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Hydroxyl Radical-Mediated Commitment of HL-60 Cells to Differentiation: Modulation of Differentiation Process by Phosphodiesterase Inhibitors

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This report shows that hydroxyl radical, generated by a Fenton reaction involving adenosine 5'-diphosphate/Fe^{2+} complex (5-15 μM) and H_2O_2 (2 μM), induced differentiation of HL-60 cells in a dose- and time-dependent manner. This is evidenced by the increases in 12-O-tetradecanoylphorbol 13-acetate- and fMLP-stimulated superoxide production capability. The cells exposed to hydroxyl radical for defined periods (24～96 hr) continued to differentiate even after the hydroxyl radical generating system had been removed. The differentiated cells displayed fMLP-stimulated calcium mobilization and increased expression of myeloid-specific antigen CD11b and CD14. The extent of the differentiation was markedly reduced by desferrioxamine (100 μM), dimethylthiourea (5 mM), *N,N'*-diphenyl-1,4-phenylenediamine (2 μM), and N-acetyl-L-cysteine (5 mM). The induction of differentiation by hydroxyl radical was enhanced by 3-isobutyl-1-methylxanthine (200 μM) and Ro-20-1724 (8 μM), and inhibited by dipyridamole (2 μM). These results suggest that hydroxyl radicals may induce commitment of HL-60 cells to differentiate into more mature cells of myelomonocytic lineage through specific signal-transduction pathway that is modulated by phosphodiesterase inhibitors.

Key Words: Differentiation, Hydroxyl Radical, Phosphodiesterase Inhibitors, Leukemia, Human

INTRODUCTION

Although reactive oxygen species (ROS) are highly cytotoxic molecules, recent evidences suggest that they may also play a role as important mediators in signal transduction. Growth factors and cytokines have been shown to induce ROS production in several types of cells (Irani et al, 1997; Bae et al, 1997; Sundaresan et al, 1995; Ohba et al, 1994). Among the physiological roles of ROS, reports have suggested the involvement of ROS in the differentiation process of leukemia cells induced by lipopolysaccharide (Kobayashi et al, 1994) platelet-activating factor (Nishihira et al, 1994) and phorbol ester (Yang & Shaio, 1994). These findings raise possibility that these ROS may play a role as a signal- transducing molecule in regulating differentiation.

Few studies, however, have specifically investigated this issue and, although hydroxyl radical, one of ROS, was suggested to induce differentiation in leukemia cells (Nagy et al, 1995; Nagy et al, 1993), only limited information on the maturation process of the cell induced by hydroxyl radical is available for now. It is unclear whether the phenotypic changes of cells in response to the challenge of hydroxyl radical are just a transient adaptation to the condition or results of terminal differentiation. Moreover, it should be clarified whether hydroxyl radical is the only

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species that mediates the differentiation. Hydroxyl radical may cause cellular damage, resulting growth inhibition (Yanagisawa-Shiota et al, 1995). Thus, the cytotoxicity of hydroxyl radical render the growth pattern, which was adopted for differentiation marker in previous studies (Nagy et al, 1995; Nagy et al, 1993), ill-fitted as an ideal parameter in differentiation study.

The present study was designed to fill these gaps currently present in the literature. Especially, to propose hypothesis that the action mechanisms of hydroxyl radical may be physiologic manner, we aimed to study whether differentiation process induced by hydroxyl radical shows the characteristics consistent with the findings of studies with other inducers.

METHODS

Chemicals

Hydrogen peroxide, ferrous sulfate, ADP, adenosine 5'-triphosphate (ATP), N-formyl-Met-Leu-Phe (fMLP), 12-O-tetradecanoylphorbol 13-acetate (TPA), dipyridamole, 3-isobutyl-1-methylxanthine (IBMX), 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol), and FURA 2-AM were purchased from the Sigma Chemical Co (St. Louis, MO). Ro-20-1724 was purchased from Calbiochem-Novabiochem Int. (La Jolla, CA). All other reagents were of the highest purity available.

