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Induction to Granulocytic Differentiation of Human Promyelocytic Leukemia Cells (HL-60) by Polyprenoic Acid (E5166)

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Abstract The effects of synthesized polyprenoic acid, E5166, on human promyelocytic leukemia cell line, HL-60, were examined. The cells continuously exposed to the drug in vitro underwent growth inhibition and granulocytic differentiation. In the culture treated with E5166, the cell cycle progression was markedly inhibited and an accumulation of the cells in G1/0 phase was apparent on day 4 when a small number of granulocyte first appeared. Subsequently, the percentage of differentiated cells in the culture increased continuously with the time of treatment and on day 7, 80% of cells acquired the morphological characteristics of mature granulocytes. The close relationship between cell kinetics and differentiation was suggested, although the mechanisms by which E5166 induces differentiation and exerts its antiproliferative effect on HL-60 are unknown.

Key Words : Differentiation, Leukemia, Polyprenoic acid, Flow cytometry, BrdUrd, Cell cycle

Introduction

Cytotoxic drugs are effective for several malignant tumors, while many tumors respond poorly to these agents. The hypothesis that the genetic transformation producing cancer cells results in dedifferentiation and a loss of growth regulation of the cells is widely accepted. In this context, as on alternative approach to the use of cytodestructive drugs for cancer therapy, the development of therapeutic agents to convert the malignant cells to more mature form with no proliferative capability is awaited with great interest. Most clinical trials of differentiation inducers have been privented because of their toxicity although the potency of retinoic acid for clinical use has been suggested.

Vitamin A and retinoids are involved in the modulation of growth and granulocytic differentiation of HL-60 cell[1,2,3]. Now, many vitamin A analogues have been synthesized with the aim of clinical application, especially for cancer treatment. A polyprenoic acid derivative, all trans-3,7, 11,15-tetramethy-2,4,6,10,14-hexadecapentaenoic acid (E5166) which was

recently synthesized[4]suppresses the process of chemical carcinogenesis of the liver in vivo[5]and inhibits cell proliferation in vitro[6]. However, little is known about the effect of the drug on cell differentiation and the relationship between cell proliferation and cell differentiation induced by this agent. The human promyelocytic leukemia cell line HL-60 is a useful system for biological and molecular analysis of myeloid differentiation and for investigating the effects of the drugs on cellular differentiation, because it undergoes differentiation to granulocytes or monocytes-macrophages in response to various compounds such as dimetyl sulfoxide (DMSO), 12-*o*-tetradecanoyl phorbol-13-acetate (TPA) and 1, 25-dihydroxyvitamin D3[7,8]. We have examined the effects of the synthesized polyprenoic acid, E5166, on cellular differentiation. In this report we show that exposure of HL-60 cells to E5166 results in differentiation into cells which closely resemble neutrophils. Furthermore, we attempt to explore the relationship between induction of granulocytic differentiation and inhibition of cell growth in terms of cell cycle kinetics.

Materials and Methods

Cell Culture : The HL-60 cells purchased from Flow Laboratoty (U.S.A.) were maintained in RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated fetal calf serum (Flow Lab., U.S.A.) at 37℃ in a humidified atomosphere of 5% CO_2 in air. The cells were subcultured in 55㎠ Falcon dishes (Falcon Plastics, U.S.A.) every 7 days. Cells were counted and sized with a Coulter counter (Coulter Electronics, U. S. A.).

Treatment with Polyprenoic Acid (E5166) : Polyprenoic acid, E5166, was generously given from Eisai Co., Ltd. (Tokyo). The drug was prepared fresh immediately prior to use ; 30 mg E5166 was dissolved in 1 ml of DMSO and 3 ml of 99% ethanol and it was added to the culture to get a desired concentration (final concentration was 7.5 μg/ml unless stated otherwise). HL-60 cells were exposed to the agent up to for 7 days, and samples were taken every 24 hours for examination of morphology and cell kinetics.

