Volume 322, number 3, 235–239 FEBS 12447 May 1993

Down-regulation of ubiquitin gene expression during differentiation of human leukemia cells

Naoki Shimbara[a], Chiharu Sato[a], Makoto Takashina[a], Tatsuo Tanaka[b], Keiji Tanaka[c] and Akira Ichihara[c]

[a]*Biomaterial Research Institute, Sakae-Ku, Yokohama 244, Japan,* [b]*Department of Biochemistry, School of Medicine, University of Ryukyus, Okinawa 903-01, Japan* and [c]*Institute for Enzyme Research, The University of Tokushima, Tokushima 770, Japan*

Received 17 March 1993; revised version received 26 March 1993

Ubiquitin, which is ligated covalently to target proteins for their acquisition of a variety of functions, is encoded by multiple unique genes in human cells: two distinct poly-ubiquitin genes with tandemly repeated sequences of 3 or 9 moieties and two mono-ubiquitin genes fused with small and large ribosomal proteins. We found that all classes of ubiquitin genes as well as the two genes encoding the ribosomal proteins S17 and L31 were expressed at abnormally high levels in various hematopoietic malignant tumor cells. In contrast, in vitro terminal differentiation of various immature leukemic cell lines, such as HL-60 promyelocytic leukemia cells and K562 erythroleukemia cells into monocytic, granulocytic and erythroid cells, induced by various agents was found to cause rapid and marked down-regulation of ubiquitin expression, irrespective of the cell type, direction of differentiation or type of signal. These findings suggest that the expressions of the multiple ubiquitin genes, coordinated with those of the ribosomal protein genes, are in a dynamic state during growth and differentiation of leukemia cells.

Gene expression; In vitro differentiation; Leukemia cell; Ubiquitin; Proteasome

1. INTRODUCTION

Ubiquitin (Ub) is an evolutionally conserved protein, consisting of 76 amino acid residues, and for post-translational modification of various proteins it is covalently conjugated to these proteins through multi-enzymatic reactions [1]. Of the many cellular processes in which the Ub system is involved, we are especially interested in cell proliferation, because during this process various important proteins related with cell cycle progression, such as cyclin, p53, c-Mos, platelet-derived growth factor receptor and T cell antigen receptor, are degraded by the Ub pathway. Ub serves as a degradation signal for proteolytic attack, and the breakdown of ubiquitinated protein is catalyzed by an ATP-dependent proteolytic complex containing a proteasome [2–4]. Recently, we found that the proteasome also plays an essential role in cell growth [4] and that the expressions of multiple proteasome genes are markedly down-regulated during in vitro induction of terminal differentiation of various types of immature human leukemic cell lines [5]. In the present study, we examined changes in the expressions of the multiple Ub genes during differentiation of leukemic cells.

2. MATERIALS AND METHODS

The materials used were as follows: [α-^{32}P]dCTP (110 TBq/mmol), and Hybond-N$^+$ nylon membranes (Amersham); TPA (12-*O*-tetradecanoylphorbol-13-acetate), retinoic acid, hemin, sphinganine and anti-Ub antibody (Sigma); 1,25-dihydroxyvitamin D3, 1,25$(OH)_2D_3$ (DuPhar, Amsterdam) and H7 (Funakoshi, Tokyo). The human cell lines HL-60 (promyelocytic cells), K562 (erythroleukemia cells), U937 (histiocytic cells) and THP1 (acute monocytic leukemia cells) were maintained as stationary suspension cultures in RPMI-1640 medium as described [5]. RNA blot hybridization was performed with 10 μg of total RNA extracted from cultured cells and ^{32}P-labelled probes as described previously [5]. For measuring the levels of four mRNAs for Ub, three cDNAs were used: one was a poly-UbB cDNA for detection of all classes of Ub mRNAs. The other two were cDNAs of about 250 bp corresponding to the C-terminal extension sequences (UBCP) of mono-UbA_{80} and mono-UbA_{52} cDNAs (for details, see [6]). cDNAs encoding the rat ribosomal proteins S17 [7] and L31 [8], the 3rd exon of the human c-*myc* gene and the cDNA of β-actin (Oncr Inc.) were also used. Immunoelectrophoretic blot analysis was carried out by the method of Towbin et al. [9]. Samples of cell extracts (50 μg) were separated by SDS-PAGE, transferred electrophoretically to a Hybond-C membrane (Amersham) and treated with anti-Ub antibodies (Sigma). [^{125}I]Protein A was used as the second antibody.