ADP/Fe^{2+} complex was obtained by combining 10 mM ADP and 1 mM ferrous sulfate in distilled/deionized H_2O and then stirring overnight at 4°C.

Cells

HL-60 cells were purchased from the American Type Culture Collection; passages 20 through 50 after the reception were used for these experiments. Cells ($2\text{-}5 \times 10^5$ cells per ml) were grown in Roswell Park Memorial Institute 1640 (RPMI 1640: GIBCO) medium supplemented with 10% FBS (HyClone), penicillin (50 U/ml), and streptomycin (50 U/ml) and on the experiment day, the medium was replaced by OPTI-MEM® (GIBCO) supplemented with 5% FBS. Substitution of OPTI-MEM® for RPMI 1640 gave identical results. Adding H_2O_2 and ADP/Fe^{2+} complexes to the culture media induced differentiation of the cells. The extent of differentiation was monitored by removing aliquots of the culture at 24 hr intervals for determination of TPA- or fMLP-stimulated superoxide generation capability and reported with Day 0 as the first day of the treatment. Cell counts were determined with a Coulter Counter (Coulter Electronic), and viability was assessed by trypan blue dye exclusion.

Induction of differentiation

Hydroxyl radicals were generated in the cultures medium by adding H_2O_2 (final concentration 2 μM) and freshly prepared Fe^{2+}, in the form of ADP/Fe^{2+} complex, at final concentrations varying from 5 to 15 μM. The ADP/Fe^{2+} complex reacts with H_2O_2 to generate hydroxyl free radicals (Biaglow et al, 1996).

Chemiluminescence assay

Superoxide production of the HL-60 cells was measured by luminol enhanced chemiluminescence recorded at the luminometer (Bio-Rad). Superoxide generating capability provides a sensitive and easily quantitative differentiation marker of leukemia cells, eliminating observer subjectivity (Levy et al, 1990). Cells were washed twice with Dulbeco's phosphate-buffered saline (PBS: 137 mM NaCl / 2.7 mM KCl / 8 mM Na_2HPO_4 / 1.5 mM KH_2PO_4 / 0.9 mM $CaCl_2$ / 0.49 mM $MgCl_2$ / 5.6 mM D-glucose / 0.33 mM sodium pyruvate) containing 0.2% bovine serum albumin (BSA) and were resuspended at 2×10^6 cells per ml. Then 300 μl aliquot of the cells and 100 μl of PBS containing luminol (100 μM) were added in cuvettes of luminometer and allowed to equilibrate for 5 min at 37°C prior to stimulation with TPA (final concentration 3 μM) or fMLP (final concentration 1 μM). Measurement of developing chemiluminescence intensity was performed through 30 min at 37°C and the results were represented on graphs with time units on the abscissa and light intensity units (mV) on the ordinate. The values in voltages of peak height per 1×10^5 viable cells were evaluated and reported as percentages of control values under RESULTS.

FACS analysis for CD11b/CD14b

Cells (1×10^6) were incubated at 4°C for 30 min with 1 μg FITC-conjugated monoclonal antibody, either anti-CD11b or anti-CD14 (Biodesign), diluted with RPMI 1640 supplemented with 5% FBS. The

cells were then washed twice with PBS, and resuspended at 10^6 cells/ml in 2% formaldehyde in PBS. Analysis of fluorescence was performed on a FACStar^{plus} (Becton Dickinson) flow cytometer.

Calcium mobilization

Cells were washed once with PBS containing 0.1% BSA and resuspended to 1×10^6 / ml in the same solution. The cells were incubated with 5 μM FURA-2 AM, together with 0.02% Pluronic F-127 at 37°C for 30 min. After loading, cells were washed to remove extracellular dye. After another 20 min of incubation at 37°C in PBS containing 0.1% BSA to allow intracellular esterase to cleave the ester bonds, the cells were washed and maintained at room temperature until use. Fluorescence was measured by spectrofluorometer (F-4010, HITACHI) using alternating excitation wavelengths of 340 and 380 nm, and an emission wavelength of 510 nm.

