiolabeled probes for 2 h at 65°C. The blots were washed once for 20 min with 2× SSC/0.1% SDS at room temperature and twice each for 15 min 0.5× SSC/0.1% SDS at 65°C. The labeled blots were exposed to X-ray film (Amersham) at −70°C with an intensifying screen.

**Cell Cycle Analysis.** Cells (2.5 × 10⁶) were seeded in 100-mm dishes. After 2 days, they were shifted to serum-free conditions for 24 h. The cells were then stimulated with 10 ng/ml IGF-I. Twenty-four hour later, the cells were harvested by trypsinization and washed once in PBS. The cells were resuspended in 50 mg/ml propidium iodide solution containing 200 μg/ml boiled RNase and incubated at room temperature for 30 min in the dark prior to flow cytometric analysis on a Becton Dickinson FACScan. Cell cycle distribution was determined with the ModFit software (Verity).

**Results**

**Induction of N-Myc Protein and N-myc mRNA by IGF-I Stimulation.** Overexpression of N-Myc protein correlates significantly with the clinical behavior and predicts outcome of neuroblastoma independently of other prognostic factors. We found an obvious induction of N-Myc protein in a KP-N-RT neuroblastoma cell line after IGF-I stimulation. To assess the mechanism involved in this induction, we performed parallel Western and Northern blot analyses of the protein and RNA samples. The cells starved in serum-free medium for 24 h express a low level of N-Myc protein. IGF-I induced an increase of N-Myc protein by 2 h and a maximum increase of 5–6-fold by 3 h, compared with values obtained for the unstimulated samples (Fig. 1A). The level of N-Myc induction was dependent on the concentration of IGF-I (Fig. 1B). A time course analysis of N-myc mRNAs after IGF-I treatment of the KP-N-RT cells showed that the induction of N-myc mRNA was seen after 1 h of stimulation with 10 ng/ml of IGF-I and peaked at 2 h (Fig. 1C). The levels of N-myc mRNA increased up to 4–5-fold, which is approximately equivalent to an increased level of protein. These findings suggest that the regulation of N-Myc protein expression by IGF-I is at the RNA level in neuroblastoma cells.

**Induction of G₁-S Cell Cycle Progression by IGF-I Stimulation.** The effect of IGF-I on cell cycle distribution was next analyzed by FACS. KP-N-RT cells were serum starved for 24 h and then stimulated with IGF-I for 24 h. Cell cycle arrest induced by serum deprivation was characterized by the presence of >80% of cells in G1, phase of the cell cycle and consequently a negligible number of cells in S phase, consistent with inhibition of G1 to S-phase progression. Incubation with IGF-I resulted in a decrease of G0-G1 phase and a marked increase of the number of cells in S-phase in all experiments, reflecting progression through the cell cycle (Fig. 1D). These results demonstrate that stimulation of IGF-I leads to cell cycle progression from G0 to S in neuroblastoma cells.

**Anti-IGF-IR Antibody, α-IR3, Blocks IGF-I-induced N-Myc Expression and Cell Cycle Progression.** To determine whether the effect of IGF-I was mediated through IGF-IR, we examined the effect of an anti-IGF-IR monoclonal antibody, α-IR3. When serum-starved cells were preincubated with α-IR3 for 1 h, prior to addition of IGF-I, there was a substantial reduction in IGF-I-induced N-Myc expression (Fig. 2A). Addition of α-IR3 also caused a corresponding decrease in IGF-I-induced cell cycle progression in a dose-dependent manner, with complete inhibition attained at 0.5 μg/ml of α-IR3 (Fig. 2B). These results demonstrate that IGF-I promotion of both IGF-I-induced N-Myc expression and cell cycle progression occurs via the IGF-IR.

**IGF-I Activation of p44/42 MAPKs (ERK1/2).** The MAPK pathway has been identified as an important signaling pathway that is activated by various growth factors. We used a phospho-specific p44/42 MAPK antibody to demonstrate IGF-I-induced tyrosine phosphorylation of these kinases in KP-N-RT cells. Serum-starved cells had a low basal level of the tyrosine-phosphorylated forms of p44/42 MAPKs. p44/42 MAPKs are rapidly phosphorylated, reaching a maximum within 30 min after stimulation with 10 ng/ml of IGF-I (Fig. 3A). The total p44/42 MAPK protein level remained constant throughout the time course assay, monitored on a paired blot of the same cell lysates by using a phospho-specific antibody. To confirm that the ERK activation mirrored the proportion of phosphorylation of these two kinases, we used the MBP as a substrate in vitro (Fig. 3B). The ERK activation mimicked the proportion of phosphorylation of these two kinases, as shown with phospho–MAPK antibody (Fig. 3A). Preincubation with the MEK1 inhibitor PD98059 inhibited IGF-I-induced phosphorylation of p44/42 MAPKs (Fig. 3C).

