

Transforming Growth Factor- β 1 Induces *junB* mRNA Accumulation, G1-Phase Arrest, and pRb Dephosphorylation in Human Leukemia HL-60 Cells

(TGF- β 1 / leukemia / HL-60)

J. PACHERNÍK^{1,2}, K. SOUČEK¹, A. HAMPL^{2,3}, J. HOFMANOVÁ¹, A. KOZUBÍK¹

¹Institute of Biophysics, Academy of Science of the Czech Republic, Brno, Czech Republic

²Laboratory of Molecular Embryology, Mendel University Brno, Brno, Czech Republic

³Developmental Biology Unit, Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, Brno, Czech Republic

Abstract. Although TGF- β 1 unambiguously functions as a regulator of hematopoietic differentiation, its significance for the development of myeloid lineage is still questionable. In this study three components of early response to TGF- β 1 treatment were investigated in human promyelocytic leukemia HL-60 cells. Changes in *junB* mRNA accumulation and pRb dephosphorylation were accompanied by accumulation of cells in G1 phase of the cell cycle. Time dependence of these changes may implicate mutual cooperation of the pRb and *junB* in the cell cycle control. It can be concluded that, although myeloid HL-60 cells are known to require rather complex cytokine stimulation to fully differentiate, they clearly possess the ability to respond to TGF- β 1.

Transforming growth factor- β 1 (TGF- β 1) is a pleiotropic cytokine belonging to the TGF- β superfamily. TGF- β -superfamily proteins participate in regulating proliferation, differentiation, and apoptosis in many cell types (Massagué, 1998). Recently, attention has been focused on the role of TGF- β 1 in hematopoiesis and leukemias. Thus far, TGF- β 1 alone has been reported to have little effect on the development of myeloid cell lineages. Kamijo et al. (1990) demonstrated that although TGF- β 1 induces differenti-

ation of human U937 and THP-1 promonocytic leukemia cell lines to macrophages, it does not affect HL-60 and ML-1 acute myeloid leukemia cell lines. Despite having no effect on myeloid cell lines by itself, when combined with dimethyl sulfoxide (DMSO), retinoic acid (RA), tumor necrosis factor- α (TNF- α), and vitamin D3, respectively, TGF- β 1 induces granulocytic and/or monocytic differentiation of HL-60 and several other myeloid leukemic cell lines (Kamijo et al., 1990; Testa et al., 1993; Turley et al., 1996; Kozubík et al., 1997). Biochemistry of such synergisms, as well as signal transduction pathways operating downstream of TGF- β 1 in leukemia cells, are far from being fully understood.

Upregulated expression of *jun*-family members, especially of *junB*, represents the primary response of TGF- β 1-sensitive cell lines (for example Mv1Lu or HaCaT) to this cytokine. Ultimately, the signals are transmitted onto various members of the cyclin-dependent kinase family, causing a decrease of their activity via association with cyclin-dependent kinase inhibitors of Kip/Cip and INK4 families. This is followed by a loss of phosphorylation of their major target – retinoblastoma protein (pRb). Since these molecules are crucial in regulating the cell cycle progression, such molecular changes finally lead to arresting cells in G1 phase of the cell cycle (for review see Massagué and Weis-Garcia, 1996; Massagué, 1998). Although various changes have been described that accompany the differentiation of myeloid cells, there is still thorough lack of data on early molecular events described above. Myeloid leukemia cell line HL-60 can give rise to either granulocyte or monocyte/macrophage cell type according to inducing agents. Therefore, it serves as a valuable model for studying differentiation processes induced by both chemical compounds and hematopoietic cytokines (Collins, 1987). Importantly, based on morphological, functional, and

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Corresponding author: Alois Kozubík, Institute of Biophysics, Academy of Science of the Czech Republic, Královopolská 135, 612 65 Brno, Czech Republic. Tel.: + 420 (5) 41517182; fax: + 420 (5) 41211293; e-mail: kozubik@ibp.cz.

Abbreviations: DMSO – dimethyl sulfoxide, PBS – phosphate-buffered saline, pRb – retinoblastoma protein, RA – retinoic acid, TGF- β 1 – transforming growth factor- β 1, TNF- α – tumor necrosis factor- α .

antigenic criteria, HL-60 cells have been concluded to be refractory to the treatment with TGF- β 1. Here, we investigated whether or not this inability to respond to TGF- β 1 is also pronounced by the absence of early molecular changes, including junB accumulation, pRb phosphorylation, and cell cycle arrest.

Material and Methods

Human myeloid leukemia HL-60 cells (from ECACC; Porton Down, Salisbury, UK) were routinely cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and gentamycin (50 μ g/ml) under standard conditions (37°C, 5% CO₂, 95% humidity). Recombinant TGF- β 1 was prepared as a stock solution (40 nM) in HCl-bovine serum albumin (all compounds from Sigma, Prague, Czech Republic). Cell numbers were determined using a Coulter Counter (model ZM). Cell viability was determined by the eosin (0.01%) dye exclusion assay. Only experiments using exponentially growing cells with viability of 90% or higher were included.

