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Enhancement by retinoid of hemin-induced differentiation of human leukemia K562 cell line

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The effect of retinoid on human leukemia K562 cell differentiation induced by hemin was examined. Retinoids (retinoic acid and synthetic retinoids [Am80 and Ch55]) dose-dependently enhanced hemin-induced erythroid differentiation of K562 cells, though these retinoids themselves did not induce the differentiation. Under optimal conditions, these retinoids caused a doubling of the population of hemin-induced differentiated cells. In addition, co-treatment of cells with hemin and retinoid led to longer maintenance of the differentiated state after the removal of hemin, which might imply aquisition of irreversibility of hemin-induced differentiation. These results suggest that the combination of retinoids with other differentiation inducers might be useful for leukemia therapy in cases where the leukemic cells are poorly responsive or unresponsive to retinoids, alone.

Hemin; K562 cell line; Retinoid; Differentiation

1. INTRODUCTION

Retinoids, namely natural and synthetic bioisosteres of retinoic acid (RA), have been shown to exhibit a variety of effects on the growth and differentiation of both normal and tumor cells [1]. Biological activities elicited by retinoids are believed to be based on the activation of their nuclear receptors, retinoic acid receptors (RARα, -β and -γ), which regulate specific gene expression. From a clinical standpoint, the differentiation-inducing activity of retinoids on acute promyeloid leukemia (APL) cells from both patients and cell lines is of particular interest [2], and has been applied to the treatment of APL [3–5]. However, in spite of the wide-ranging biological activities of retinoids, their clinical utilization is restricted at present to APL therapy.

The K562 cell line was established by Lozzio and Lozzio from a patient with chronic myeloid leukemia (CML) in terminal blast crisis [6] and this cell line can be induced to undergo erythroid differentiation by various compounds, including hemin [7]. However, it is not responsive to retinoids, though the cells do contain RARs [8].

To explore the therapeutic potential of retinoids in some types of leukemia other than APL, in this study, we have investigated the effects of retinoids on hemin-induced differentiation of K562 cell line. As retinoids, we chose retinoic acid (RA) and two synthetic retinoids (4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenylcarbamoyl)-benzoic acid, Am80 [9] and (E)-4-[3-(3,5-di-*tert*-butylphenyl)-3-oxo-1-propenyl]benzoic acid, Ch55 [10]; structures shown in Fig. 3). Both Am80 and Ch55 are potent retinoids which show higher activity than RA in human leukemia HL-60 cell differentiation-induction assay [9–12].

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Abbreviations: Am80, 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenylcarbamoyl)benzoic acid; Ch55, (E)-4-[3-(3,5-di-*tert*-butylphenyl)-3-oxo-1-propenyl]benzoic acid; RA, all-*trans*-retinoic acid; APL, acute promyeloid leukemia.

2. MATERIALS AND METHODS

2.1. *Cells and culture conditions*

The K562 cell line used in this work, which was provided by Prof. Takashi Tsuruo (Institute of Molecular and Cellular Biosciences, University of Tokyo), was maintained in RPMI 1640 supplemented with 5% fetal bovine serum. The cultures were incubated at 37°C in a 5% CO_2 humidified incubator. They were subcultured every 2–4 days to maintain them in a state of logarithmic growth.

2.2 *Chemicals*

Solutions of RA, Am80 and Ch55 were prepared by dissolving each drug in absolute ethanol. Hemin was dissolved a mixture of ethanol and pyridine (4:1, v/v). Addition of drugs to cell cultures was performed so as to make the final concentration of organic solvent no higher than 0.5% v/v.

2.3. *Assay of erythroid differentiation*

K562 cells (4×10^4/ml) were incubated with hemin in the presence or absence of a retinoid for 4 days. The erythroid differentiation of the cells was scored by benzidine staining according to the procedure reported by Cooper et al. [13].

2.4. *Method of drug removal*

The incubated cells were pelleted by low-speed centifugation (750 rpm) and washed twice with PBS. The washed cell pellet was resuspended in a new medium and reincubated for 4 days, followed by benzidine staining.