3. RESULTS

3.1. *Levels of mRNAs transcribed from 4 classes of Ub genes during differentiation of leukemia cells*

On Northern blot analysis with Ub-cDNA, three distinct mRNAs of 2.6, 1.2 and 0.6 kb were detected in total RNAs extracted from human HL-60 leukemia cells with poly-UbC, poly-UbB and two mono-UbAs, respectively [6], whereas with the UBCP-UbA_{80} and UBCP-UbA_{52} probes, corresponding to small and large ribosomal proteins, respectively [10], mRNAs with approximately similar lengths of 0.6 kb were detected (Fig.

Correspondence address: K. Takana, Institute for Enzyme Research, The University of Tokushima, Tokushima 770, Japan.

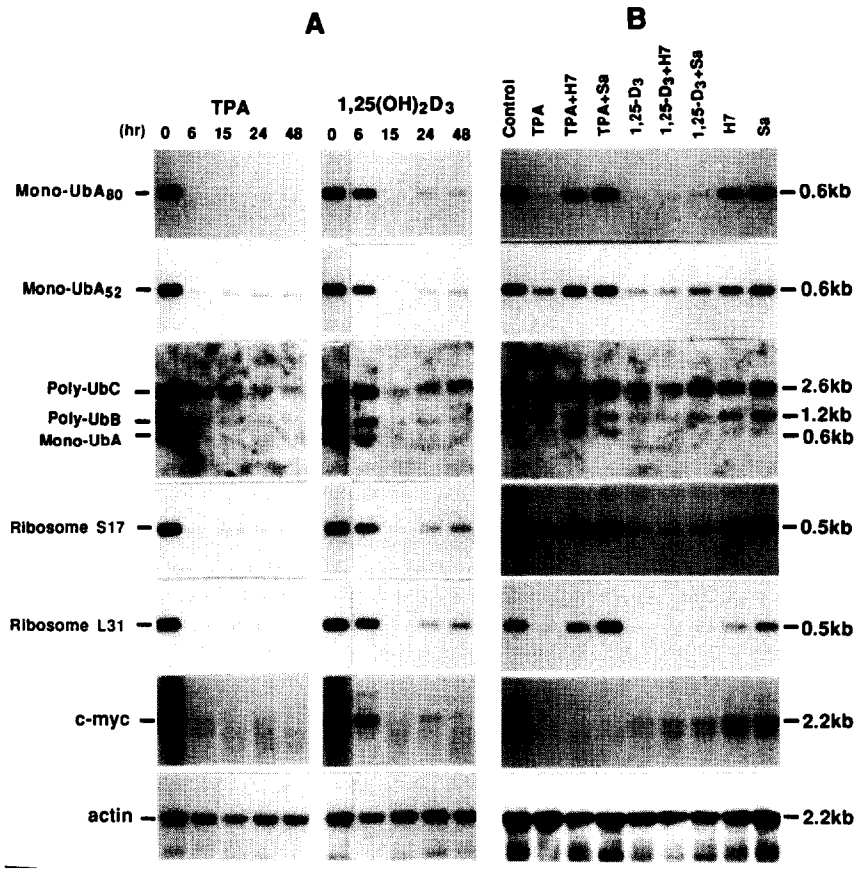

Fig. 1. Down-regulation of the expression of mRNAs encoding Ub during monocytic differentiation of promyelocytic leukemic HL-60 cells. (A) Time course: HL-60 cells (3×10^5 cells/ml) were treated with 5×10^{-8} M TPA (left panel) or 5×10^{-7} M $1,25(OH)_2D_3$ (right panel) for the indicated times. (B) Effects of C-kinase inhibitors: HL-60 cells were treated for 48 h with TPA or $1,25(OH)_2D_3$ ($1,25\text{-}D_3$) in the presence or absence of 18 μM H7 or 6 μM sphinganine (Sa). Total RNA (10 μg) extracted from these cells was used for Northern blot analysis with three classes of Ub cDNAs, c-*myc* and actin as probes.

