Differentiation Inducing Effects of 2-Chloro-3-amino-1,4-naphthoquinone on Human Leukemia HL-60

Kyong Ah BAE* and Se Young CHOUNG
Department of Hygicnic Chemistry, College of Pharmacy, Kyung Hee University, 1 Hoegidong, Dongdaemoon-ku, Seoul 130–701, Korea. Received December 4, 1995; accepted February 2, 1996

There are some highly cytotoxic anticancer compounds inducing differentiation of cancer cells to normal cells at below highly cytotoxic concentration. Naphthoquinone derivatives having cytotoxic effects on cancer cell were tested to learn whether they have differentiation inducing activity in human leukemia HL-60 cells or not. When HL-60 cells were treated with 2-chloro-3-amino-1,4-naphthoquinone (CANQ) for four days, differentiation-related phenotypes such as nitroblue tetrazolium (NBT)-reducing ability and phagocytosis were induced. These differentiation markers were increased by cotreatment with 1,25-dihydroxyvitamin D3, which is a well-known inducer of differentiation in HL-60 cells. To evaluate the route of differentiation induced by CANQ, we examined naphthol AS-D chloroacetate esterase and α-naphthylacetate esterase activities and changes in cellular size and granularity.

Treatment of HL-60 cells with CANQ for four days resulted in an 82.4% increase in α-naphthylacetate esterase activity in spite of a 0.2% increase in naphthol AS-D chloroacetate esterase activity. The size of cells in cell mass was larger and granularity was more decreased than untreated cells. These results indicate that HL-60 cells were induced to differentiate into macrophage-like cells.

Key words 1,4-naphthoquinone; HL-60; differentiation; macrophage

The human promyelocytic leukemia cell line, HL-60,1) has been a useful model to study proliferation and differentiation. HL-60 cells are differentiatively bipotent precursor cells2) which are capable of selectively undergoing either myeloid or monocytic differentiation in response to a variety of agents, including vitamins and their analogues and cytokines.3–9) 12-0-Tetradecanoylphorbol-13-acetate (TPA), 1,25-dihydroxyvitamin D3 (1,25-(OH)2D3), and γ-interferon have been shown to induce the differentiation of HL-60 cells toward macrophages whereas retinoids and dimethyl sulfoxide were able to differentiate the cells toward granulocytes.10,11) It has been reported that protein kinase A (PKA) acts as a positive regulatory signal in the process of HL-60 cell differentiation.12) Anthracycline antibiotics such as marcellomycin and aclacinomycin A that have quinone moeity in their structure are known to induce differentiation of HL-60 at concentrations lower than that required to exert cytotoxic effects on cancer cells.13)

Naphthoquinone (NQ) compounds are known to have various kinds of pharmacological effects such as broad spectrum of antibiotic effects on virus, bacteria and fungus and anticancer activities. They also have physiological functions such as anticoagulating activity.14–17)

Recently, we reported novel NQ compounds that have anticancer activity as well as antibiotic, antifungal and anticoagulating activity.18–20) Some of the NQ compounds inhibited phosphodiesterase (PDE) activity which may result in the increase of cAMP levels and thereby activation of PKA. These findings suggested that the NQ compounds may have differentiation inducing effects in HL-60 cells since it has been reported that PKA acts as a positive regulatory signal in the process of HL-60 cell differentiation.12) Therefore, the present investigation was undertaken to address whether the NQ compounds have differentiation inducing effects in HL-60 cells or not. 1,25-(OH)2D3, a well-known differentiation inducing agent of HL-60 was also tested for comparison.

MATERIALS AND METHODS

Materials RPMI 1640 medium and fetal bovine serum were obtained from Gibco Laboratories (Grand Island, NY), 1,25-(OH)2D3, nitroblue tetrazolium (NBT), N-formyl-Met-Leu-Phe (fMLP), latex beads, penicillin and streptomycin were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture HL-60 cells obtained from the Korean Cell Line Bank (KCLB) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37°C in an atmosphere of 5% CO2. The cells were passaged when they reached a density of approximately 2.0 × 106 cells/ml to a density of 0.2 × 106 cells/ml or 0.1 × 106 cells/ml with fresh medium.