Total RNA was isolated from 10⁷ cells by a single-step modification of standard Chomczynski's protocol (Xie and Rothblum, 1991). Twenty micrograms of total RNA were separated on a 1.5% agarose/formaldehyde gel, capillary-transferred onto a positively charged nylon membrane (Qiagen, Hilden, Germany) and probed. The probe was an 1.46 kb fragment of junB cDNA cloned in pGEM (gift from Dr. A. Valette; C.N.R.S., Toulouse, France). The probe was labeled using Boehringer-Mannheim (B/M) non-radioactive DIG kit and hybridized according to the protocol suggested by the manufacturer (Roche, Mannheim, Germany). Transcripts were visualized with Attophos substrate (Amersham Pharmacia, Vienna, Austria) and analyzed by a STORM 860 densitometer (Molecular Dynamics, Sunnyvale, CA).

Cell cycle analysis: upon their collection, cells were washed with phosphate-buffered saline (PBS) (pH 7.4), and stained with Vindelov's solution (Vindelov, 1977) for 30 min at 37°C. For each measurement, 2 \times 10⁴ cells were collected and analyzed by flow cytometry (FACSCalibur, Becton-Dickinson, San José, CA; 488 nm laser beam for excitation). The doublets were determined in FL-2 area vs. FL-2 width dot-plot. The data were analyzed using ModFit Software (Verify Software House, Topsham, ME) and statistically tested by ANOVA followed by Bonferroni's multiple comparison test.

For pRb Western blot analysis cells were washed in PBS (pH 7.4), and lysed in 50 mM Tris-HCl (pH 7.5) buffer containing 1% sodium dodecyl sulphate and 20% glycerol. The amounts of total protein in lysates were determined using the DC Protein assay kit (Bio-Rad, Vienna, Austria). Lysates were supplemented with bromphenol blue and 2-mercaptoethanol to final concentrations of 0.01% and 1%, respectively. Equal

amounts of total protein (20 μ g) were subjected to 7.5% SDS-PAGE (C = 0.1, T = 10). Proteins were transferred onto polyvinylidene fluoride membranes (Immobilon-P, Sigma, Prague, Czech Republic). The membranes were blocked in 5% nonfat milk for 1 h and then incubated with mouse monoclonal antibody against human pRb (14001A; Pharmingen, San Diego, CA) at room temperature for 1 h. Anti-pRb immunoglobulins were visualized using rabbit anti-mouse IgG labeled with horseradish peroxidase (A 9044; Sigma, Prague, Czech Republic) and enhanced chemiluminescence reagent (ECL+; Amersham Pharmacia, Vienna, Austria).

Results and Discussion

As we have shown previously, the concentrations of TGF- β 1 higher than 200 pM did not augment its effect on proliferation (Kozubík et al., 1997). Here, continuous exposure to 200 pM TGF- β 1 induced HL-60 cells to rapidly but transiently upregulate the amount of junB mRNA. As documented in Fig. 1, at 1 h after the beginning of treatment the level of junB mRNA was increased about 3-fold, but only about 2-fold at 3 h, and it dropped down to the level similar to that of nontreated controls at 24 h. Together, these data demonstrate that TGF- β 1 alone induces rapid accumulation of the early response gene junB in myeloid leukemia cells HL-60. Previously, various differentiation-inducing chemical compounds were shown to stimulate the accumulation of junB mRNA in HL-60. This accumulation was fast within macrophage/monocyte differentiation induced with phorbol ester, vitamin D₃, or sodium butyrate and slow within granulocyte differentiation induced with DMSO or RA (Mollinedo and Naranjo 1991, Mollinedo et al. 1993). In this context, the fast accumulation of junB mRNA upon the exposure to TGF- β 1 described here further supports the notion that this cytokine functions as an inducer and/or participant in monocyte/macrophage differentiation, as has been suggested previously (Testa et al., 1993; Turley et al., 1996).

TGF- β 1 has a potential to inhibit the progression of the cell cycle in certain cell types (Reynisdóttir et al., 1995). Here we show that also in HL-60 cells, exposure to TGF- β 1 leads to a significant accumulation of cells in G₁ phase that is proportional to the decreased number of cells in S phase (Fig. 2). However, the maximal redistribution of cells in cell cycle phases is reached after 12 h of TGF- β 1 treatment (~60% in G₁ phase; ~20% in S phase), and then it is maintained essentially unchanged. In other words, HL-60 cells never respond to TGF- β 1 by complete G₁ arrest. Correspondingly, only partial dephosphorylation of pRb occurs after both 12-hour and 24-hour TGF- β 1 treatments (Fig. 3). Notably, the cell cycle characteristics described here fit well with our previous findings that TGF- β 1 causes only minor changes in the dynamics of multiplication of HL-60 cells (Kozubík et al., 1997).