Measurement of DNA Content : The cells were stained with propidium iodide (PI, Calbiochem-Bering Co., U.S.A.) by the method previously described[6]. Briefly, the cells were centrifuged and suspended in 2 ml of phosphate buffered saline (PBS) containing 0.1% Triton X-100 and 0.1% RNase. PI was added into the suspension to get a final concentration of 50 μg/ml. Prior to flow cytometric measurment, the suspension was filtered to remove cell clumps by 43 μm nylon mesh. The fluorescence from PI was measured using FACS Analyzer (Becton Dickinson Co., U.S.A.) in a linear scale mode. The stained HL-60 cells were excited at a 485-nm line from a mercury arc lamp and red fluorescence was collected through a 580-nm long-pass filter. Routinely, 1×10^4 cells were measured and 256-channel histograms were generated as DNA content distributions. A histogram analysis was perfromed by using a computer program[9].

Cell Cycle Analysis : To estimate the durations of the cell cycle (Tc) and S phase (Ts), FLSm (Fraction of Labeled mid S phase cells) method was employed as described previously[10]. Briefly, HL-60 cells exposed to E5166 for 3 days or unexposed to the drug were labeled with 15 μM bromodeoxyuridine (BrdUrd, Sigma Chemical Co., U.S.A.) at 37℃. Ten minutes later, the cells were wahed with a warm medium, recultured in the BrdUrd-free and E5166 contained medium and taken every 1 to 2 hours up to 38 hours. The cells fixed in 70% ethanol were treated with 4N HCl at 20℃ for 20 minutes. FITC staining was carried out by an indirect immunofluorescence technique using monoclonal anti-BrdUrd antibody (Becton Dickinson Co., U.S.A.) and FITC conjugated anti-BrdUrd antibody (Cappel Lab., U.S.A.). Furthermore, staining of DNA was performed with 5 μg/ml PI. For control, the unlabeled cells were also stained by the same manner as the cells labeled with BrdUrd. Tc and Ts can be determined from a FLSm curve which were generated by plotting the percentage of BrdUrd labeled cells in a narrow window defined in the mid S phase of the DNA hitogram against the time after labeling. Labeling index (LI) was calculated by the following equation,

LI=the number of labeled cells in the window/the number of cells in the window×100(%).

Results

Morphological Alteration in Cells Exposed to E5166 : The culture was sampled daily for observation of morphological changes and flow cytometry. No differentiated cells were recognizable until the 3rd day after addition of E5166 into the medium. On day 4 a small number of the cells with morphological characteristics of neutrophils first appeared in the culture exposed to the drug (Fig. 1). Subsequently, the proportion of granulocytic differentiated cells rapidly increased and reached to more than 50% on day 7 (Table I). In contrast, the differentiated cells were seldom found in the control cultures even after the culture for 7 days ; most of untreated cells (over 90%) showed promyelocytic or blastic features.

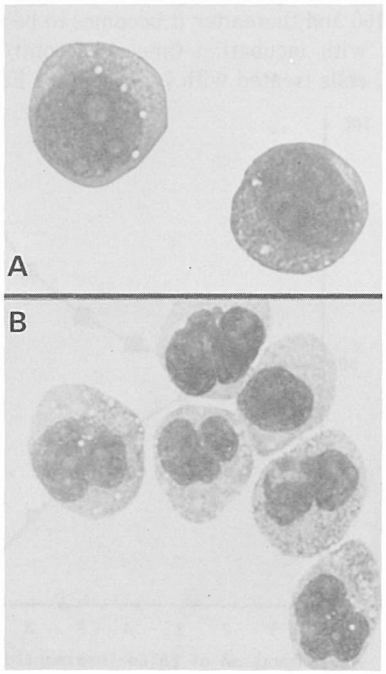

Fig. 1 Promyelocytic leukemia cells (HL-60) cultured in normal medium (A). The cells exposed to E5166 for 7 days show morphological chracteristics of mature granulocytes (B). Wright-Giemsa stain, x1000.

