

Differentiation of HL-60 cells is promoted by H-toxin of *Clostridium septicum*

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Abstract H-toxin of *Clostridium septicum* potentiated dimethyl sulfoxide (DMSO)-induced differentiation of human promyelocytic leukemia HL-60 cells which were monitored by nuclear morphology and production of oxidative radicals. But, H-toxin did not induce differentiation of HL-60 cells in the absence of DMSO. These phenomena were not observed by staphylococcal leukocidin, a cytotoxin affecting to HL-60 cells. In HL-60 cells, ADP-ribosylation of 118, 93, 75 and 58 kDa membrane proteins was observed, but the ADP-ribosylation was not detected either in differentiated HL-60 cells by DMSO or in normal polymorphonuclear leukocytes of human. When the membranes of HL-60 cells were incubated with H-toxin, ADP-ribosylation of the membrane proteins was inhibited. Such suppressive effects on ADP-ribosylation were not observed by DMSO or staphylococcal leukocidin. These data suggest that inhibition of the ADP-ribosylation by H-toxin may play an important role in potentiation of DMSO-induced differentiation of HL-60 cells.

Key words: Differentiation; HL-60 cell; *Clostridium septicum*; H-toxin; ADP-ribosylation

1. Introduction

H-toxin is a novel hemolytic toxin produced by *Clostridium septicum*. Its molecular weight estimated by SDS-PAGE is about 31,000 [1]. H-toxin was found to be cytotoxic to HL-60 cells [2]. In this report we showed that a sublethal dose of H-toxin potentiated the differentiation of HL-60 cells induced by DMSO. To clarify this phenomena, we investigated the effect of H-toxin on the endogenous mono-ADP-ribosylation of membrane proteins; because we thought that the first interaction of the toxin to HL-60 cells occurred in membranes and mono-ADP-ribosylation is an important posttranslational modification of proteins. Recently, the existence of ADP-ribosyltransferases in eukaryote cells was reported and some of them were purified [3–7]. We have studied the endogenous ADP-ribosylation in HL-60 cells [8]. In the HL-60 cell membrane, several proteins were ADP-ribosylated. These acceptor proteins of ADP-ribose were found not to be GTP binding proteins from the results of azido GTP binding assay. One of the acceptor proteins, with a molecular weight of 38,000, was found to be glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [9]. Covalent binding of ADP-ribose and NAD⁺ to GAPDH was recently reported [10,11]. The rest of the proteins were not identified yet and the role of endogenous ADP-ribosylation of such proteins are not known. In this report we showed that H-toxin suppressed endogenous ADP-ribosylation of 118, 93, 75 and 58 kDa membrane proteins and promoted the differentiation of HL-60 cells induced by DMSO. Further, we studied that the ADP-ribosylation of these membrane proteins was diminished in association with differentiation by DMSO. There is only one report which suggested that endogenous mono-ADP-ribosylation was correlated to differentiation. They showed that the inhibitor of the ADP-ribosylation suppressed differentiation of murine leukemic cell line induced by granulocyte colony-stimulating factor [12]. On the contrary, in this report, we showed by using H-toxin that inhibition of

the mono-ADP-ribosylation correlates to the differentiation of HL-60 cells.

2. Materials and methods

2.1. Chemicals

[³²P]NAD (spec. act. 800 Ci/mmol) was purchased from New England Nuclear and [adenine-¹⁴C]NAD (spec. act. 272 mCi/mmol) was from Amersham. Reagents for electrophoresis were obtained from Bio-Rad. All other chemicals used were of analytical grade.

2.2. HL-60 cell culture and differentiation by DMSO

HL-60 cells were cultivated in RPMI 1640 medium supplemented with 10% fetal calf serum. For the differentiation, the indicated concentration of DMSO and/or H-toxin was added to HL-60 cells (1×10^5 /ml) and incubated at 37°C for several days in a CO₂ incubator. Each cell culture was harvested and membranes were prepared as described elsewhere [8].

2.3. Assay of ADP-ribosylation

80 µg of membranes were incubated for 60 min at 37°C in a volume of 200 µl containing 100 mM Tris buffer (pH 7.5), 10 mM thymidine, 1 mM EDTA, 5 mM MgCl₂, 2 mM dithiothreitol and 1 µM [³²P]NAD (2.5 µCi). Reactions were terminated by the addition of 800 µl of 10% trichloroacetic acid, kept for 30 min at 4°C and centrifuged for 10 min at 7000 × g. The pellets were dissolved in 1% SDS/5% mercaptoethanol, heated for 10 min at 60°C and resolved in SDS polyacrylamide gel (10%) electrophoresis (SDS-PAGE) by the method of Laemmli [13]. Gels were stained with Coomassie brilliant blue and autoradiographed with a Kodak X-Omat film with an intensifying screen at –80°C for 20 h or analyzed by a Bio-imaging analyzer Bas 2000 (Fuji photofilm corp.).

