Korean J. Zool. 39:47-53, 1996

Expression of the Heat Shock Proteins and Glucose-Regulated Proteins during Phorbol 12-Myristate 13-Acetate-Induced Megakaryocytic Differentiation of K562 Erythroleukemia Cells

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We examined the expression of the heat shock proteins (HSPs) and glucose-regulated proteins (GRPs) during phorbol 12-myristate 13-acetate (PMA)-induced megakaryocytic differentiation of human erythroleukemia K562 cells. PMA-treated K562 cells showed a cell growth arrest and alteration in morphology and patterns of gpIIIa and *c-myc* expression, characteristic of megakaryocytic differentiation. During the megakaryocytic differentiation, HSP90A, HSP90B, and HSP28 mRNA and protein levels markedly decreased, while GRP78/BiP and GRP94 mRNA levels were enhanced. On the other hand, HSP70A and HSP70B mRNA levels were reduced, but HSP70 protein levels were not changed by PMA treatment. These results suggest specific roles for the HSPs and GRPs in K562 cell proliferation and megakaryocytic differentiation.

KEY WORDS: Heat Shock Proteins, Glucose-Regulated Proteins, K562 Differentiation, Megakaryocytes, Phorbol 12-Myristate 13-Acetate

The synthesis of a group of proteins is highly induced in response to various environmental stresses such as high growth temperature or glucose starvation (Welch, 1990). The stress-inducible protein families include the HSPs and GRPs. Depending on the nature of the stress, the HSPs and GRPs can be induced simultaneously, reciprocally or individually (Welch, 1990). The expression of the HSPs is enhanced in cells exposed to high growth temperature or heavy metal, whereas the synthesis of the GRPs is induced by glucose starvation or perturbation of Ca^{2+} homeostasis. Increased synthesis of both the HSPs and GRPs occurs in cells treated with amino acid analogs. Several studies have shown that the expression of the stress proteins is also altered during cellular differentiation. When murine teratocarcinoma stem cells differentiate into parietal endoderm cells by retinoic acid and dibutyryl cAMP, an increase in HSP90 mRNA level is observed (Kohda *et al.*, 1991). In addition, the expression of HSP70 and HSP28 is enhanced as K562 erythroleukemia cells differentiate into erythrocytes by hemin (Theodorakis *et al.*, 1989; Mivechi *et al.*, 1994). Granulocytic differentiation of HL-60 leukemia cells by either DMSO or N-methylformamide markedly reduces HSP90 and HSP70 mRNA levels (Beere *et al.*, 1993; Yufu *et*

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al., 1989). However, the exact function of the HSPs in cellular differentiation is not clearly understood.

In this study, we examined the changes in expression of the HSPs and GRPs during megakaryocytic differentiation of human leukemia K562 cells. K562 cells differentiate into cells with megakaryocytic characteristics by a tumor promoting phorbol ester, PMA. Megakaryocytic differentiation is accompanied by the increased expression of megakaryocyte-specific antigen, gpIIb/IIIa and the concomitant disappearance of erythrocyte-specific marker, hemoglobin (Hocevar *et al.*, 1992). Decreased expression of *c-myc* (Colamonici *et al.*, 1986) and enhanced expression of *c-jun* and *c-fos* (Alitalo *et al.*, 1990) are also observed in PMA-treated K562 cells. In this study we show that the expression of the HSPs and GRPs is differentially regulated during PMA-induced megakaryocytic differentiation of K562 cells, suggesting specific roles for the HSPs and GRPs in K562 cell proliferation and megakaryocytic differentiation.

Materials and Methods

Cell Culture and PMA Treatment

K562 cells were obtained from Aichi Cancer Institute, Nagoya, Japan and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 1% penicillin-streptomycin in a 37°C incubator with 5% CO_2. PMA (Sigma Chemical Co., St. Louis, MO) was prepared as a concentrated stock in DMSO. K562 cells plated at a density of 4×10^5cells/ml were treated with 50 nM PMA, and cultured for 4 days without medium change. Viable cells were determined by trypan blue dye exclusion and counted every 24 h. Cell viability was >95% in all cultures.