Cell Volume : Cell volume measured by Coulter counter decreased rapidly with the duration of the treatment with E5166 even when neutrophilic differentiation was not yet demonstrated morphologically (Fig. 2). On day 6, the cells exposed to the drug were about half of the control cells in size. The untreated cells also decreased slightly in size during the 6-day culture.

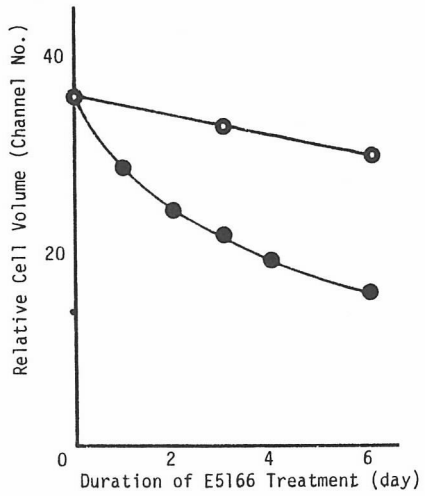

Fig. 2 HL-60 cells treated with E5166 rapidly decrease in size (●) and on day 6 the cell size is falf of the cotrol (○).

Cell kinetics : For the control cells, the doubling time was about 29 hours under the culture condition in our laboratory. This experiment was carried out using exponentially growing cells. The cell proliferation was retarded by the treatment with E5166. This appeared to be dose-dependent (Fig. 3). The inhibition of cell growth was not recognized during the first day of the treatment, but after exposure to the drug for 2 days the increase in cell number became to be delayed (Fig. 4). The retardation of cell proliferation appeared after only one or two doubling in cell number. The alteration of the cell cycle compartments determined from FCM also occurred in tandem with the delay of cell proliferation during the culture in E5166. The exposure to the agent resulted in the increase in the fraction of G1/0 phase

associated with reduction of the S phase fraction. An G1/0 accumulation of the cells increased with time after treatment with the drug and the proportion of G1/0 phase cells reached 90% of the cells on day 7 (Fig. 5). In contrast, the compartments of the cell cycle for the control culture was virtually maintained at a level of the start of the experiment.

Tc and Ts can be estimated graphically from FLSm curves generated by plotting labeling indices in the mid S phase portion against the time after BrdUrd labeling, described previously [10]. For the control cells in exponentially growing condition, Tc and Ts were 20 and 12 hours, respectively (Fig. 6).

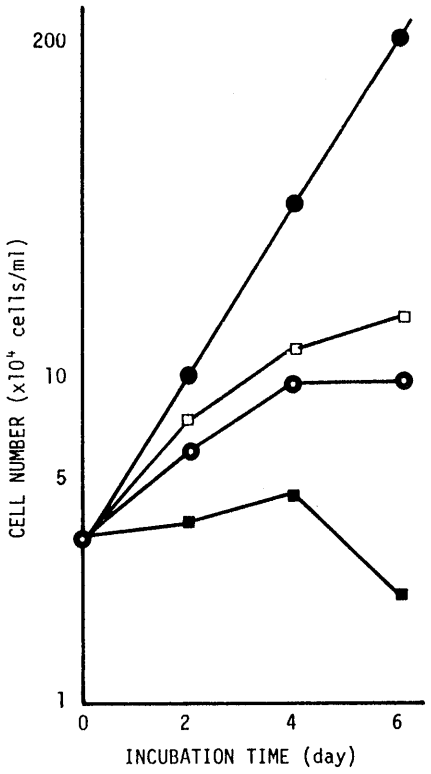

Fig. 3 The control cells proliferate in exponential manner with doubling time of 29 hours (●). Retardation of cell proliferation is induced by treatment with E5166. Concentration of E5166 : □ ; 4 μg/ml, ○ ; 8 μg/ml, ■ ; 16 μg/ml.