2.4. H-toxin and staphylococcal leukocidin

H-toxin was purified from culture media of *Clostridium septicum* #44 strain according to Takano et al. [1]. Staphylococcal leukocidin was purified by the methods of Noda et al. [14].

2.5. Assay of chemiluminescence

Cells were cultivated for 4 days in various conditions. 2×10^6 cells were suspended in 1 ml of MEM including 100 ng/ml of TPA and lucigenin (25.5 µg/ml). The chemiluminescence was measured at 37°C in a 6 channel Biolumat LB 9505 chemiluminometer.

2.6. Assay of NAD glycohydrolase activity

Membranes were incubated with [adenine-¹⁴C]NAD in 100 mM

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Tris-HCl (pH 7.5) buffer at 37°C for 60 min in a total volume of 100 μ l and reactions were terminated by the addition of 100 μ l of ethanol and filtrated by centrimer 10000 (Sanko Junyaku Co. Ltd.). Aliquots of the filtrate were applied to spots on a polyethyleneimine-cellulose plate using capillary tubes. As a standard, ADP-ribose was applied to the same plate and revealed under UV light (254 nm). [14 C]NAD was also run as a standard and detected by a Bio-imaging analyzer. The plate was developed by using first, 1M-acetic acid, until the solvent front came to the origin and then 0.9 M acetic acid/0.3 M LiCl until the solvent was within 1 cm of the end of the plate. The radioactivity was detected by a Bio-imaging analyzer.

3. Results

3.1. Effects of H-toxin on the differentiation of HL-60 cells induced by DMSO

HL-60 cells can be induced to differentiate to mature myeloid cells by 1.25% of DMSO [15]. H-toxin was cytotoxic to HL-60 cells [2] and minimum effective cytotoxic dose to HL-60 cells was 5 μ g/ml. When HL-60 cells were treated with 2.5 μ g/ml of H-toxin alone, no morphological change by phase contrast microscopy was observed. However, combination of 2.5 μ g/ml of H-toxin and 0.75% of DMSO (which concentration did not induce differentiation of most cells) suppressed cell growth significantly (data not shown). Cytospin preparation of 4 days culture was stained with May-Giemsa and examined. The stages of differentiation of each cell culture are shown in Table 1. In untreated HL-60 cells and HL-60 cells treated with H-toxin, most cells were promyelocytes. In the cells treated with 0.75% DMSO, 12% of cells became myelocytes but the rest were promyelocytes. However, in the cells treated with 0.75% DMSO and 2.5 μ g/ml of H-toxin, promyelocytes decreased to 15% and about 80% cells were myelocytes or metamyelocytes. Moreover, band and segmented cells appeared, although it was only 5.5%. These data showed that H-toxin potentiated the differentiation by DMSO morphologically. Further investigation about those cells was planned to find out whether those cells were functionally differentiated cells. Production of oxidative radicals is one of the marker of functional differentiation. Therefore, chemiluminescence reactions were examined. As shown in Fig. 1, untreated HL-60 cells and HL-60 cells treated with H-toxin alone did not induce chemiluminescence at all. On the other hand, in the cells treated with 0.75% of DMSO, a slight increase of chemiluminescence reaction was observed. However, combined treatment of both reagents significantly