**PD98059 Inhibits IGF-I-induced N-Myc Expression and Cell Cycle Progression.** To address the role of MAPK pathway in IGF-I-induced N-Myc expression and cell cycle progression, serum-starved cells were pretreated with MEK1 inhibitor PD98059 for 1 h before IGF-I stimulation. Treatment with PD98059 impaired IGF-I-mediated induction of N-Myc expression in a dose-dependent manner with complete inhibition attained at 50 μM PD98059 (Fig. 4A). PD98059 also inhibited the induction of N-myc mRNA in response to IGF-I (Fig. 4B). Furthermore, treatment with PD98059 inhibited IGF-I-induced cell cycle progression in a dose-dependent manner (Fig. 4C). However, its inhibition of cell cycle progression was not complete, even in the presence of 50 μM PD98059. These results demonstrate that the MAPK pathway is required for both IGF-I-dependent induction of N-myc expression and cell cycle progression. Because PD98059 could not completely block cell cycle progression, it is possible that IGF-I may also function through some unknown pathways.
IGF-IR downstream signal cascade partially, other than MAPK, to accelerate progression into S phase.

Discussion

Amplification of N-myc is found in ~20–30% of neuroblastomas and is one of the most powerful prognostic factors predicting the poor prognosis (3). The levels of N-Myc protein and mRNA expressed in neuroblastoma tissue correlate well with grade of differentiation and clinical prognosis (4, 5). Direct evidence of N-myc contributing to tumorigenesis has been obtained from transgenic mice overexpressing N-Myc, which show a high incidence of neuroblastoma (6). Enforced expression of N-Myc has been reported to accelerate cell cycle progression (10) and enhance the malignant phenotype of neuroblastoma cells (25).

IGF-I and IGF-II have shown to be growth/survival factors for neuroblastoma (11–13). Several lines of evidence indicate that IGF-IR function may be particularly important in the pathogenesis of neuroblastoma (14, 15). Inhibition of IGF-IR expression in neuroblastoma cells has been shown to induce the regression of established tumor in mice (16). Inversely, overexpression of the IGF-IR in neuroblastoma cells appears to prevent apoptosis and enhance neuroblastoma tumorigenesis (17).

Both N-Myc expression and the IGF-I system, therefore, appear important in the pathogenesis and development of neuroblastoma. A recent report by Chambery et al. (18) has demonstrated a transcriptional regulation of IGF-IR by N-myc using an N-myc-transfected neuroblastoma cell line. Whether IGF-I regulates expression of N-myc has not been addressed. Here, we demonstrate that IGF-I induced the expression of N-myc mRNA and N-Myc protein. Induction was dependent on the concentration of IGF-I. N-Myc protein levels rose 5–6-fold by 3 h after IGF-I stimulation with parallel increases in mRNA expression by 2 h.

The MAPK signal transduction pathway can be activated in response to a wide variety of extracellular stimuli including IGF-I (26). Signaling via the IGF-IR is an important contributor to the malignant phenotype of many tumor types (27). The activation of Ras/Raf/MEK/ MAPK and PI3K/Akt cascades has been implicated in IGF-IR signal transduction, leading to cell proliferation, differentiation, antiapoptosis, and tumor development. These MAPKs can phosphorylate a variety of substrates, including transcription factors, that control cell growth (28). We therefore assessed the contribution of the MAPK pathway signaling in IGF-I induction of N-Myc. The dual specificity kinase MEK1 phosphorylates p44 MAPK (ERK1) and p42 MAPK (ERK2) on both a threonine and a tyrosine residue (Thr 202 and Tyr 204) to activate pathways that regulate the proliferation and differentiation in diverse cell types including human malignant cells (26, 29, 30). In KP-N-RT human neuroblastoma cells, treatment with IGF-I induced concentration- and time-dependent phosphorylation of p44/42 MAPKs. IGF-I induced a rapid phosphorylation of p44/42 MAPKs that was detected at 5 min, peaked at 30 min, and then