1). Interestingly, the levels of all Ub mRNAs were quite low in human peripheral lymphocytes and monocytes (data not shown), suggesting that abnormally high expression of multiple Ub genes is closely related to rapid proliferation of hematopoietic cells. As shown in Fig. 1A (left panel), treatment of HL-60 cells with TPA caused marked down-regulation of the levels of mRNAs transcribed from all classes of Ub genes, although the decrease of the mRNA encoding the largest poly-UbC was relatively small. This down-regulation was rapid, indicating half-lives of less than 3 h during stimulation, suggesting rapid turnover of the mRNAs. Similar down-regulations of the Ub mRNA levels were observed during monocytic differentiation induced by $1,25(OH)_2D_3$, although the disappearance of the mRNAs was slower than during treatment with TPA (Fig. 2A, right panel). Thus down-regulation of Ub expression seems to be independent of the type of signal.

Intracellular transduction of the signal of TPA is known to be mediated by protein kinase C, so we examined whether down-regulation of Ub by $1,25(OH)_2D_3$ was mediated through a similar C-kinase pathway. For this, we tested the effects of C-kinase inhibitors, such as H7 [7] and sphinganine [8] on the down-regulation of Ub expression during treatment of HL-60 cells with these inducing agents. As shown in Fig. 1B, down-regulation of Ub genes in HL-60 cells induced by TPA was markedly blocked by the presence of H7 or sphinganine, but these two inhibitors had no effect on the decrease of the mRNA levels of Ub during $1,25(OH)_2D_3$-induced monocytic differentiation of these cells except for partial inhibition of poly-UbC gene expression by sphinganine. These findings suggest that protein kinase C is involved in the TPA-mediated pathway of down-regulation of Ub genes, but not in the $1,25(OH)_2D_3$-dependent pathway. Thus, the mechanisms of down-regulations of Ub genes induced by TPA and $1,25(OH)_2D_3$ seem to be different. These two inhibitors alone had no effect on the mRNA levels of Ubs, suggesting that down-regulation of Ub expression is not dependent on the intracellular pathway for signal transduction evoked by TPA and $1,25(OH)_2D_3$ and that differentiation of the cells itself is important for their down-regulating effects.

It was of interest to determine whether TPA-induced

down-regulation of Ubs was specific for HL-60 cells. To examine this, we measured alterations of the Ub mRNA levels during TPA-induced monocytic differentiation of other types of cells. As shown in Fig. 2A, TPA caused marked decreases in the levels of multiple Ub mRNAs in U937, THP1 and K562 cells, suggesting that down-regulation of Ub gene expression induced by TPA is independent of the cell type. We also examined whether down-regulation of Ubs was specific to monocytic differentiation of cells by studies on HL-60 cells treated with DMSO and retinoic acid to induce their differentiation into granulocytic cells, and K562 cells treated with *n*-butyric acid (BuAc) and hemin to induce their erythroid differentiation. Results showed marked down-regulation of Ubs during both granulocytic differentiation of HL-60 cells (Fig. 2B), and erythroid differentiation of K562 cells (Fig. 2C), indicating that down-regulation of the family of Ub genes is independent of the direction of differentiation.