RESULTS

Functional changes in HL-60 cells induced by hydroxyl radicals

The TPA-stimulated superoxide production was measured for 5 days after a single treatment with H_2O_2 and various concentrations of ADP/Fe^{2+} (Fig. 1). There were apparent increases of TPA-stimulated superoxide generating capability with time-course from Day 2. The time-course curves showed most steep increases between Day 2 and Day 3, and a plateau at Day 5, except in the lowest dose in which the a peak was shown at Day 3. The degrees of the increase of superoxide generating capability were dependent on the concentration of ADP/Fe^{2+} complex. Viability was between 77% to 93% on Day 5. ATP/Fe^{2+} induced larger increase of TPA-stimulated superoxide generating capability than the same dose of ADP/Fe^{2+} (Fig. 1). As the expression of formylated peptide receptors is a functional characteristic of mature phagocytes (Lloyds & Hallett, 1995), chemiluminescence generation and Ca^{2+} mobilization stimulated by fMLP were also determined (Fig. 2, 3). Compared to the appearances of increase in TPA-stimulated superoxide generating capability, that of fMLP-stimulated showed the delayed time-course. On Day

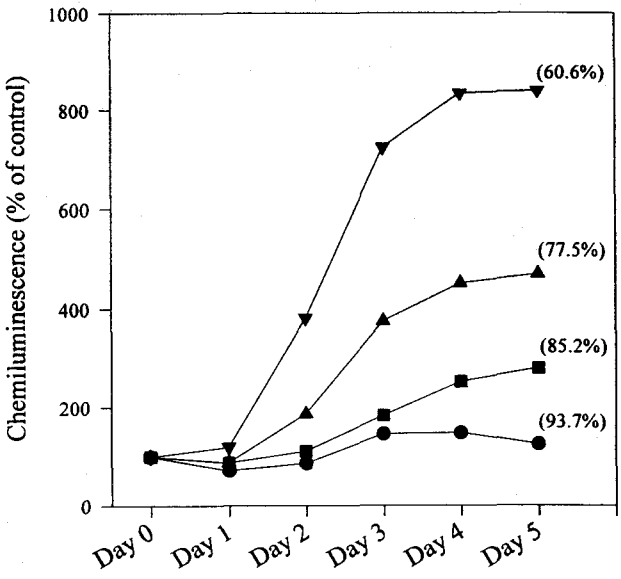

Fig. 1. Maximal generation of luminol-enhanced chemiluminescence stimulated by 2 μM TPA in HL-60 cells during 5 days of culture in media with 2 μM H_2O_2 and variable concentrations of ADP/Fe^{2+} or ATP/Fe^{2+}. The numbers in parentheses are percentages of viable cells on Day 5. ●; 2.5 μM ADP/Fe^{2+}, ■; 5 μM ADP/Fe^{2+}, ▲; 10 μM ADP/Fe^{2+}, ▼; 10 μM ATP/Fe^{2+}.