Fig. 1. Induction of N-Myc protein and N-myc mRNA by IGF-I stimulation. A, induction of N-Myc protein. Serum-starved cells were incubated with 10 ng/ml IGF-I for the times indicated. Values are means from three separate experiments; bars, SE. Western blots were analyzed by laser densitometry. The levels of N-Myc protein were normalized against β-actin. B, concentration-dependent induction of N-Myc by IGF-I. C, induction of N-myc mRNA. Total RNA was extracted from quiescent cells that had been treated with 10 ng/ml IGF-I for times indicated and then analyzed by Northern blotting for levels of N-myc mRNA and levels of the control message β-actin with specific probes. Densitometric quantitation of normalized ratio of N-myc:β-actin (mean; n = 3; bars, SE). D, induction of G1/S cell cycle progression by IGF-I stimulation. Serum-starved cells were stimulated with IGF-I for 24 h. After propidium iodide staining, cell cycle distribution was determined (mean; n = 3; bars, SE).

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decreased at 60 min. The specific MEK1 inhibitor PD98059 blocked IGF-I-stimulated p44/42 MAPK phosphorylation; consequently, we studied the effect of PD98059 on N-Myc induction by IGF-I. Preincubation of the cells with PD98059 blocked IGF-I-induced N-Myc expression. Furthermore, PD98059 also inhibited cell cycle progression by IGF-I. These effects of IGF-I were also blocked by a neutralizing antibody against IGF-IR, α-IR3. The results presented here demonstrate that IGF-I-dependent transcriptional induction of N-Myc is mediated through activation of the MAPK signaling pathway.

The stimulation of IGF-IR by IGF-I can induce N-Myc expression, which is thought to play a role in G1 to S-phase transition. The addition of α-IR3 or PD98059 blocked not only N-Myc induction but also cell cycle progression in IGF-I-stimulated cells. An early report indicated that N-Myc accelerated progression into S-phase during the first 5 h after serum stimulation of quiescent SH-EP neuroblastoma cells (10). In the current report, cell cycle progression was significant at 24 h after IGF-I stimulation. Both α-IR3 at 0.5 μg/ml and PD98059 at 50 μM completely blocked N-Myc induction. In contrast, whereas α-IR3 inhibited nearly 100%, PD98059 was less effective in inhibiting progression to S-phase. Because PD98059 could not completely block cell cycle progression, it is possible that IGF-I may also function through some unknown IGF-IR downstream signal cascade partially, other than MAPK, to accelerate progression into S-phase. Together, these data suggest that IGF-I may induce cell cycle progression by both direct effects on N-Myc transcription, mediated through the MAPK pathway, and through effects independent of N-Myc and ERK1/2 activation.

Previous studies have demonstrated that IGF-I promotes neuronal differentiation through activation of the MAPK pathway in SH-SY5Y, a non-N-myc-amplified neuroblastoma cell line (31, 32). In contrast, using N-myc-amplified KP-N-RT cells, we observed the IGF-I promotes cell cycle progression after up-regulation of N-Myc but not differentiation. Potentially, the level of IRS-1, one of the major substrates of the IGF-IR, expression could determine that the IGF-I signal promotes differentiation or proliferation. SH-SY5Y cells have been reported to lack IRS-1 and differentiate in response to IGF-I stimulation (32). Similar results have been reported by Valentinis et al. (33), who demonstrated that lack of IRS-1 promotes IGF-I-mediated...
through the IGF-IR/MAPK pathway. Together with a previous report indicating transcriptional regulation of IGF-IR expression by N-myc (18), both the IGF system and N-myc seem to up-regulate each other, which could result in progression of neuroblastoma through a positive feedback mechanism. The IGF-IR/MAPK signal pathway has clinical linkage for the progression of neuroblastoma by not only direct cell cycle progression but also N-myc up-regulation. Our results support for the idea of targeting the IGF-IR/MEK/MAPK pathway in a mechanism-based therapeutic approach in the management of N-myc-amplified neuroblastoma, in agreement with other reports (16, 17).

References


Fig. 4. A, PD98059 inhibits IGF-I-induced N-Myc protein. Serum-starved cells were incubated for 1 h with PD98059 (0–50 μM) before a 3-h incubation with 10 ng/ml IGF-I (mean; n = 3; bars, SD). B, inhibition of IGF-I-induced N-myc mRNA by α-IR3 or PD98059. Serum-starved cells were either left untreated or preincubated with 0.5 μg/ml of α-IR3 or 50 μM of PD98059 for 1 h and then stimulated with IGF-I for 2 h. Bars, SE. C, PD98059 inhibits IGF-I-induced G1/S cell cycle progression. Serum-starved cells were incubated for 1 h with PD98059 before a 24-h incubation with 10 ng/ml IGF-I (mean; n = 3; bars, SE).


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