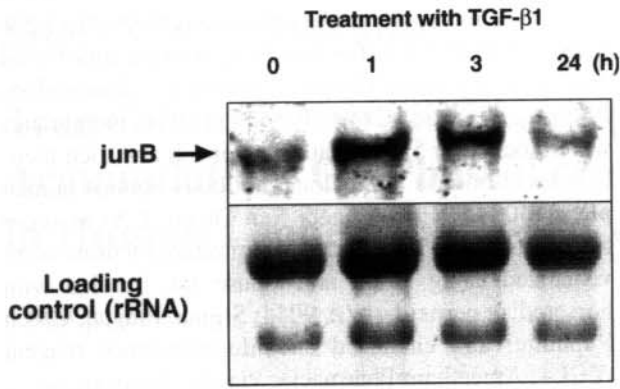


Fig. 1. Accumulation of junB protooncogene in TGF- β 1-treated HL-60 cells. Cells were exposed continuously to 200 pM TGF- β 1 and the levels of junB mRNA were determined by Northern blot analysis in unstimulated – control cells (0 h) and 1, 3, and 24 h after the beginning of the treatment of cells with TGF- β 1. Total RNA visualized by ethidium bromide staining is shown to demonstrate equal loading. The data are representative of at least three independent experiments.

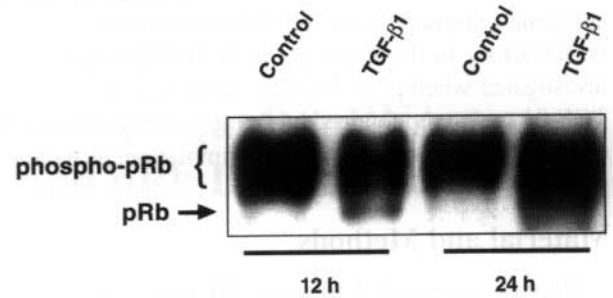


Fig. 3. The changes in the phosphorylation status of pRb in HL-60 cells upon their treatment with TGF- β 1. Cells were cultured with and without TGF- β 1 for 12 and 24 h, respectively. Cells cultivated for 12 or 24 h, respectively, without TGF- β 1 served as a control. Cell lysates were Western-blot analyzed for pRb. The data are representative of at least three independent experiments.

Taken together, our results demonstrate for the first time that also in myeloid leukemia cells, here represented by the HL-60 cell line, TGF- β 1 is capable to induce the molecular changes that are typically associated with entry into differentiation of blood cell lineages, including 1) accumulation of the early response gene junB, 2) accumulation of cells in G1 phase of the cell cycle, and 3) dephosphorylation of pRb. Based on these findings, we conclude that TGF- β 1 signal transduction in myeloid cells is probably realized by a similar way as in other TGF- β 1-responding cells. Thus, the insufficiency of TGF- β 1 to induce differentiation in the myeloid cell lineage reported previously must be due to the requirement of this cell type for signals also from TGF- β 1-independent regulatory pathways. As is obvious from the data mentioned above, the pathways regulated by TNF- α or vitamin D3 might be the potential candidates.

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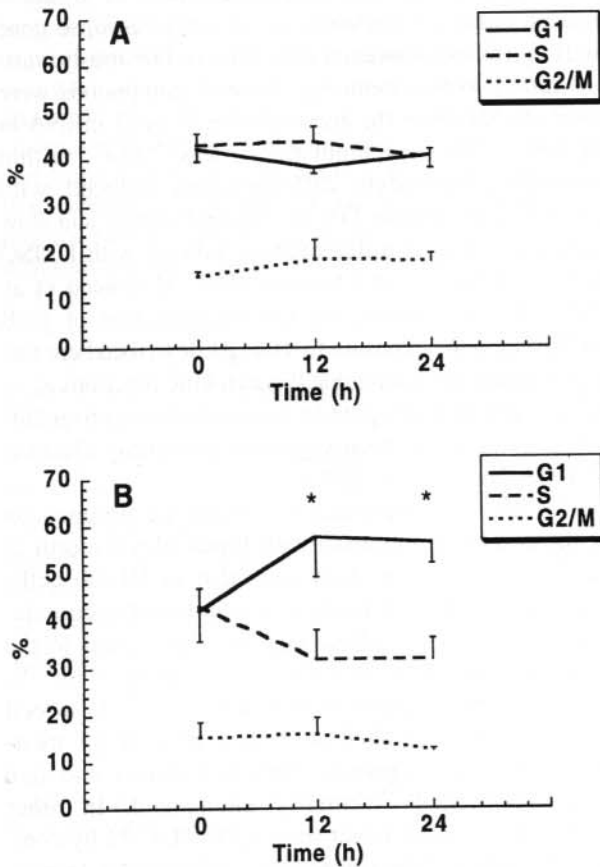


Fig. 2. The effect of TGF- β 1 on the progression of the cell cycle in HL-60 cells. Cells were cultured without (A) and with (B) 200 pM TGF- β 1 for 12 and 24 h, respectively. Symbol [*] represents the values that are significantly different from nontreated controls, $P < 0.05$.

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