SDS-PAGE and Western Blot Analysis

Cells were washed in cold phosphate-buffered saline, lysed in SDS-sample buffer by heating at 100°C for 10 min and cellular proteins were separated by SDS-PAGE. The resulting gels were either stained with Coomassie Blue or transferred to nitrocellulose paper. For Western blotting (Towbin *et al.*, 1979), the membrane was blocked with 5% nonfat milk in TBS-T for 1 h at room temperature. Membranes were then incubated with either anti-HSP90 MoAb (AC88, a gift from Dr. D. O. Toft, Mayo Medical School, Rochester, Minnesota), anti-HSP73 MoAb (1B5, StressGen, Victoria, British Columbia, Canada), anti-HSP72 MoAb (C92), anti-HSP72/73 MoAb (N27), anti-ERp72 PoAb (gifts from Dr. W. J. Welch, University of California, San Francisco, CA), or anti-HSP28 PoAb (a gift from Dr. J. Landry, Laval University, Quebec, Canada) for 1-2 h at room temperature. After washing in TBS-T three times, the blot was incubated with peroxidase-conjugated secondary antibody and the antibody-specific proteins were visualized by the enhanced chemiluminescence detection system according to the recommended procedure (Amersham Corp.).

Northern Blot Analysis

Total RNA was isolated using TRI reagent according to manufacturer's protocol (Molecular Research Center Inc., Cincinnati, OH) and electrophoresed on a denaturing gel (1% agarose, 2.2 M formaldehyde). For Northern blotting (Maniatis *et al.*, 1989), RNA was transferred onto nylon membrane (Zeta-Probe membrane, Bio-Rad, CA) and hybridized with either human HSP90A, HSP90B, HSP70B, HSP28 (StressGen, Victoria, British Columbia, Canada), human HSP70A (a gift from Dr. R. I. Morimoto, Northwestern University, IL), hamster GRP94, GRP78/BiP (gifts from Dr. A. S. Lee, Univ. of Southern California, Los Angeles, CA) or *c-myc* (a gift from Dr. S. H. Kim, Pusan National University, Korea) cDNA probes which had been labeled with [α-^{32}P]dCTP (Amersham Corp.) by random priming. The filter was then exposed to X-ray film for 2-5 days. The quantification of the autoradiogram was carried out using a densitometric scanner (TLC CS-930; Shimadzu Co., Japan).

Immunofluorescence microscopy

To determine the expression of gpIIIa, K562 cells were washed with phosphate-buffered saline, attached on cover slips with poly-L-lysine (Sigma Chemical Co.) and fixed in 3.7% formaldehyde for 30 min. Staining was performed using fluorescein

isothiocyanate-conjugated gpIIIa MoAb (DAKO Corp.) diluted 1:200 for 1 h in the dark. After staining, specimens were analyzed with a confocal imaging system using a 400 × objective lens with 3 zoom (Carl-Zeiss LSM410 Invert).

Results

Megakaryocytic differentiation of K562 cells

To induce the megakaryocytic differentiation of K562 cells, the cells were incubated in the presence of 50 nM PMA for up to 4 days. As shown in Fig. 1A, PMA-treated cells became slightly larger and flatter than untreated cells, aggregated and were more adherent to the plastic of culture flasks, a characteristic of megakaryocytic differentiation (Bütler *et al.*, 1990). PMA treatment also caused an immediate growth arrest (Fig. 1B), although cell viability was not affected as determined by trypan blue dye exclusion. Direct immunofluorescent spectroscopy observation showed an extensive expression of gpIIIa on the surface of PMA-treated K562 cells (data not shown). A significant reduction in *c-myc* expression was also observed after 6 h of PMA treatment (Fig. 2). These results clearly indicate that K562 cells are induced to differentiate into megakaryocytes by PMA treatment.

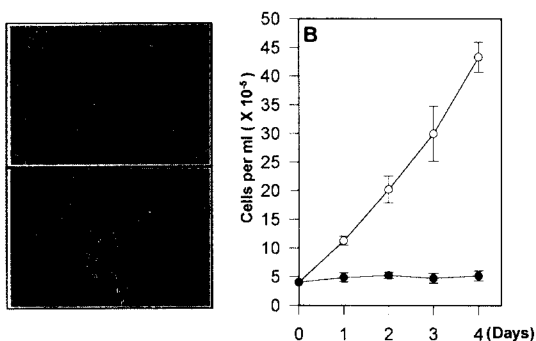

Fig. 1. Morphology and growth rate of PMA-treated K562 cells. A. K562 cells were cultured in the absence (-PMA) or presence of 50nM PMA in RPMI 1640 growth medium for 24 h (+PMA). × 100. B. Growth curve of K562 cells. K562 cells plated at a density of 4 × 10^5cells/ml were incubated in the absence (-○-○-) or presence (-●-●-) of 50 nM PMA for 4 days and counted by hemocytometer.

