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Latent Form of Transforming Growth Factor-β1 Acts as a Potent Growth Inhibitor on a Human Erythroleukemia Cell Line

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SUMMARY: Recombinant latent form of transforming growth factor-β1 (L-TGF-β1) is activated by various chemical treatments, including acidification and heating. However, cellular mechanisms that release transforming growth factor-β (TGF-β) in an active form have not been fully elucidated. Investigated herein are the effects of L-TGF-β1 on various leukemic cell lines. Heat-activated L-TGF-β1 inhibited colony formation of U937, KG-1 and HL-60, whereas untreated L-TGF-β1 had only a marginal effect on these cells. In contrast, colony formation of human erythroleukemia cell line (HEL) was markedly inhibited by both heat-activated and untreated L-TGF-β1. *In vitro* incubation of L-TGF-β1 with HEL cells did not release the active form in the culture supernatants. These results suggest that HEL cells are capable of activating L-TGF-β1, but only in a cell-associated manner. Since HEL cells produce L-TGF-β1, it may act as an autocrine negative growth factor on these cells.

Transforming growth factor-β (TGF-β) is a 25 kDa dimeric protein with multiple functions on cellular growth and differentiation in many cells (for review, see Ref. 1). TGF-β belongs to a family of peptides; thus far, five closely related peptides have been identified. TGF-β, which was first purified from human platelets, was defined as TGF-β1. cDNA cloning of TGF-β1 disclosed that TGF-β1 is synthesized from 390 amino acid precursor protein and released as a dimer of the C-terminal 112 amino acids. TGF-β is a potent growth inhibitor on most normal and some transformed cells, including epithelial cells, endothelial cells, hematopoietic progenitor cells and blood lymphocytes. TGF-β1 is secreted as a latent high molecular weight complex (latent form of TGF-β1; L-TGF-β1) from producer cells; it must be activated before it binds to cell surface receptors (2).

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The abbreviations used are: TGF-β1, transforming growth factor-β1; L-TGF-β1, latent form of TGF-β1; TGF-β1-BP, TGF-β1-binding protein; TGF-β1-LAP, TGF-β1-latency associated peptide; FBS, fetal bovine serum.

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Three different forms of L-TGF-β1 have been identified (3). Platelet-derived L-TGF-β1, a large latent TGF-β1 complex, is composed of three different subunits: mature TGF-β1, the N-terminal remnant of the TGF-β1 precursor, and a novel 125-160 kDa TGF-β1-binding protein (TGF-β1-BP) (4-6). In contrast, L-TGF-β1 obtained from recombinant materials is a small latent complex, composed of only mature TGF-β1 and the N-terminal remnant of the TGF-β1 precursor (3,7). The N-terminal remnant of the TGF-β1 precursor is responsible for TGF-β1 latency; thus, it was denoted as a TGF-β1-latency associated peptide (TGF-β1-LAP). The function of TGF-β1-BP remains to be elucidated. The other form of L-TGF-β1 is a complex associated with α_2-macroglobulin, which may act as a scavenger for TGF-β *in vivo* (8,9).

TGF-β is activated by various chemical and enzymatic treatments such as extreme pH values, chaotropic agents, plasmin (10) and glycosidases (11). However, activation mechanisms of L-TGF-β1 *in vivo* are not fully elucidated. In this communication, we show that L-TGF-β1 acts as a potent growth inhibitor on a leukemic cell line, HEL. The molecular mechanism for the activation is discussed.

MATERIALS AND METHODS

Reagents. Recombinant L-TGF-β1 was obtained from CHO cells expressing human preproTGF-β1. It was concentrated 100-fold by an Amicon concentrator. Material purity was approximately 10 % (Ichijo, H. *et al.*, manuscript in preparation). Human TGF-β1 was purchased from R & D Systems, Inc. (MN, USA).

Cell Culture. HEL, U937, KG-1 and HL-60 were cultured in RPMI 1640 medium supplemented with 10 % fetal bovine serum (FBS). Mink lung epithelial cells (CCL-64) were cultured in MEM in 10 % FBS and subcultured every four days after digestion with trypsin/EDTA solution (Gibco Laboratories).

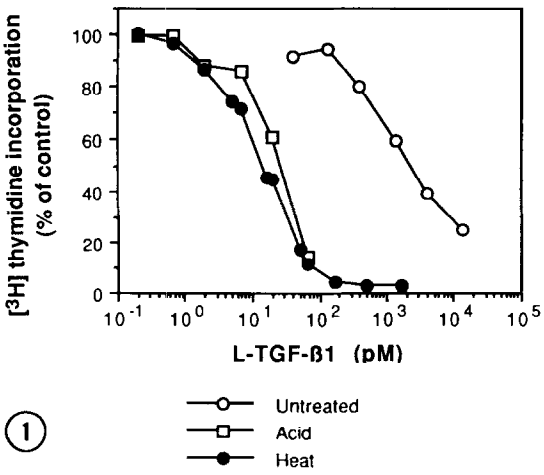
Chemical Activation of L-TGF-β1. Activation of L-TGF-β1 by acid was performed by adding equal amounts of 1 M HCl. Materials were incubated for 30 min at room temperature and then neutralized by 1 M NaOH and 1 M Hepes buffer (pH 7.4). Heat actvation was carried out by incubating L-TGF-β1 at 85°C for 10 min.