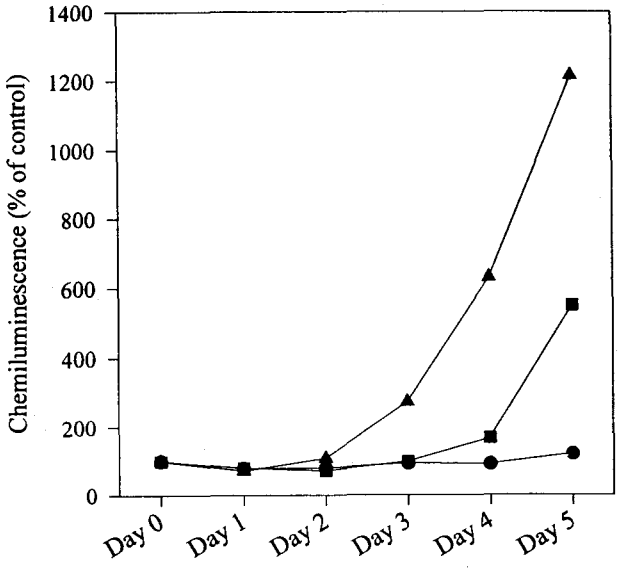

Fig. 2. Maximal generation of luminol-enhanced chemiluminescence stimulated by 1 mM fMLP in HL-60 cells during 5 days of culture in media with 2 μM H_2O_2 and variable concentrations of ADP/Fe^{2+}. ●; 2.5 μM ADP/Fe^{2+}, ■; 5 μM ADP/Fe^{2+}, ▲; 10 μM ADP/Fe^{2+}.

4, cells, treated with 2 μM H_2O_2 and 10 μM ADP/Fe^{2+}, showed intracellular calcium mobilization in response to the challenge with fMLP. In undifferentiated HL-60 cells, calcium mobilization was not induced with this dose. They do, however, respond to ATP (100 μM), which served as a positive control when fMLP-dependent calcium mobilization was not observed. In the next experiments, we used 2 μM H_2O_2 and 10 μM ADP/Fe^{2+} as a hydroxyl radical generating system.

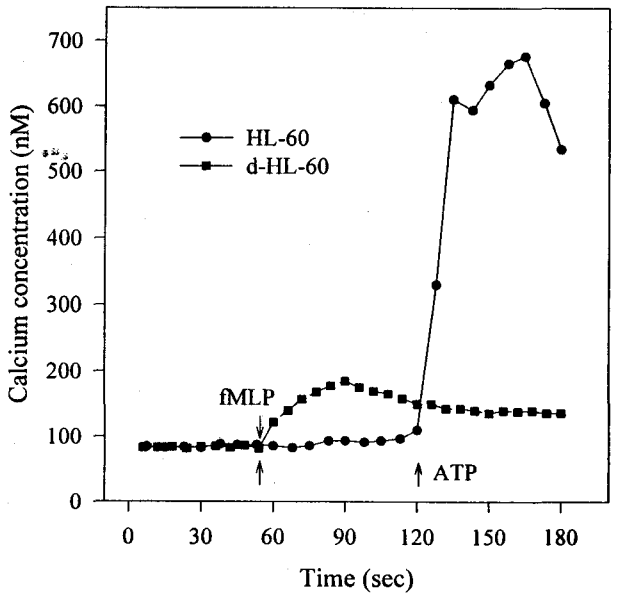

Fig. 3. Intracellular calcium mobilization of cells in response to challenge of fMLP. The cells were cultured in medium supplemented with 10 μM ADP/Fe^{2+} and 2 μM H_2O_2 (d-HL-60) or without the hydroxyl radical generating system (HL-60) for 4 days. ATP was served as a positive control when fMLP-dependent calcium mobilization was not observed. fMLP; 50 nM, ATP; 100 μM.

Increase of CD11b/CD14 cell surface antigen expression

The CD11b and CD14 antigens are markers associated with mature myeloid cell, and their expression is correspondingly low on undifferentiated HL-60 cells (Rosmarin et al, 1989; Simmons et al, 1989). Induction of differentiation in HL-60 cells along with either the monocytic or granulocytic pathway results in an increase in the expression of these antigens, with the increase in CD14 expression being especially characteristic of monocytic differentiation (Simmons et al, 1989). Analysis of CD11b and CD14 antigen was performed with cells which had been exposed to the hydroxyl radical generating system for 48 or 96 hr after a total of 4 days of culturing. FACS analysis revealed increases in the number of CD11b and CD14 antigen positive cells as well as in the levels of expression in an exposing time dependent manner (Fig. 4).