3.2. *Levels of mRNAs encoding S17 and L31 ribosomal proteins during differentiation of leukemia cells*

As mono-Ub genes are fused with ribosomal protein genes, we examined whether the expression of genes encoding other ribosomal proteins is coordinated to those of mono-Ub genes. For this, we compared the mRNA levels of two ribosomal proteins, S17 and L31, with those of mono-Ub mRNAs and found that these two types of mRNAs decreased similarly on treatment with various inducing reagents, irrespective of the cell type, direction of differentiation or type of signal (Figs. 1 and 2). Interestingly, the effect of C-kinase inhibitors, such as H7 and sphinganine, on the expression of these ribosomal proteins was also similar to those of the two mono-Ub genes, suggesting that down-regulation of the expression of ribosomal protein genes encoded by the mono-Ub genes is coordinated with those of other ribosomal proteins. In contrast, the level of actin mRNA did not change significantly during any of the treatments examined.

3.3. *In vitro differentiation of human leukemia cell lines*

During these experiments, we examined differentiation of cells in several ways. We found microscopically that marked morphological changes occurred on treatments with various inducers, as indicated by aggregation of the cells and their tight attachment to the culture dishes (data not shown). Rapid down-regulation of the mRNA levels of the proto-oncogene c-*myc* has been used as a marker of differentiation in a blood-cell culture system [13]. We also observed a decrease in c-*myc* mRNA levels during induction of differentiation of all leukemic cells examined (Figs. 1 and 2). However, unlike the effect of C-kinase inhibitors such as H7 or sphinganine on the down-regulation of expression of ubiquitin and ribosomal genes, these inhibitors did not prevent down-regulation of the mRNA level of c-*myc*

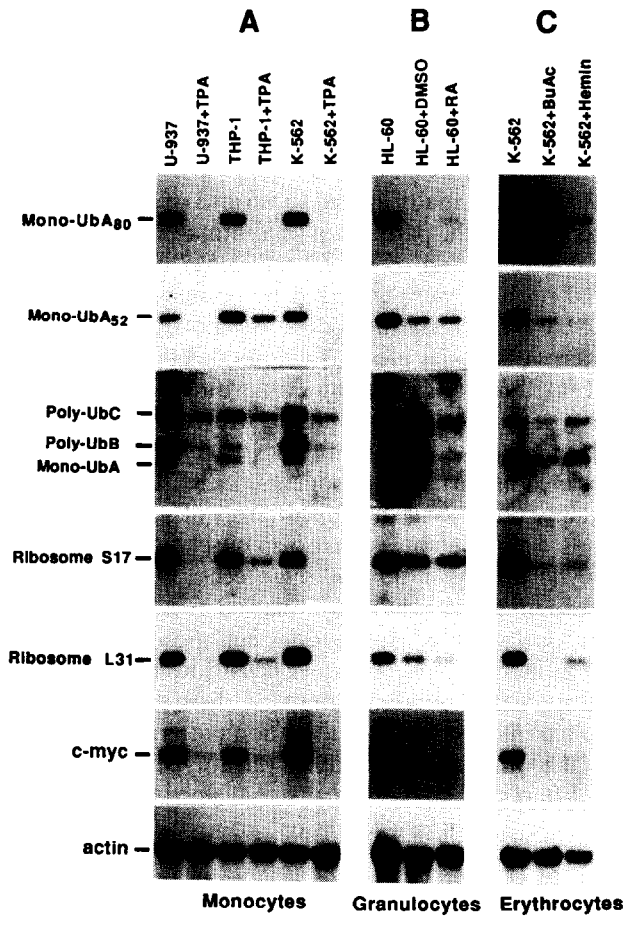

Fig. 2. Effects of cell type and direction of differentiation on down-regulation of expressions of multiple Ub genes. (A) U937, THP1 and K562 cells (3×10^5 cells/ml) were treated for 72 h with TPA. (B) HL-60 cells were treated for 72 h with 1.25% DMSO or 1.0×10^{-6} M retinoic acid (RA). (C) K562 cells were treated for 120 h with 1.4×10^{-2} M *n*-butyric acid (BuAc) or 5×10^{-5}M hemin. Northern blot analysis was performed as for Fig. 1. The direction of differentiation of the cells examined is shown at the bottom.

by TPA (Fig. 1B), suggesting that TPA down-regulates the c-*myc* gene by another pathway not involving C-kinase. Interestingly, these C-kinase inhibitors caused partial inhibition of the down-regulation of c-*myc* induced by $1,25(OH)_2D_3$, which is consistent with a report by others [14]. Thus, the mechanisms of down-regulation of c-*myc* gene expression induced by TPA and $1,25(OH)_2D_3$ seem to be different.