Assay for Growth Inhibition Cells were seeded at 2 × 103 cells/ml and incubated for 4 d with 2-chloro-3-amino-1,4-naphthoquinone (CANQ) and/or 1,25-(OH)2D3 at various concentrations ranging from 10–7 m to 10–10 m. The percentage of growth inhibition was calculated as described.20

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growth \text{ inhibition (\%)} = \left(1 - \frac{T}{C}\right) \times 100
\]

\( T \): number of cells of treated culture
\( C \): number of cells of control culture

Differentiation of HL-60 Human leukemia HL-60 cells (2 × 105 cells/ml) were incubated with CANQ (Fig. 1) and/or 1,25-(OH)2D3 at the final concentration of 10–7 m for 4 d.

1) NBT Reduction Test: The percentage of HL-60 cells capable of reducing NBT was determined by counting the number of cells which contained precipitated formazan particle after the cells were incubated with NBT (1.0 mg/ml)
PBS) for 30 min at 37°C, fMLP (10^{-6} M) was used as stimulator for the formation of formazan. 

2) Phagocytosis: Phagocytic activity was determined by measuring the capacity of the cells to engulf polystyrene latex particles (average diameter: 0.8 μm). HL-60 cells were suspended in serum free medium containing 0.2% latex particles and incubated at 37°C for 4 h. On completion of the incubation, the cells were washed three times with PBS. The cells containing particles were scored as phagocytic cells.

3) Esterase Activity Test: A smear preparation was chemically stained for x-naphthyl acetate esterase and naphthyl AS-D chloroacetate esterase by the standard technique as described previously. 

Changes in Size and Granulation HL-60 cells were incubated with CANQ (10^{-7} M) and/or 1,25-(OH)_{2}D_{3} (10^{-7} M) for 7 d. After incubation, the cells were washed with PBS and assayed with a Facscan analyzer (Becmann Co., Ltd.).

RESULTS

Cell Growth Inhibition The chemical structure of CANQ is shown in Fig. 1. 1,25-(OH)_{2}D_{3} is one of the well-known differentiation inducers of HL-60. In order to measure the growth inhibitory effects of CANQ and 1,25-(OH)_{2}D_{3} on HL-60 cells and to determine whether there is an interaction of CANQ with 1,25-(OH)_{2}D_{3} or not, we compared their growth inhibition effects on the cells separately and in combination. Treatment of HL-60 cells with 1,25-(OH)_{2}D_{3} at the concentration ranging from 10^{-7} M to 10^{-10} M led to dose dependent inhibition of the growth of the cells (Fig. 2). CANQ at a concentration of 10^{-7} M to 10^{-10} M also had an inhibitory effect on the growth rate of the cells in a dose-dependent manner. CANQ exhibited a similar degree of inhibition on HL-60 cell growth as 1,25-(OH)_{2}D_{3} did. When HL-60 cells were treated with CANQ in combination with 1,25-(OH)_{2}D_{3}, the percentage of growth inhibition at a concentration of 10^{-10} M did not increase, but at the range of 10^{-9} M—10^{-7} M it increased approximately two-fold.

NBT Reduction Test During differentiation, cells normally acquire the ability to produce superoxide anion when they are activated by physiological stimuli such as the chemotactic peptide fMLP. HL-60 cells that are differentiated by dimethylsulfoxide treatment appear to produce superoxide anion when stimulated by fMLP. Figure 3 shows the effects of CANQ (10^{-7} M) and/or 1,25-(OH)_{2}D_{3} (10^{-7} M) on the differentiation of HL-60 as measured by the ability of the cells to reduce NBT, when incubated for 4 d. Approximately 12.7% of the untreated HL-60 cells were positive, whereas 42% of HL-60 cells became stainable with NBT after treatment with CANQ (10^{-7} M). When HL-60 cells were treated with 1,25-(OH)_{2}D_{3} (10^{-7} M) alone or with both CANQ (10^{-7} M) and 1,25-(OH)_{2}D_{3} (10^{-7} M), positive cells accounted for 27.3% and 55.1%, respectively (Fig. 3).