Exposure time necessary for optimal HL-60 differentiation

To determine how long an exposure to hydroxyl radical was required for HL-60 cells to differentiate, we cultured cells in the presence of hydroxyl radical

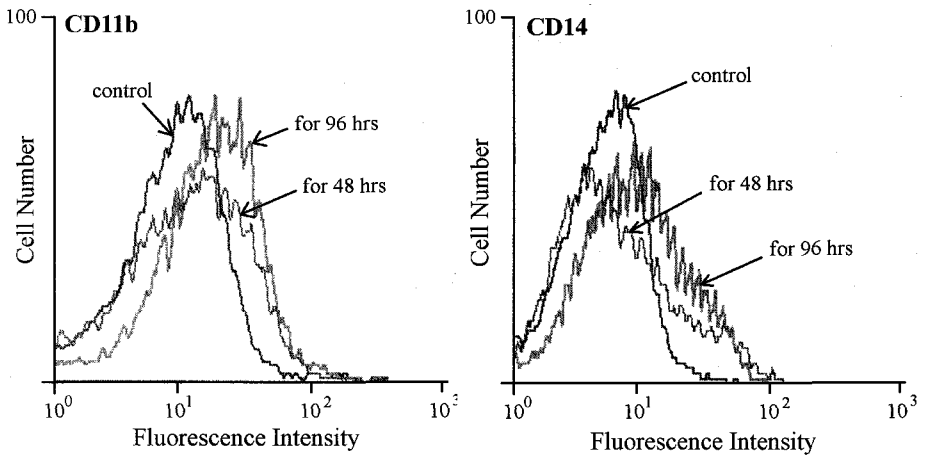

Fig. 4. Representative histograms of FACS analysis of CD antigens in HL-60 cells. The cells were exposed to H_2O_2 (2 μM) and ADP/Fe^{2+} (10 μM) for indicated periods of a total of 4 days culture.

generating system for various time periods. After being exposed to hydroxyl radical, cells were washed and then resuspended in hydroxyl radical generating system-free medium (Fig. 5). Exposure for 24 hr was enough to produce minimal increase in TPA-stimulated superoxide generating capability. These increases were obvious from Day 2, and this is 24 hr after hydroxyl radical generating system was removed. Prolonging the time of exposure up to Day 4, higher increases in oxidative burst capability were observed. The time-course curves revealed that differentiation process of HL-60 cells continued, even after the hydroxyl radical generating system was not present. In cells exposed for 24 hr, a decline was shown after the maximum differentiation. Presumably, this is due to proliferation of uncommitted cell.

Effect of radical scavenger on the differentiation

A reciprocal test of involvement of hydroxyl radical in the differentiation induction is to examine whether a treatment with antioxidant can influence differentiation. After exposure to hydroxyl radical generating system for 24 hr in the presence or absence of various antioxidants and iron chelator, cells were washed and cultured for further 48 hr (Fig. 6). Dimethylthiourea is a hydroxyl radical scavenger (Halliwell & Gutteridge, 1990). N-acetyl-L cysteine and *N,N'*-diphenyl-*p*-phenylenediamine serve as a scavenger for ROS, and N-acetyl-L cysteine is also a precursor for gluthathione. Desferrioxamine, metal-chelating agents to bind iron which otherwise might participate in the Fenton reaction, had been demonstrated to be a scavenger of hydroxyl radical (Halliwell, 1985). They all inhibited the differentiation.