Expression of the HSPs and GRPs during megakaryocytic differentiation of K562 cells

In order to examine the changes in HSPs expression during PMA-induced megakaryocytic differentiation of K562 cells, the cells were treated with PMA for 4 days and the cellular proteins were examined by SDS-PAGE and Western blot analysis. As shown in Fig. 3, HSP90 and HSP28 protein levels abruptly decreased upon treatment of PMA. On the other hand, the levels of both inducible HSP70 (referred to as HSP72) and cognate HSP70 (referred to as HSP73) which were identified with C92 (anti-HSP72) and 1B5 (anti-HSP73) MoAbs, respectively, were not changed by PMA treatment (Figs. 3D and E). No significant change in HSP72 and HSP73 levels was also observed by Western blotting with N27 MoAb that recognizes both HSP72 and HSP73 (Fig. 3F). Similarly, the level of ERp72, one of resident endoplasmic reticulum stress proteins

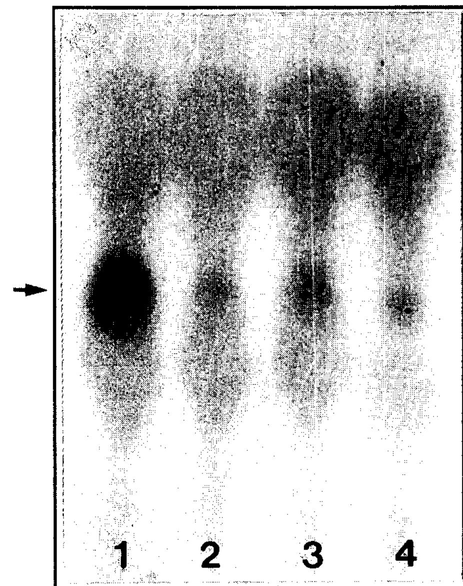

Fig. 2. Northern blot analysis of *c-myc* expression in PMA-treated K562 cells. K562 cells were cultured in the absence (lane 1) or presence of 50 nM PMA for 6 (lane 2), 12 (lane 3) or 24 h (lane 4). Total RNA was extracted, electrophoresed, and hybridized with *c-myc* cDNA probe.

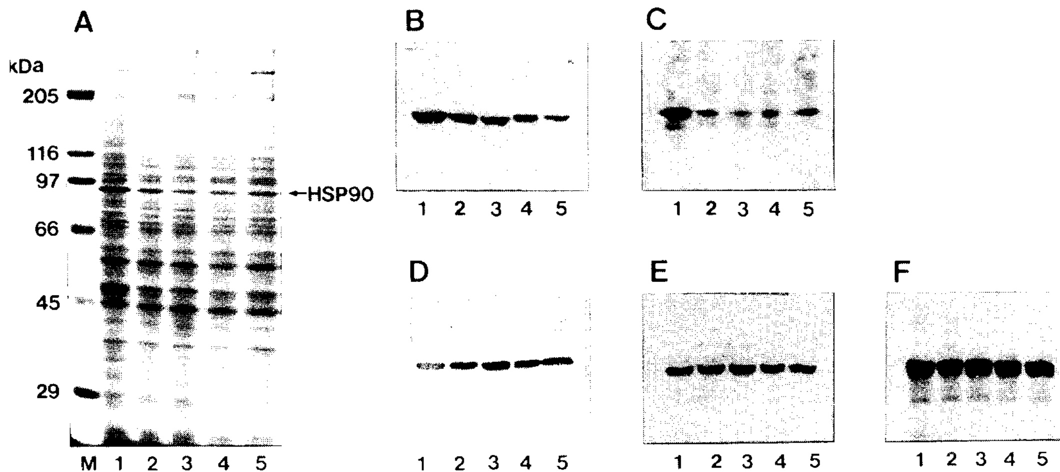

Fig. 3. Western blot analysis of the HSP protein levels during PMA-induced K562 cells differentiation. K562 cells were cultured in the absence (lane 1) or presence of 50 nM PMA for 1 (lane 2), 2 (lane 3), 3 (lane 4) or 4 days (lane 5). The cellular proteins were analyzed by SDS-PAGE and the resulting gels were then either stained with Coomassie Blue (panel A) or blotted with either anti-HSP90 (AC88, panel B), anti-HSP28 (panel C), anti-HSP73 (1B5, lane D), anti-HSP72 (C92, panel E), anti-HSP72/73 (N27, panel F).

(Dorner *et al.*, 1990), was not changed by PMA treatment (data not shown).