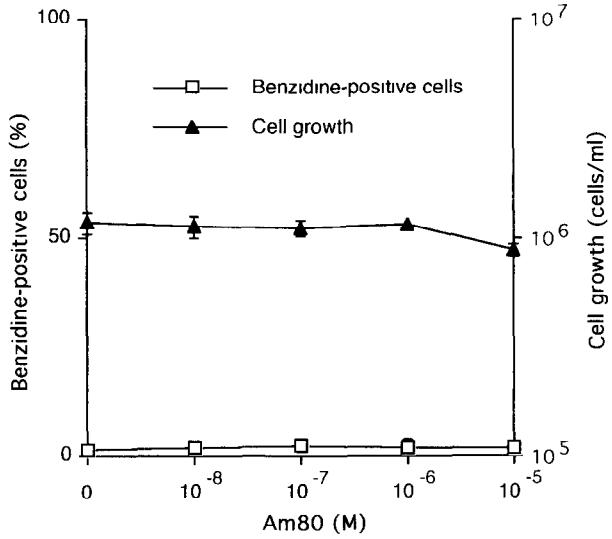

Fig. 1. Effect of Am80 on K562 cell growth, and differentiation-inducing ability of Am80. K562 cells (4×10^4/ml) were incubated for 4 days in the presence of the indicated concentration of Am80, and then counted and assayed for erythroid differentiation by benzidine staining. The total cell number (▲) (right), and the percentage of benzidine-positive cells (□) (left) are shown.

3. RESULTS

3.1. *Effect of Am80 on K562 cell growth and its differentiability*

The effects of Am80 on K562 cell growth and on the inducibility of K562 cell differentiation are shown in Fig. 1. Am80 itself did not inhibit K562 cell growth at concentrations of up to 1 μM. This result is consistent with the very low toxicity of the compound [9,11,12]. Further, Am80 possessed no differentiation-inducing activity on K562 cells at concentrations up to 10 μM. RA and Ch55 also failed to induce K562 cell differentiation (data not shown). Therefore, it can be concluded that the retinoids had no effect on K562 cell growth/differentiation in the concentration ranges examined.

3.2. *Effect of Am80 on K562 cell differentiation induction by hemin*

Though Am80 itself had no ability to induce erythroid differentiation of K562 cells, it dramatically augmented the population of hemin-induced benzidine-positive K562 cells (Fig. 2). The benzidine-positive cells induced from K562 cells by co-treatment with hemin and Am80 were microscopically indistinguishable from those induced by treatment with hemin alone. This enhancement of hemin-induced K562 cell differentiation by Am80 could be observed at Am80 concentrations as low as 10 nM and was dose-dependent up to 1 μM. The maximum augmentation was observed at 1 μM Am80 and 2.5 μM hemin, and amounted to approximately 200%. It was observed that when the hemin concentration was comparatively low, Am80 augmented K562 cell differentiation more efficiently.

3.3. *Effect of retinoids other than Am80*

We also evaluated the ability of RA and Ch55 to augment hemin-induced K562 cell differentiation (Fig. 3). As we had expected, RA and Ch55 augmented hemin-induced differentiation of K562 cells as effectively as Am80. The concentration giving approximately 100% augmentation of the hemin-induced differentiation was 1 μM for RA and Am80, and 100 nM for Ch55. This order of potency is in good coincidence with that of their activity to induce HL-60 cell differentiation and that of their binding affinity toward RARα/-β [9–12].

3.4. *Effect of retinoid on maintenance of the differentiated state of K562 cells*

The induction of K562 cell differentiation by hemin is reversible [14], so it was of interest to investigate the effect of Am80 on the kinetics of hemin-induced differentiation. K562 cells were exposed to 10 μM hemin in the presence or absence of 1 μM Am80 for 4 days. The cells were then washed, resuspended in fresh medium with or without Am80, and assayed for benzidine-positive cells after reincubation for 4 days. After the removal of hemin, the majority of the cells differentiated by hemin alone reverted to the undifferentiated state, as reported [14]. On the other hand, approximately 50% of those differentiated by hemin in the presence of Am80 retained their differentiated state, whether Am80 was contained in the reincubation medium or not (Fig. 4). Consequently, co-treatment of cells with hemin and Am80 resulted in longer maintenance of the differentiated state after the removal of hemin.