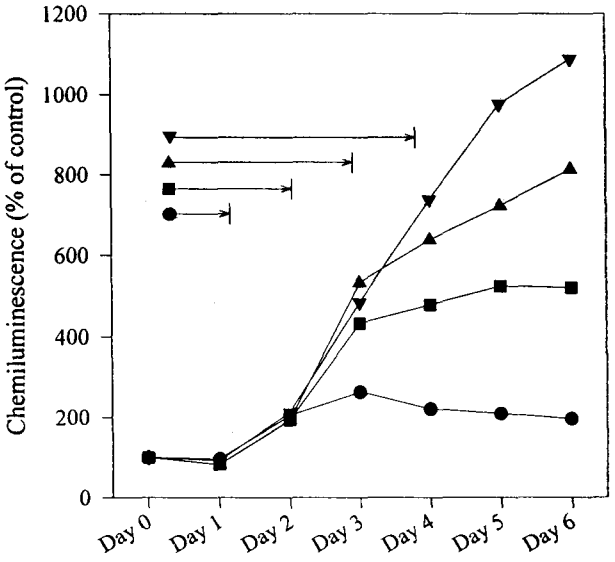

Fig. 5. Maximal generation of luminol-enhanced chemiluminescence stimulated by TPA in HL-60 cells during 6 days of culture. The cells were cultured in medium mixed with H_2O_2 (2 μM) and ADP/Fe^{2+} (10 μM) for various periods indicated by symbol-arrows, washed, and then cultured in succession in medium without the hydroxyl radical generating system.

Reciprocal regulation of the differentiation by phosphodiesterase inhibitors

The induced maturation of HL-60 cells by several inducers (Chaplinski & Niedel, 1982; Boss, 1989; Fontana et al, 1985) is associated with the effects that increase the intracellular cyclic nucleotides. To prove

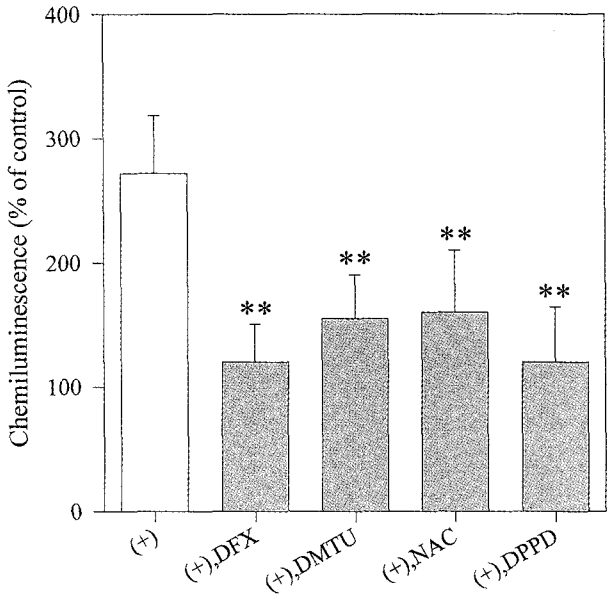

Fig. 6. Effects of various radical scavengers and iron chelator on the increase of chemiluminescence in response to TPA in hydroxyl radical-treated HL-60 cells. The cells were exposed to ADP/Fe^{2+} (10 μM) and H_2O_2 (2 μM), and without (positive control)/with radical scavengers or iron chelator for 1 day, washed, and then cultured without hydroxyl radical generating system for further 2 days. DFX; 100M desferrioxamine, DMTU; 5 mM dimethylthiourea, NAC; 5 mM N-acetyl-L-cysteine, DPPD; 2 μM N,N'-diphenyl-1,4-phenylenediamine. Values are mean standard error. **; $P<0.01$ from positive control.

that this signal-transduction machinery is involved in the hydroxyl radical-induced differentiation mechanism, we studied its effect on the differentiation of phosphodiesterase (PDE) inhibitors. After exposure to hydroxyl radical (1 μM H_2O_2 and 5 μM ADP/Fe^{2+}) for 24 hr in the presence or absence of PDE inhibitors, cells were washed and cultured for further 48 hr (Fig. 7). The study was performed in culture medium supplemented with 2% FBS. A nonspecific inhibitor of PDE, IBMX, and a selective inhibitor of cAMP-specific PDE, Ro-20-1724, combined with hydroxyl radical increased superoxide generating capability to 600% and 800% respectively at Day 2. In the absence of any other agents, Ro-20-1724, IBMX or hydroxyl radical alone increased superoxide generating capability by 130%, 260% and 400% respectively. However, a selective inhibitor of cGMP-specific PDE, dipyridamole, significantly inhibited hydroxyl radical-induced differentiation. Moreover, dipyridamole reversed inhibition of cell growth by hydroxyl radical (data not shown).