The changes in HSP and GRP mRNA levels during the megakaryocytic differentiation of K562 cells were determined by Northern blot analysis. As shown in Fig. 4, HSP90A, HSP90B, and HSP28 mRNA levels decreased approximately 2-fold following the K562 cell differentiation. The expression of inducible HSP70A and HSP70B genes was also reduced during the cell differentiation, unlike no change in HSP70 protein level. On the other hand, GRP94 and GRP78/BiP mRNA levels increased to a peak level until 3 days of PMA treatment, and slightly decreased thereafter. Since the expression of GRPs can be induced by glucose starvation (Welch, 1990), we examined if the GRP expression is induced in PMA-untreated K562 cells when cultured for 4 days without medium change. Under these conditions, GRP94 and GRP78 mRNA levels were not induced (data not shown), suggesting that the GRP induction is not due to glucose starvation but rather related to the megakaryocytic differentiation of K562 cells. These results provide the first evidence for the differential expression of the HSPs and GRPs during PMA-induced megakaryocytic differentiation of K562 cells.

Discussion

K562 cells can be induced to differentiate into megakaryocytes by PMA (Hocevar *et al.*, 1992; Colamonici *et al.*, 1986; Alitalo *et al.*, 1990). Megakaryocytic differentiation of K562 cells was accompanied by the extensive cell surface expression of megakaryocyte-specific antigen, gpIIIa, and a change in cell morphology (Fig. 1A). PMA treatment caused an almost immediate growth arrest of the cells occurring during the first 24 h of treatment (Fig. 1B). Cell growth arrest may be correlated to the down-regulation of *c-myc* expression which occurs during the megakaryocytic differentiation of K562 cells (Fig. 2) (Colamonici *et al.*, 1986). *C-myc* has been suggested to be involved in proliferation of a variety of mammalian cells as a positive regulator of cell growth (Bishop *et al.*, 1991).

In this study, we showed the differential expression of the HSPs and GRPs during the megakaryocytic differentiation of K562 cells. Treatment of K562 cells with PMA caused an abrupt decrease in HSP90A, HSP90B, and

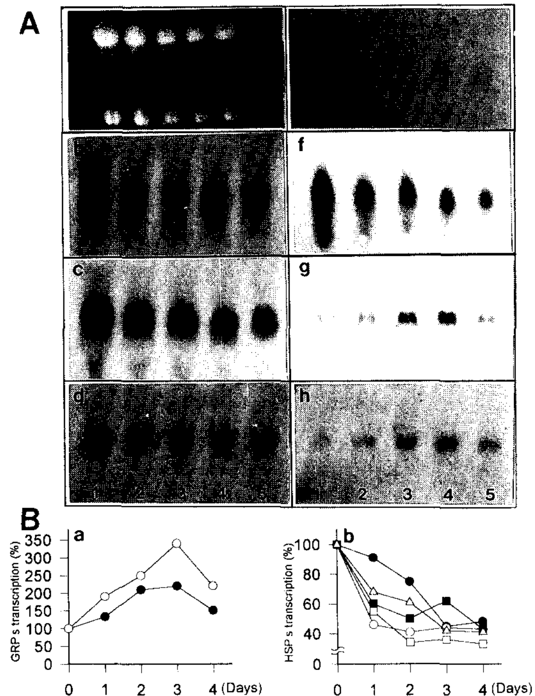

Fig. 4. Northern blot analysis of the HSP and GRP expression during PMA-induced K562 cell differentiation. A. Autoradiogram of Northern blot. Total RNA was purified from K562 cells cultured in the absence (lane 1) or presence of 50 nM PMA for 1 (lane 2), 2 (lane 3), 3 (lane 4), or 4 days (lane 5) and blotted with cDNA probes for HSP90A (b), HSP90B (c), HSP70A (d), HSP70B (e), HSP28 (f), GRP94 (g) and GRP78/BiP (h). Ethidium bromide-stained RNA pattern is shown in panel a. B. Densitometric scanning of Northern blot. HSP and GRP mRNA levels shown in the autoradiogram in panel A were quantified by densitometric scanning. The values are shown relative to those of control cells. In panel a, ○, GRP94; ●, GRP78/BiP. In panel b. ○, HSP90A; ●, HSP90B; □, HSP70A; ■, HSP70B; △, HSP28.

HSP28 mRNA and protein levels and a concomitant increment in GRP78/BiP and GRP94 mRNA levels (Figs. 3 and 4). Such changes in the HSPs and GRPs expression were not observed in PMA-treated other cells such as HeLa cells and primary cultured chick muscle cells (data not shown), thereby indicative of specific relationship between the expression of the HSPs/GRPs and growth/differentiation of K562 cells. Interestingly, during the megakaryocytic differentiation, HSP70 mRNA and protein levels did not appear to be correlated. HSP70 transcript levels were markedly reduced, but its protein levels remained constant (Figs. 3 and 4). The reason for this phenomenon is not clear. HSP70 protein may be stabilized or protected from proteolysis in K562 cells. Similarly, it was recently reported that HSP28 protein levels are in discord with its mRNA levels in retinoic acid-induced granulocytic differentiation of NB4 leukemia cell line (Spector *et al.*, 1995).