Monocytic and granulocytic differentiations were detected by measuring the change in activity for reduction of nitroblue tetrazolium (NBT), and erythroid differentiation was monitored as the production of hemoglobin staining with benzidine. After 3 days treatment, almost all the HL-60 cells were NBT-positive in cultures with TPA, and 55–95% were NBT-positive in cultures with $1,25(OH)_2D_3$, retinoic acid or DMSO (data not shown). Cultures of unstimulated control HL-60 cells contained less than 5% NBT-positive cells. Hemin and BuAc

caused similar inductions of differentiation of K562 cells, as judged by measurement of benzidine-positive cells (data not shown).

3.4. *Patterns of ubiquitinated proteins during differentiation of leukemia cells*

As Ub is normally ligated to various cellular proteins, we compared the patterns of ubiquitinated proteins during differentiation of HL-60 and K562 cells. Ubiquitinated proteins were analyzed by immunoblotting with anti-Ub polyclonal antibodies. As shown in Fig. 3, the patterns of cellular proteins ubiquitinated in HL-60 cells and in K562 cells were somewhat different, suggesting cell type-specific ubiquitination. Moreover, differentiation of HL-60 cells into monocytic cells caused disappearance of certain components, increases in intensity of many bands and the appearances of some faint new bands. Similar changes were also observed during granulocytic differentiation of these cells. Differentiation-dependent changes in ubiquitination of cellular proteins were also observed during differentiation of K562 cells into monocytic and erythroid cells. Interestingly, similar monocytic differentiations of these two cell types resulted in different ubiquitination patterns, suggesting that the terminal-differentiated phenotypes of these cells are different. The biological significance of differentiation-specific ubiquitinations of various cellular proteins is unknown.

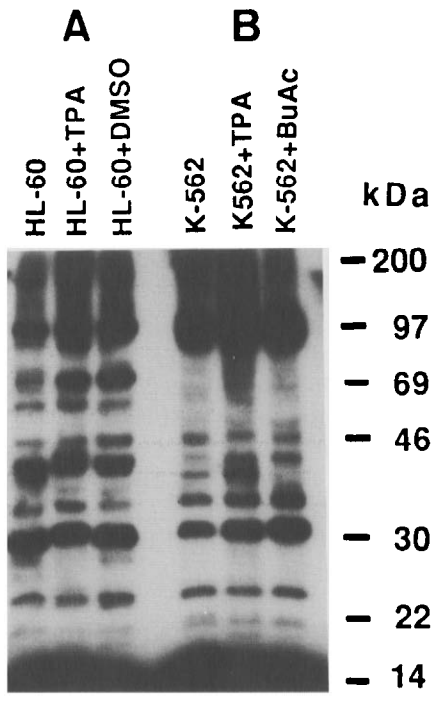

Fig. 3. Detection of ubiquitinated proteins in crude extracts from leukemic cells by immuno-blot analysis. Panels A and B show results with extracts of HL-60 and K562 cells, respectively. The cells were treated with various inducers, as for Figs. 1 and 2. Samples of 50 μg of cell proteins were subjected to SDS-PAGE. Immunoblot analysis was carried out as described in section 2. The positions of M_r marker proteins are shown at the right.