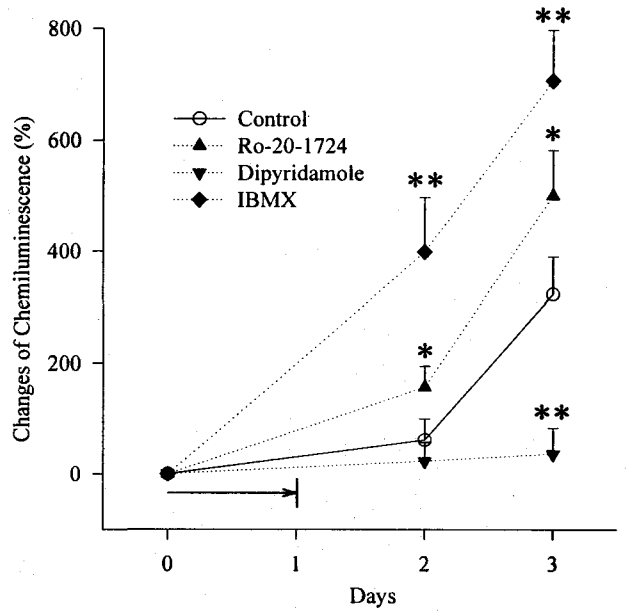

Fig. 7. Effects of phosphodiesterase inhibitors on the increase of chemiluminescence in response to TPA in hydroxyl radical-treated HL-60 cells. The cells were exposed to 2 μM H2O2 and 5 μM ADP/Fe^{2+} with phosphodiesterase inhibitors for 1 day (indicated by arrow), washed, and then cultured in medium for another 2 days without hydroxyl radical producing agents. Ro-20-1724: 8 μM, IBMX: 200 μM dipyridamole: 2 μM. Each point represents mean$\pm$standard error. *:$P<0.05$, **:$P<0.01$ compared to control.

DISCUSSION

In a well-defined and systemic fashion, this study showed that hydroxyl radical triggered HL-60 cells to differentiate into more mature cells of myelomonocyte lineage in a dose- and time-dependent manner. The cells showed normal functional characteristics of mature phagocyte, including respiratory burst, fMLP receptor expression and CD antigen expression. The involvement of hydroxyl radical was clearly demonstrated by showing that the differentiation was inhibited by antioxidants, including hydroxyl radical specific scavenger, and iron chelator.

To study in nontoxic condition, the concentrations, proven to be subcytotoxic to HL-60 (Yanagisawa-Shiota et al, 1995), of hydrogen peroxide was adopted. The high percentage of viable cells eliminated the possibility that the hydroxyl radical-induced differentiation was a result of selective enrichment for differentiated cells.

The extent of differentiation was more dependent on the concentration of ADP/Fe^{2+} complex than H_2O_2. The generation of hydroxyl radical by Fe^{2+} was very rapid (Biaglow et al, 1996). Thus H_2O_2 which was added to culture seems to be consumed within a short period of time. Reducing agents in culture medium (O'Connell et al, 1986) and superoxide (Halliwell & Gutteridge, 1981) produced during the cellular respiration seem to participate in continuous generation of hydroxyl radical by recycling Fe^{3+} to Fe^{2+}, which may be the major contributor in inducing the differentiation.