The changes in expression of the HSPs and GRPs during cell differentiation have been shown in a variety of eucaryotic cell differentiation systems. Treatment of K562 cells with PMA decreases the HSP90 and HSP28 gene expression (Figs. 3 and 4), whereas treatment of HL-60 cells with the phorbol ester increases HSP70A and HSP28 levels (Mivechi *et al.*, 1994). HSP70 and HSP28 levels are enhanced during hemin-induced erythrocytic differentiation of K562 cells (Theodorakis *et al.*, 1989; Mivechi *et al.*, 1994). Further, granulocytic differentiation of HL-60 cells reduces HSP90 and HSP70 mRNA levels (Beere *et al.*, 1993; Yufu *et al.*, 1989). Thus, this different expression of the stress proteins indicate specific and distinct roles for the HSPs and GRPs in different types of leukemic cell differentiation.

Since HSP90 is one of the proteins highly expressed in untreated K562 cells, HSP90 overexpression may be required for K562 cell proliferation. Enhanced expression of HSP90 has been observed after oncogene transformation of cell lines and in a number of cancer cells which are continuously growing (Lebeau *et al.*, 1991; Gress *et al.*, 1994). In addition, HSP90 is known to be associated with many regulatory proteins such as steroid hormone receptor, protein kinases and oncogene proteins that are involved in cell proliferation and transformation (Welch, 1990). Thus, HSP90 may participate in cell transformation by interacting with oncogene proteins or other regulatory proteins. It is also possible that HSP90 may function as a negative regulator of cell differentiation.

HSP28 that is down-regulated by PMA treatment may be also implicated in the maintenance of K562 cell phenotype. HSP28 is known to function as molecular chaperone (Jakob

et al., 1993) and exert an specific inhibitory effect on actin polymerization (Miron *et al.*, 1991). During PMA-induced megakaryocytic differentiation of K562 cells, G-actin is known to be polymerized to F-actin (Bütler *et al.*, 1990). Thus, down regulation of HSP28 may be linked to actin polymerization in K562 cells.

Unlike HSP90 and HSP28, GRP78/BiP and GRP94 expression increased by PMA treatment (Fig. 4), thus indicating that the GRPs may be related to K562 cell differentiation. GRP78/BiP and GRP94 also function as molecular chaperone within the endoplasmic reticulum. During the megakaryocytic differentiation, the synthesis of many secretory and surface proteins such as IL-6, GM/CSF and gpIIb/IIIa are elevated (Hocevar *et al.,* 1992). GRPs may be involved in the folding and/or assembly of these secretory and surface proteins, although it is not demonstrated whether the GRPs interact with these proteins. Further understanding of the functions of HSPs and GRPs as well as the molecular mechanism of regulation of their expression will provide a better understanding of the events responsible for cell growth and differentiation.

Acknowledgements

We thanks Drs. A. S. Lee, R. I. Morimoto, S. H. Kim, D. O. Toft, J. Landry, and W. J. Welch for providing us with cDNA probes and antibodies.

This work was supported by grant (94-0401-10-01-3) from the Korea Science and Engineering Foundation and the Baisc Science Research Institute Program (BSRI-94-4409), Ministry of Education, Korea.

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(Accepted November 24, 1995)

K562 백혈구암 세포의 Phorbol 12-Myristate 13-Acetate에 의한 대핵세포로의 분화과정에서 Heat Shock Proteins와 Glucose-Regulated Proteins의 발현

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K562 사람 백혈구암 세포에서 phorbol 12-myristate 13-acetate(PMA)에 의한 대핵세포로의 분화과정에서 heat shock proteins(HSPs)와 glucose-regulated proteins(GRPs)의 발현을 조사하였다. PMA에 의한 K562 세포의 분화 특징은 세포성장의 억제, 형태학적 변화, gpIIIa의 발현 증가, *c-myc* 발현의 감소 등으로 나타난다. PMA에 의한 대핵세포 분화과정에서, HSP90A, HSP90B 그리고 HSP28 mRNA와 단백질 합성은 현저히 감소하는 반면, GRP78/BiP와 GRP94의 mRNA 합성은 증가하였다. 한편, HSP70A와 HSP70B의 mRNA 합성은 감소하였지만, HSP70 단백질의 합성은 변함없었다. 이러한 결과는 HSPs와 GRPs가 K562 세포의 증식 또는 대핵세포 분화과정에서 특이한 역할을 할 것임을 시사하고 있다.