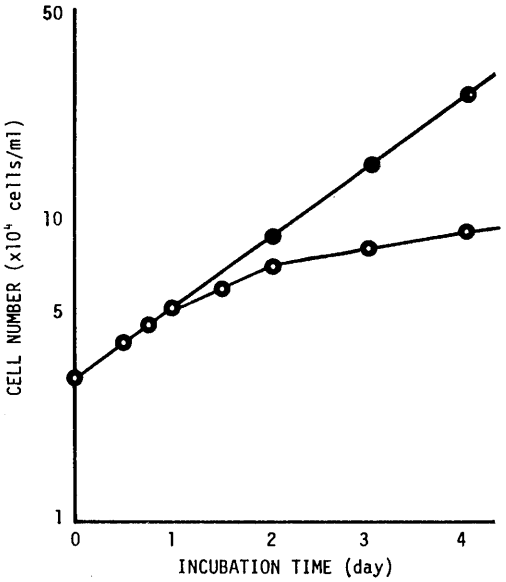

Fig. 4 Retardation of cell growth is not evident until the 2nd day of treatment with E5166 and thereafter it becomes to be apparent with incubation time. ● ; control and ○ ; cells treated with 7.5 μg/ml of E5166.

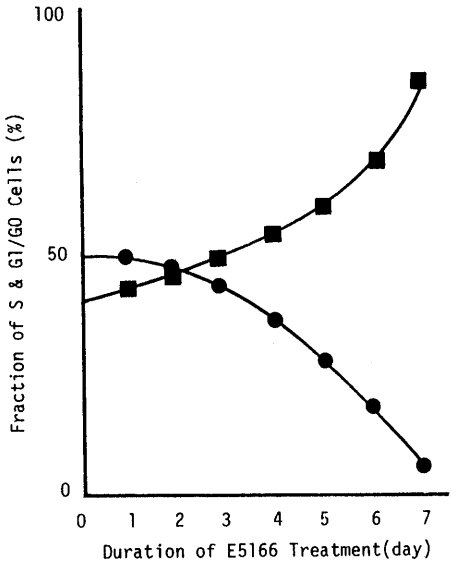

Fig. 5 Decrease in S fraction (●) and increase in G1/0 fraction (■) which are determined by FCM become evident with the duration of E5166 treatment. On day 7, the percentage of G1/0 fraction reaches 90% of cells.

In contrast, for the cells treated with E5166 for 3 days prior to the labeling with BrdUrd no second peak appeared. For this interval, the cells passed at least two division cycle. Until on day 2 no difference in proliferation rate was evident between treated and control cultures, suggesting that cell growth rate was initially unaffected (Fig. 4). In the cultures treated with E5166, the cells with neutrophilic features were first recognized on day 4 when marked perturbation of the cell cycle progression and considerable G1/0 accumulation of the treated cells had been already demonstrated.