In present study, we aimed to know hydroxyl radical commit HL-60 cells to myeloid differentiation. Exposure to dimethyl sulfoxide (DMSO) for 12 hr is the minimal requirement to promote a stochastic commitment to terminal differentiation in a fraction of HL-60cells (Tarella et al, 1982). However, 72 hr of a latent period occurs between induction by DMSO and appearance of the earliest functions (Tarella et al, 1982). Our study demonstrates that 24 hr-exposure to hydroxyl radical was sufficient to induce detectable increase in TPA-stimulated superoxide generating capability. 48 hr of latent period was noted between induction period by hydroxyl radical generating system and the appearance of differentiated function. The expressions of functional differentiation markers appeared from Day 2, even after the hydroxyl radical generating system had been removed. This helps distinguish two sequential events.

The first event is transition from an uncommitted to a committed state, which is driven by hydroxyl radical, and then the expression of a differentiated phenotype. This second event probably does not require hydroxyl radical.

Although the involvement of hydroxyl radical in the differentiation of leukemia cells has been suggested (Nishihira et al, 1994; Nagy et al, 1993; Nagy et al, 1995), there is no evidence that prove the relationship between hydroxyl radical and known signal-transduction pathways. Several investigators have demonstrated that agents that are known to increase intracellular cAMP levels induce the differentiation of HL-60 cells (Chaplinski & Niedel, 1982; Olsson et al, 1982). In addition, these agents interact with retinoids and DMSO in a synergistic fashion in the induction of differentiation (Chaplinski & Niedel, 1982; Fontana et al, 1985; Olsson et al, 1982). Exposure of HL-60 cells to retinoic acid and dimethyl formamide result in the elevation of cAMP-dependent protein kinase with phosphorylation of specific substrates during the differentiation pathway (Fontana et al, 1984). Thus cAMP may be a common mediator in response to chemically dissimilar inducers (Chaplinski & Niedel, 1982; Fontana et al, 1985). In the present study, we showed that hydroxyl radical-induced differentiation was potentiated with a nonspecific PDE inhibitor and a selective cAMP-specific PDE inhibitor. IBMX is more effective than Ro-20-1724, which is consistent with the previous finding that elevation of both cAMP and cGMP above a critical level is necessary for inducing HL-60 cell differentiation (Bang et al, 1994). Although, cGMP signal transduction pathway involved in inducing differentiation of HL-60 cells, dipyridamole, a selective inhibitor of cGMP-specific PDE, significantly inhibited hydroxyl radical-induced differentiation. A possible explanation is free radical scavenging effect of dipyridamole (De La Cruz et al, 1994).

It is difficult to extrapolate our results to speculations regarding the role of hydroxyl radical in vivo, because nearly all the iron is located in enzymes, heam-containing proteins, or in specific iron-binding proteins such as transferrin, ferritin, and lactoferrin. These protein-bound irons are known to be incapable of catalyzing hydroxyl radical formation. However, 'transit pool' of intracellular iron, in which iron attached to phosphate ester (such as ATP, ADP, or GTP), is redox-active (Biaglow et al, 1996). Thus, hydrogen peroxide formed intracellularly by growth factors or cytokines (Irani et al, 1997; Bae et al, 1997; Sundaresan et al, 1995; Ohba et al, 1994) may react with redox-active iron to produce hydroxyl radical.

In summary, we first described following features which suggest that hydroxyl radical induces differentiation of HL-60 cells in very sophisticated manner: (Irani et al, 1997) a clear relationship between a dose, exposure time of inducer and the extent of differentiation; (Bae et al, 1997) a latent period and commitment of cells to terminal differentiation; (Sundaresan et al, 1995) expression of normal functional phenotypes of mature phagocytes; (Ohba et al, 1994) synergistic interaction with PDE inhibitors. We propose that hydroxyl radical initiate physiologic differentiation process through specific signaling pathway that is shared by some other differentiation inducers rather than by randomly altering molecular conformation, causing intermolecular cross linking which is proposed by previous studies (Nagy et al, 1993; Nagy et al, 1995).

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