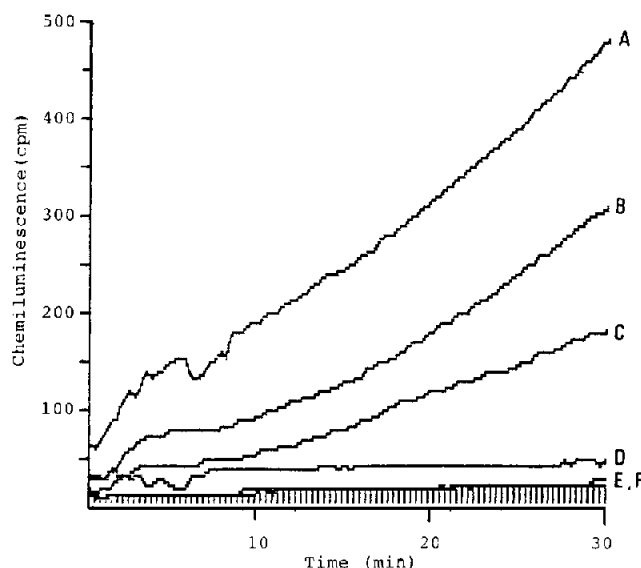


Fig. 1. Changes in chemiluminescence of HL-60 cells treated with DMSO or H-toxin. HL-60 cells were cultured in indicated amounts of H-toxin in the presence or absence of DMSO for 4 days and chemiluminescence were measured in each culture as described in section 2. 2.5 μ g/ml of H-toxin plus 0.75% of DMSO (A), 1.25 μ g/ml of H-toxin plus 0.75% of DMSO (B), 0.75% of DMSO (C), 2.5 μ g/ml of H-toxin (D), 1.25 μ g/ml of H-toxin (E), untreated (F).

induced chemiluminescence production. These results showed that H-toxin potentiated the differentiation of HL-60 cells not only morphologically but also functionally. Staphylococcal leukocidin is another cytotoxin affecting to HL-60 cells [16]. Therefore, we examined whether it promoted the DMSO-induced differentiation of HL-60 cells as well as H-toxin, but there were no effect of the leukocidin on the differentiation of HL-60 cells (data not shown).

3.2. Effect of H-toxin on the endogenous ADP-ribosylation of HL-60 cells

HL-60 cell membrane proteins were incubated with various concentrations of H-toxin in a reaction mixture including [32 P]NAD described in section 2. This reaction mixture contained 10 mM thymidine to inhibit poly-ADP-ribosylation. Radiolabeled proteins were separated by SDS-PAGE and detected by a Bio-imaging analyzer (Fuji photofilm corp.). Those labeled proteins were mono-ADP-ribosylated because they were hydrolyzed by snake venom phosphodiesterase [8]. As shown in Fig. 2, H-toxin suppressed endogenous ADP-ribosylation of 118, 93, 75 and 58 kDa proteins does dependently (lanes 4–6) and the percentages of the suppression to each proteins by 5 μ g/ml of H-toxin were 64, 45, 23 and 16%, respectively. However, the ADP-ribosylation of a 38 kDa protein, recently found to be GAPDH [9], was not suppressed. By contrast, any effects on ADP-ribosylation were not observed when the membranes were incubated with 1.25% of DMSO (lanes 1, 4). Furthermore, when the membranes were treated with both DMSO and H-toxin, DMSO did not change the inhibitory effect that H-toxin had on the ADP-ribosylation of several proteins (lanes 1–3). Staphylococcal leukocidin did not suppress endogenous ADP-ribosylation (data not shown). These data suggested that such suppression is derived from the specific activity of H-toxin.

Table 1
Differential counts of HL-60 cells after incubation with H-toxin and dimethylsulfoxide

Treatment	Cell type (%)			
	Promyelocyte	Myelocyte	Meta-myelocyte	Band and Segmented cells
Control	98.4	0.8	0.8	0
H-toxin (2.5 μ g/ml)	96.7	3.3	0	0
DMSO (0.75%)	87.8	12.2	0	0
H-toxin (2.5 μ g/ml) + DMSO (0.75%)	15.1	46.6	32.9	5.5

HL-60 cells were incubated in the presence of the indicated concentration of inducing agent. After 4 days, differential counts were performed in May-Giemsa-stained cytospin preparations of the cell suspensions. The numbers given represent the average of duplicate experiments.

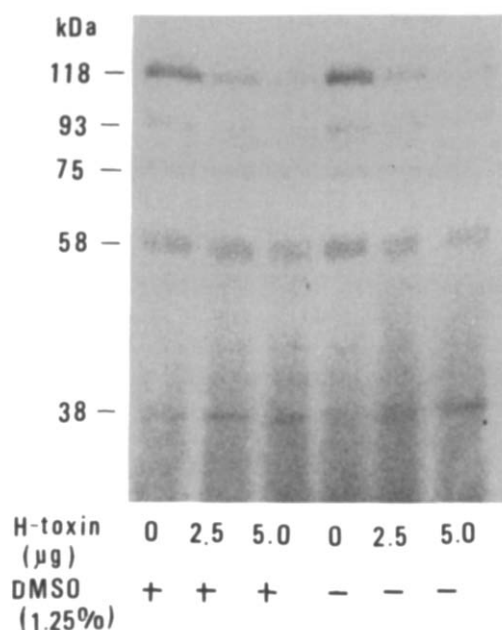


Fig. 2. Effects of H-toxin and/or DMSO on the endogenous ADP-ribosylation of HL-60 cell membrane proteins. ADP-ribosylation assays in the membranes (80 µg protein) were performed as described in section 2 with various concentrations of H-toxin in the presence (lanes 1–3) or absence of 1.25% of DMSO (lanes 4–6) for 60 min at 37°C. ADP-ribosylated proteins were separated by SDS-PAGE and detected by a Bio-imaging analyzer. Similar results were obtained in five other experiments.