Phagocytosis Treatment of HL-60 cells with CANQ (10^{-7} M) and/or 1,25-(OH)_{2}D_{3} (10^{-7} M) for 4 d led to increase of phagocytic activity of the cells. Approximately 10.3% of untreated HL-60 cells were phagocytic cells, whereas in HL-60 cells treated with CANQ (10^{-7} M) or 1,25-(OH)_{2}D_{3} (10^{-7} M) phagocytic cells accounted for 47.4% and 39.9%, respectively (Fig. 4).

Naphthol AS-D Chloroacetate Esterase and x-Naphthyl Acetate Esterase Activities Naphthol AS-D chloroace-
Table 1. Effects of CANQ and/or 1,25-(OH)₂D₃ on the Differentiation of HL-60 Cells

<table>
<thead>
<tr>
<th>Inducer</th>
<th>Naphthol AS-D chloroacetate esterase activity (%)</th>
<th>Naphthylacetate esterase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0 ± 0.0</td>
<td>9.4 ± 0.35</td>
</tr>
<tr>
<td>1,25-(OH)₂D₃</td>
<td>0.0 ± 0.0</td>
<td>58.7 ± 6.65</td>
</tr>
<tr>
<td>CANQ</td>
<td>0.2 ± 0.1</td>
<td>82.4 ± 8.26</td>
</tr>
<tr>
<td>1,25-(OH)₂D₃ + CANQ</td>
<td>0.0 ± 0.0</td>
<td>84.2 ± 6.28</td>
</tr>
</tbody>
</table>

HL-60 cells were cultured for 96 h in the presence of 1,25-(OH)₂D₃ (10⁻⁷ M) and/or CANQ (10⁻⁷ M). Each value represents the mean ± S.E. of three separate experiments. (10⁻⁷ M) and 1,25-(OH)₂D₃ (10⁻⁷ M), they also showed the enlargement in cellular size and less granulation.

DISCUSSION

The application of most currently used anticancer agents has been limited due to their broad side effects, primarily, cytotoxicities to normal cells that block metabolism or signal transduction. Therefore, a therapy which induces differentiation of malignant cells to normal cells other than by killing cancer cells has become one of the new areas of cancer chemotherapy. Leukemia has been considered as the best target for differentiation therapy. Recently some highly cytotoxic anticancer compounds have been reported, for example, anthracycline which induces differentiation of leukemia cells at lower concentration than that having a cytotoxic effect. Thus, these agents can be used in treatment of cancer with lower side effects.

In the present investigation, NQ derivatives which have cytotoxic effects on cancer cells were tested for differentiation inducing activity; they proved to have lower cytotoxic effects on normal LLC-PK1 cells and red blood cells than on cancer cells (data not shown). We attempted to determine whether the compounds have differentiation inducing activity in human leukemia HL-60 using various differentiation markers.

CANQ produced dramatic changes in morphology and in expression of differentiation markers in HL-60 cells: NBT reducing ability, phagocytic activity, and the appearance of N-naphthylacetate esterase activity. The cells treated with CANQ became larger and more hypogranular than untreated control cells. NBT reducing ability and phagocytic activity were also increased by treatment of HL-60 cells with CANQ. CANQ compared favorably with 1,25-(OH)₂D₃ in both these features. N-Naphthyl acetate esterase activity was significantly increased by treatment with CANQ, but not with naphthol AS-D chloroacetate esterase. Most of the cells were found to be differentiated into macrophage-like cells by treatment with CANQ.

Taken together, results demonstrated that NQ is a new inducer of differentiation in human leukemia HL-60 cells causing their change into macrophage-like cells.

At present, the mechanism by which CANQ induces differentiation of HL-60 cells is not known. The effect seems to be related to its inhibitory effect on PDE,
which may lead to an increase of cAMP levels accompanied by the activation of PKA. PKA may thus have a crucial role in the regulation of HL-60 cell differentiation. Further detailed studies concerning the mechanism of action of NQ derivatives on HL-60 cell differentiation are going on.

Acknowledgments We are grateful to Professor Chung Kyu Ryu for the generous supply of 2-chloro-3-amino-1,4-naphthoquinone. This study was supported by a grant from the Korean Science and Engineering Foundation and partly by a grant from Kyung Hee University.

REFERENCES