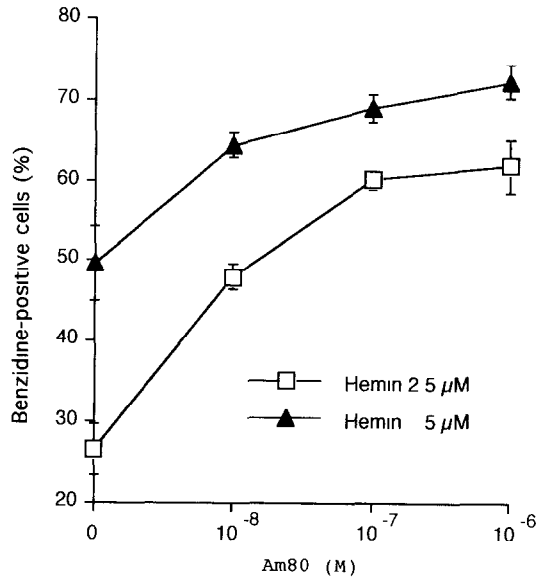

Fig. 2. Effect of Am80 on hemin-induced K562 cell differentiation. K562 cells (4×10^4/ml) were incubated for 4 days in the presence of 2.5 μM (□) or 5 μM (▲) hemin with the indicated concentration of Am80, followed by assay of benzidine staining. The percentage of benzidine-positive cells is shown.

4. DISCUSSION

In this work, we examined the effect of retinoids on hemin-induced differentiation of the K562 cell line. We found that retinoids augmented the differentiation of K562 cells induced by hemin, though the retinoids themselves are neither differentiation-inducing nor toxic toward K562 cells. In other words, hemin conferred upon K562 cells a retinoid-responsiveness as regards erythroid differentiation. This ability to augment hemin-induced differentiation of K562 cells seems to be a general property of retinoids because RA and Ch55 showed similar effects to Am80. Considering the existence of RARα and -β in K562 cells [8] and the established selectivity of these retinoids for retinoid-binding proteins [11,12], i.e. (1) Am80 and Ch55 are RAR-specific (no binding to retinoid × receptors (RXRs)), (2) Ch55 does not bind CRABP, and (3) Am80 is RARα/-β-selective (no binding to RARγ), the effects of these retinoids are presumably elicited by the activation of RARα and/or RARβ. The order of potency of these retinoids to augment the hemin-induced K562 cell differentiation is the same as that of their affinity for RARα/-β.