3.3. Changes in the endogenous ADP-ribosylation of membrane proteins during differentiation by DMSO

Changes in the endogenous ADP-ribosylation following differentiation by DMSO were investigated. HL-60 cells were treated with 1.25% of DMSO to differentiate into mature myelocytes [15,16]. The cells treated with DMSO for 3 and 6 days were centrifuged and each membrane fraction was prepared, and then ADP-ribosylation reaction was performed as described in section 2. As shown in Fig. 3A, in untreated HL-60 cells, 118, 93, 75, 58 and 38 kDa proteins were mainly ADP-ribosylated. Following differentiation, the ADP-ribosylation of 118, 83, 75 and 58 kDa proteins was decreased. There were time differences in the disappearance of ADP-ribosylation. The ADP-ribosylation of a 58 kDa protein disappeared first and that of a 118 kDa was last. NAD glycohydrolase activity which cleaves NAD into nicotinamide and ADP-ribose is one of the factor to decrease ADP-ribosylation. There is a report that NAD glycohydrolase activity was induced in membranes of retinoic acid-treated HL-60 cells and that cholera or pertussis toxins failed to ADP-ribosylate Gαs to Gαi, respectively, in the membranes [17]. Therefore, NAD glycohydrolase activity in membranes of DMSO-treated cells was examined using [¹⁴C]NAD and degradation of the NAD was analyzed by thin layer chromatography. A newly synthesized product following incubation was identified as ADP-ribose. Rf values of NAD and ADP-ribose was 0.70, 0.38, respectively. NAD glycohydrolase activities in the membranes of HL-60 cells and DMSO treated HL-60 cells were 40 and 4 fmol/min/µg protein, respectively. These data showed that NAD glycohydrolase activity was decreased by the treatment of DMSO, suggesting that the

decrease of ADP-ribosylation in DMSO-treated cells did not depend on the NAD glycohydrolase activity.

The endogenous ADP-ribosylation of the membranes of human PMN was compared to that of HL-60 cells. In the case of PMN, only ADP-ribosylation of a 38 kDa protein was detected and the other ADP-ribosylation, which was found in HL-60 cells, was not observed (Fig. 3B).

3.4. Changes in ADP-ribosylation in the membranes of HL-60 cells differentiated by H-toxin and DMSO

Above data showed that decreases of ADP-ribosylation correlated to differentiation. Then, we examined if decreases of the ADP-ribosylation also occurred in the membranes of differentiated HL-60 cells by both 0.75% of DMSO and 2.5 µg/ml of H-toxin. Membranes were prepared from the cells treated with both 0.75% of DMSO and 2.5 µg/ml H-toxin for 4 days and ADP-ribosylation of the membranes was compared with that of untreated HL-60 cells. As shown in Fig. 4, in the membranes treated with both H-toxin and DMSO, decreases of ADP-ribosylation of 118, 93, 75 and 58 kDa proteins were observed.

4. Discussion

In this report, we showed that *Clostridium septicum* H-toxin promoted the differentiation of HL-60 cells in the presence of DMSO. Our recent results showed that H-toxin also promoted differentiation of HL-60 cells induced by retinoic acid. This