4. DISCUSSION

The differentiation-dependent down-regulation of multiple Ub genes in immature human leukemic cells observed in this work resembles that of the proteasome that we reported before [5]. The expression of these Ub and proteasome genes may well be coordinated, because these two types of gene products are involved in the same degradation system, consisting of selective recognition of target proteins by Ub and their subsequent destruction by the proteasome [2–4]. Moreover, differentiation of leukemic cells not only caused down-regulation of expression of the poly-Ub genes that increase the cellular Ub pool, but also of the mono-Ub genes. The latter effect may be related to ribosome biogenesis, because mono-Ub genes produce ribosomal proteins as well as the Ub moiety [1,10], and because the mRNA levels of two ribosomal proteins, S17 and L31, changed in a similar fashion to those of the mono-Ub genes (Figs. 1 and 2). Previous studies have shown that the ribosome content is high in rapidly growing cells with high protein-synthesizing activity, and that differentiation of leukemic cells is always associated with withdrawal from the cell proliferation. Errors of transcription and translation could be high during protein synthesis in rapidly proliferating cells with an increase in the possibility of the generation of abnormal proteins. The observed growth-dependent decrease in the mRNAs producing Ub and proteasomes [5] in cells may result in the loss of an ATP/Ub-dependent proteolytic system responsible for maintaining cellular homeostasis by preventing accumulation of abnormal proteins generated by biosynthetic errors.

In the present study, we found that ubiquitination of proteins showed cell-specific patterns (Fig. 3), which were not affected by Ub gene expression. Previously, we reported specific ubiquitinated proteins in cancerous parts of human renal cell carcinomas [6], suggesting that specific ubiquitination occurs commonly during carcinogenesis or differentiation in various cells. At present, it is unknown whether the heterogeneity in size of ubiquitinated proteins is due to mono-ubiquitination of different cellular proteins or multi-ubiquitination of the same proteins. However, the latter possibility is unlikely, because multi-ubiquitinated proteins are believed to be degraded rapidly by the proteasome system in cells, and normal intermediates of proteolysis cannot be detected in the cells. Thus, the ubiquitination of cellular proteins shown in Fig. 3 may differ from the acquisition of a degradation signal. Therefore, it is of importance to identify and characterize the proteins ubiquitinated in a proliferation- and differentiation-specific manner.

REFERENCES

[1] Finley, D. and Chau, V. (1991) Annu. Rev. Cell Biol. 7, 25–69.
[2] Goldberg, A.L. (1992) Eur. J. Biochem. 204, 9–23.
[3] Hershko, A. and Ciechanover, A. (1992) Annu. Rev. Biochem. 61, 761–807.
[4] Tanaka, K., Tamura, T., Yoshimura, T. and Ichihara, A. (1992) New Biologist 4, 1–14.
[5] Shimbara, N., Orino, E., Sone, S., Ogura, T., Takashina, M., Shono, M., Tamura, T., Tanaka, K. and Ichihara, A. (1992) J. Biol. Chem. 267, 18100–18109.
[6] Kanayama, H., Aki, M., Tanaka, K., Kagawa, S., Miyaji, H., Satoh, M., Okada, F., Sato, S., Shimbara, N. and Ichihara, A. (1991) Cancer Res. 51, 4467–4485.
[7] Nakanishi, O., Oyanagi, M., Kuwano, Y., Tanaka, T., Nakayama, T., Mitsui, H., Nabeshima, Y. and Ogata, K. (1985) Gene 35, 289–296.
[8] Tanaka, T., Kuwano, Y., Kuzumaki, T., Ishikawa, K. and Ogata, K. (1987) Eur. J. Biochem. 162, 45–48.
[9] Towbin, H.S., Staehelin, J. and Gordo, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[10] Finely, D., Bartel, B. and Varshavsky, A. (1989) Nature 338, 394–401.
[11] Hidaka, H., Inagaki, M., Kawamoto, S. and Sasaki, Y. (1984) Biochemistry 23, 5036–5041.
[12] Merrill, A.H., Sereni, A.M. Jr., Stevens, V.L., Hannun, Y.A., Bell, R.M. and Kinkade, J.M. Jr. (1986) J. Biol. Chem. 261, 12610–12615.
[13] Siebenlist, U., Bressler, P. and Kelly, K. (1988) Mol. Cell. Biol. 8, 867–874.
[14] Simpson, R.U., Hsu, T., Wendt, M.D. and Tayler, J.M. (1989) J. Biol. Chem. 264, 19710–19715.