Leukemic Colony Assay. Colony formation assay of leukemic cell lines was performed by the method of Minden *et al.* (12) with a modification. Briefly, 1 x 10^3 cells were cultured in 24-well tissue culture plates (Falcon) in 0.5 ml of α-MEM (Gibco Laboratories) with 20 % FBS, 0.8 % methylcellulose and various concentrations of heat-activated or untreated L-TGF-β1. Cultures were incubated at 37°C in a humidified atmosphere of 5 % CO_2. After 4-6 days of incubation, colonies consist of more than 20 cells were counted under inverted microscope.

Growth Inhibition Assay for TGF-β. Standard bioassay for TGF-β was carried out using CCL-64 cells as target cells (13). CCL-64 cells were transferred to MEM containing 1 % FBS in 24-well tissue culture plates. After 24-48 h of incubation, test samples were added. Twenty hours later, 0.2 μCi of [^{3}H] thymidine (6.7 Ci/mmol, New England Nuclear) was added, and incubation was continued for another 4 h. The ^{3}H radioactivity incorporated into DNA was determined as previously described (14).

RESULTS

Activation of Recombinant L-TGF-β1 by Heat and Acid. L-TGF-β1 is known to be activated by various chemical treatments, such as acid (less than pH 3.5) (4). We have



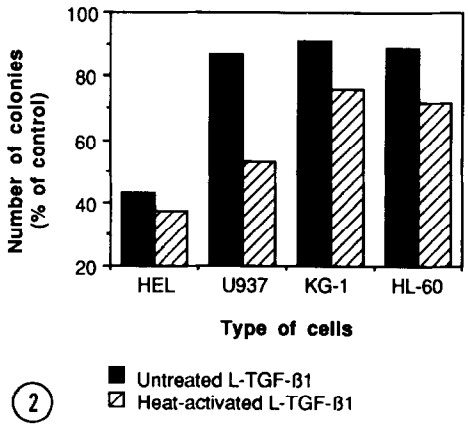


Fig. 1. Activation of L-TGF-β1 by Acid and Heat. For acid activation, L-TGF-β1 was treated with 1 M HCl for 30 min at room temperature, and neutralized by 1 M NaOH and 1 M Hepes (pH 7.4). For heat activation, L-TGF-β1 was incubated at 85°C for 10 min. Growth inhibition assay for TGF-β was performed using CCL-64 cells as target cells.

Fig. 2. Effects of L-TGF-β1 on Leukemic Colony Formation. Heat-activated or untreated L-TGF-β1 (1.2 nM) was added to various leukemic cells lines. Blast colonies consist of more than 20 cells were counted after 4-6 days under inverted microscope.

confirmed that heating at 85°C for 10 min also activates L-TGF-β1 (Fig. 1). Degree of activation varied, but was almost identical to the acidification. Untreated material inhibited the growth of CCL-64 cells when added at concentrations exceeding 100-fold, which was analogous with the results obtained from platelet-derived L-TGF-β1 (11). Since heat-activated L-TGF-β1 does not have high ion concentrations, chemical activation of L-TGF-β1 was carried out by heating in further experiments.

Effects of active and latent forms of TGF-β1 on Leukemic Colony Formation. It is known that active form of TGF-β inhibits soft agar colony formation of leukemic cell lines (15). We have tested the effect of L-TGF-β1 on various leukemic cell lines. Heat-activated L-TGF-β1 inhibited colony formation in all cell lines investigated. Growth of HEL and U937 cells was markedly inhibited by heat-activated L-TGF-β1, whereas growth inhibition of KG-1 and HL-60 cells was moderate (Fig. 2). Untreated L-TGF-β1 had a marginal effect on the growth of U937, KG-1 and HL-60 cells, but it efficiently inhibited colony formation of HEL cells. Fig. 3 shows the dose-responsive effect of heat-activated and untreated L-TGF-β1 on HEL and U937 cells. The effects of untreated L-TGF-β1 on HEL cells were almost identical to those of activated L-TGF-β1. Furthermore, results in human TGF-β1, which does not contain TGF-β1-LAP, were nearly identical to those of heat-activated and untreated L-TGF-β1 , although human TGF-β1 was approximately 3 times more potent in a molar base (Fig. 4).