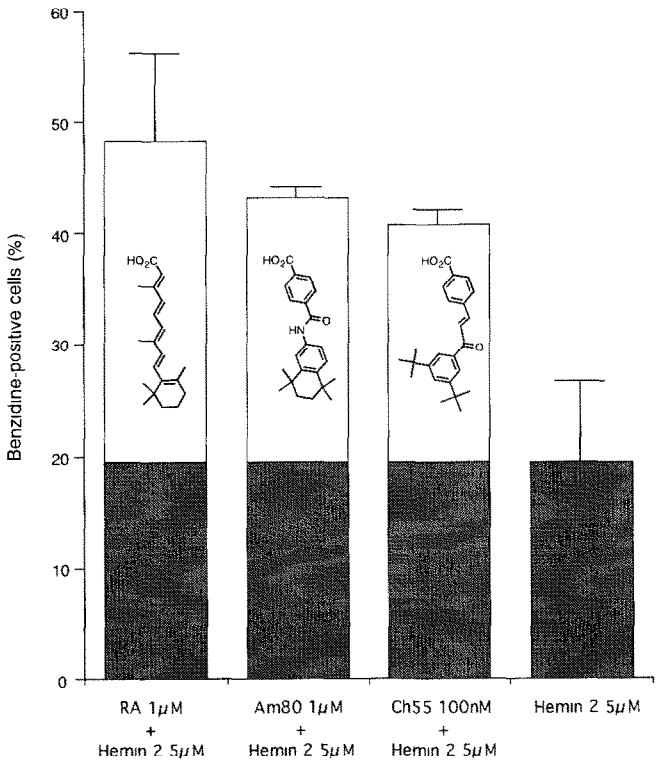

Fig. 3. Effect of the three retinoids on hemin-induced K562 differentiation. K562 cells (4×10^4/ml) were incubated for 4 days in the presence of 2.5 μM hemin in the presence or absence of the indicated concentration of a retinoid, and then assayed by benzidine staining. The percentage of benzidine-positive cells is shown. Shadowed portions indicate the percentage (19.4%) of differentiated cells induced by hemin alone.

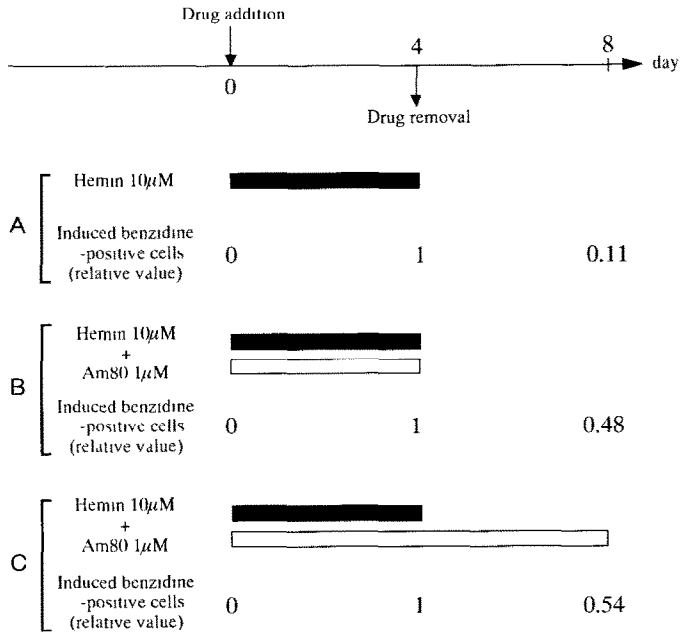

Fig. 4. Effect of retinoid on the kinetics of hemin-induced K562 cell differentiation after removal of the drugs. K562 cells (4×10^4/ml) were incubated for 4 days in the presence of 10 μM hemin with (A) or without (B,C) 1 μM Am80, then washed twice in PBS and reincubated for 4 days with (C) or without (A,B) 1 μM Am80. The cells were then assayed by benzidine staining. The values are relative values based on the number of differentiated cells in 1 ml at the end of the first 4-day incubation at the indicated concentration, taken as 1 unit. Closed bars and open bars indicate the presence of hemin (10 μM) and Am80 (1 μM), respectively, in the incubation media.

In addition, retinoids influenced the kinetics of hemin-induced differentiation after the removal of the drugs, that is, hemin-induced differentiation of K562 cells seemed to acquire irreversibility in the presence of Am80, at least in part. There might exist a retinoid-responsive gene(s) which confers irreversibility on hemin-induced K562 cell differentiation. It was reported that hemin does not affect c-*myc* expression in K562 cells [15], though c-*myc* expression is known to be suppressed in various cell lines in the course of induction of terminal differentiation. For example, RA, Am80 and Ch55 strongly suppressed c-*myc* expression in HL-60 cells prior to terminal differentiation [16]. Identification of such retinoid responsive-gene(s) (one of the candidates being c-*myc*) is in progress.

Our results presented here would suggest that retinoids in combination with other differentiation inducers could be useful for leukemia therapy in cases where the leukemic cells are not responsive to retinoid, alone.

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