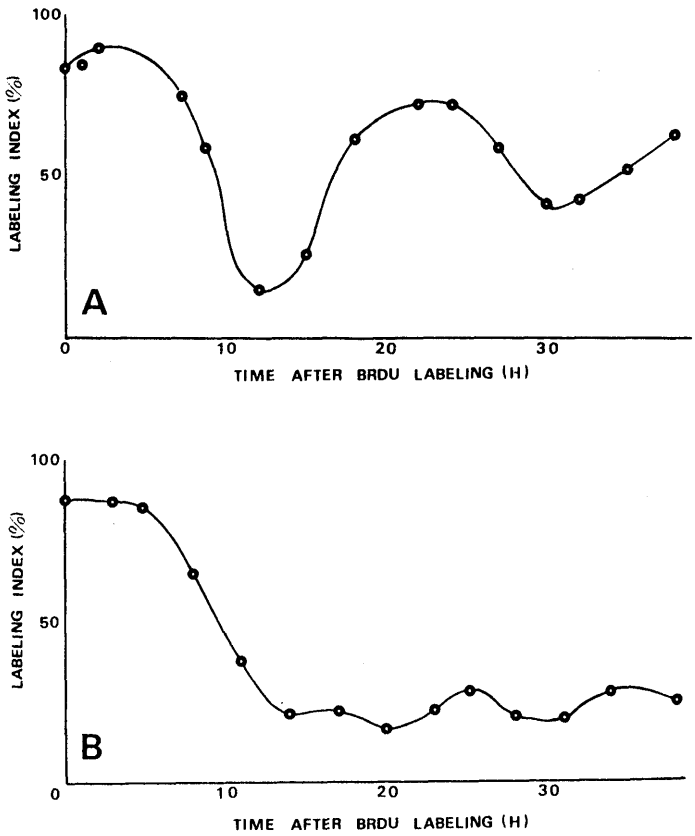

Fig. 6 FLSm curves of the control (A) and treated (B) cells. Graphic extractions of the cell cycle paramenters for control cells. Tc is the time from the 50% of labeling index on the descending part of the first wave to the 50% of labeling index on the descending portion of the next wave and Ts is the between 50%-points of labeling index on the second wave. Tc and Ts are 20 and 12 hours, respectively. On the other hand, the second peak is not demonstrated in FLSm curve of the cells treated with the drug for 3 days, suggesting no re-entry of the labeled cells into S phase.

Discussion

It is well-known that HL-60 cells have a dual capability to differentiate into either granulocyted or monocytes, according to the mode of simulation. Vitamin A analogues induce the cells to differentiate to morphologically mature granulocytes [7,8]. As might be be expected, HL-60 cells exposed to the synthesized polyprenoic acid, E5166, underwent marked inhibition of cell growth and granulocytic differentiation. With the addition of the drug, the rate of cell growth decreased apparently until day 2 and after this time G1/0 accumulation of cells became definite. The differentiated myeloid cells similar to neutrophils first appeared on day 4 and subsequently the proportion of differentiated cells rapidly increased with the incubation time. It is difficult to determine the precise period necessary for commitment to undergo granulocytic differentiation in this experiment with E5166. Between the initiation of E5166 treatment and the appearance of the differentiated granulocytes, however, there is a 4-day lagperiod corresponding to the interval for which the treated cells traverse at least 2 division cycle. This may be due to the reason why the "switch" into the differentiation pathway occurs during a restricted stage of the cell cycle [11]. It is also possible to consider an alternative explanation that as part of the cellular repsonse to this compound considerable time is necessary to generate more critical cellular components, e. g., "receptors", which induce alterations in the cell nucleus for induction of differentiation [14,15].

In the chase experiment using BrdUrd, FLSm curve obtained from the consecutive FCM DNA-BrdUrd bivariate distributions provided detail informations about the progression of the cell cycle[10]. In contrast to the control culture, there was no second peak of the FLSm curve in the cells exposed to E5166 for 3 days prior to BrdUrd pulse labeling. The S phase cells can complete DNA synthesis and traverse the cell cycle form G2M to G1/0 phases at almost the same rate as the control until day 3 since the growth rate of the cells is not different from the control cultures for this period. After this time, however, the labeled cells which arrive in G1/0 phase can no longer enter another S phase. Thus, the cells exposed to E5166 for longer than 4 day pile up in G1/0 successively after cell division. On day 4, although the G1 phase cells were counted more than 70%, differentiated cells were only about 10% of cell population. These results imply that an appearance of differentiated granulocytes is preceded by G1/0 accumulation. It is also likely that the cells, once committed by E5166 to differentiation, are arrested at G1/0 phase, not at random throughout the cycle. Boyd and Metcalf demonstrated that all differentiated cells induced by sodium butyrate were also arrested in G1/0 and that most cells completed one division in the presence of butyrate and sometime several division before loss of proliferative potential [11]. Significant terminal cell differentiation occurs only after a minimum exposure to inducer of two division cycles [8,13]. On the other hand, Ferrero and his colleagues reported that entire process of granulocytic differentiation did not require cell division and that DNA synthesis and induced maturation was independent events [12]. Granulocytic differentiation of HL-60 cells induced by E5166 is closely related to cell kinetics, although the possibility cannot be exluded that growth inhibiton simply reflect the cell differentiation by E5166.

It would be worthwhile to begin the experiments for therapeutic trial of malignant tumor with this agent since antineoplastic and differentiation capabilitites of the agent are demonstrated in this experiment.

Acknowledgements

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