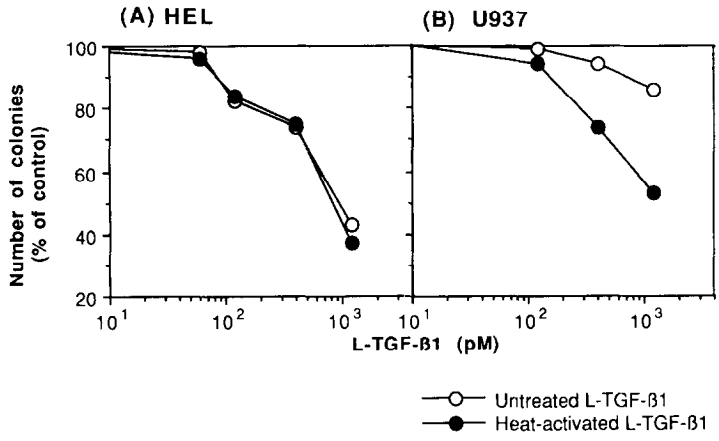


Fig. 3. Dose-Responsive Effects of L-TGF-β1 on Leukemic Colony Formation on HEL and U937 Cells. Various concentrations of heat-activated or untreated L-TGF-β1 were added to (A) HEL and (B) U937 cells, and blast colony formation was examined.

Activation of Recombinant L-TGF-β1 by HEL Cells. To determine whether the growth inhibitory effect of L-TGF-β1 on HEL cells are due to activation by this cell line in culture, we incubated L-TGF-β1 with HEL cells and then examined the amount of activated TGF-β1 by adding the culture supernatants to CCL-64 cells. HEL cells did not significantly activate L-TGF-β1 after incubation for 8 h at 37°C (data not shown).

DISCUSSION

TGF-β is known as one of the most potent inhibitors for cellular growth. It is produced in various animal tissues, and most cell types have the receptors for TGF-β (16). Thus, one of the most important steps for regulation of TGF-β action occurs in the activation step. It is known that TGF-β1, 2 and 3 are produced as latent forms. Activation of TGF-β1 by chemical treatments is well characterized. Extreme pH values, heat, and chaotropic

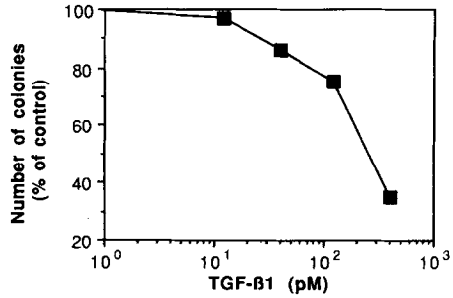


Fig. 4. Effects of TGF-β1 on HEL Cells. Various concentrations of human TGF-β1 (R & D Systems, Inc.) were added to HEL cells, and blast colonies were counted.

agents efficiently activate L-TGF-β1. Since TGF-β1-LAP is responsible for TGF-β1 latency, structural changes in this molecule are believed to activate TGF-β1. Several enzymes are also known to activate L-TGF-β1 (2,10). We have previously shown that the carbohydrate structures in TGF-β1-LAP are crucial to its latency (11). It was also shown that cultured cells, such as osteoclasts (17) and endothelial cells co-cultured with pericytes or smooth muscle cells (18,19), can activate L-TGF-β. It has been suggested that the acidic enviroment produced by osteoclasts was important for activation (17); however, the pH value of the conditioned medium was not low. Plasmin was considered responsible for the activation induced by endothelial cells and pericytes (19).

In this study, we have shown that L-TGF-β1 is as potent as heat-activated L-TGF-β1 on HEL cells. This is the first observation that exogenously added L-TGF-β1 has potent growth inhibitory activity. Human TGF-β1, which lacks TGF-β1-LAP, showed similar growth inhibitory effects. Thus, growth inhibition by L-TGF-β1 appears to be induced by TGF-β1 via activation but not by other molecules such as TGF-β1-LAP. The molecular mechanisms for the activation of L-TGF-β1 by HEL cells remain to be elucidated. This cell line was originally established from a patient with erythroleukemia, but it has some properties of megakaryocytic cells (20). Thus, enzymes that are relatively specific for erythroid and/or megakaryocytoid lineages might be responsible for the activation. Since obvious activation of L-TGF-β1 was not observed after incubation with this cell line, it is possible that activation occurs only in a cell-associated manner and that activated TGF-β1 was not released from the cells into the conditioned medium.

It is noteworthy that HEL cells produce large quantities of TGF-β1 (21, and Miyazono, K. *et al.*, unpublished data). Thus, it is highly possible that the TGF-β1 produced by HEL cells is self-activated and acts as a growth inhibitor in an autocrine fashion. Our results indicate that L-TGF-β1 is a potent growth inhibitor for certain cell types and that this ubiquitous molecule with powerful effects may play an important role on *in vivo* cellular growth.

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