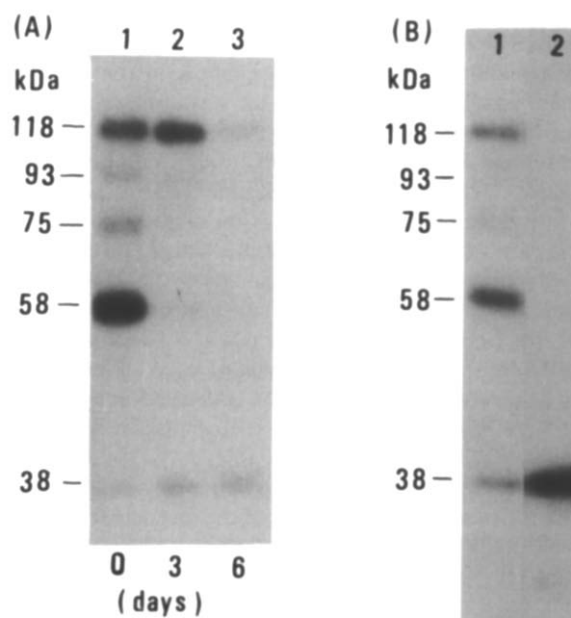


Fig. 3. (A) Changes in the ADP-ribosylation on the various differentiated stages of HL-60 cells. Cells were treated with 1.25% of DMSO for 0 (lane 1), 3 (lane 2) and 6 (lane 3) days and membranes were prepared from each cell culture. 80 µg of each membrane preparation was incubated with [³²P]NAD for 60 min at 37°C as described in section 2. ADP-ribosylated proteins were separated by SDS-PAGE and detected by autoradiography. The autoradiogram was shown. Similar results were obtained in five other experiments. (B) Comparison of the ADP-ribosylation of membranes of HL-60 cells (lane 1) with that of human PMN (lane 2). PMN was prepared from human peripheral blood by Ficol density gradient and membranes were prepared by the same method as HL-60 cells. ADP-ribosylation was assayed and the autoradiogram was shown. This experiment was done twice and the results were almost identical.

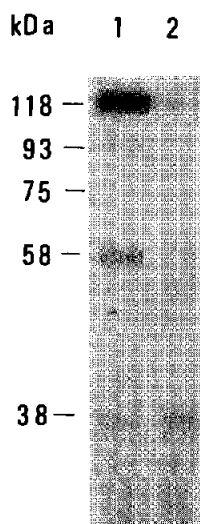


Fig. 4. Changes in the ADP-ribosylation following differentiation by H-toxin in the presence of DMSO. HL-60 cells were treated with 2.5 μ g/ml of H-toxin in the presence of 0.75% of DMSO for 4 days and membranes were prepared. 80 μ g of the membrane preparation was incubated with [32 P]NAD for 60 min at 37°C as described in section 2. ADP-ribosylated proteins were separated by SDS-PAGE and detected by a Bio-imaging analyzer. HL-60 cells (lane 1), HL-60 cells treated with H-toxin and DMSO (lane 2). This experiment was carried out three times and the results were almost identical.

effect did not occur by staphylococcal leukocidin, another cytotoxin to HL-60 cells, suggesting that this effect is specific to H-toxin. One of the biochemical changes in the membrane proteins of HL-60 cells by H-toxin was suppression of endogenous ADP-ribosylation of 118, 93, 75 and 58 kDa proteins and no other toxins including staphylococcal leukocidin did not have such an effect as far as we examined. Furthermore we found that the ADP-ribosylation of those membrane proteins decreased in association with differentiation of HL-60 cells into myeloid pathway and that in human PMN, the ADP-ribosylation was not detected. These results suggested that the suppression of the ADP-ribosylation related to the differentiation and that the suppressive effect of H-toxin of the ADP-ribosylation might be involved in the promotion of DMSO-induced differentiation of HL-60 cells. However, H-toxin itself did not induce differentiation of HL-60 cells in the absence of DMSO, suggesting that suppression of ADP-ribosylation was not enough to commit differentiation. It seems that the first signal which was triggered by DMSO was necessary.

In the HL-60 cell membrane, ADP-ribosylation of a 38 kDa protein was also observed but the extent of the ADP-ribosylation was not decreased following differentiation in contrast to what we found in the case of ADP-ribosylations of 118, 93, 75 and 58 kDa proteins. The 38 kDa protein was identified as

GAPDH and found to be ADP-ribosylated by ADP-ribose or NAD nonenzymatically [9]. It was also reported that NAD itself was covalently bound to GAPDH [10,11]. Therefore, ADP-ribosylation of the 38 kDa protein may not have occurred by endogenous ADP-ribosyltransferase activity and this may be the reason why the extent of the ADP-ribosylation was not decreased.

NAD glycohydrolase activity which cleaves NAD, a substrate of the ADP-ribosyltransferase, did not increase in DMSO-treated cells. Therefore, decrease of ADP-ribosyltransferase activity or that of the amount of acceptor proteins may account for the decreases of the extent of the ADP-ribosylations of 118, 93, 75 and 58 kDa proteins. H-toxin may directly suppress the activity of ADP-ribosyltransferase in the membranes. To clarify this, identification of acceptor proteins and purification of ADP-ribosyltransferases are to be worked on next.

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