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Synergistic Action of Activin A and Hexamethylene Bisacetamide in Differentiation of Murine Erythroleukemia Cells[1]

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ABSTRACT

Activin A, a peptide homologous to transforming growth factor β, induced erythroid differentiation of murine erythroleukemia cells in a dose-dependent manner, as judged by the proportion of benzidine-positive cells. The extent of differentiation induced by activin A depended on the cell density in the initial inoculum; the percentage of benzidine-positive cells markedly decreased at an increasing cell density. In contrast, the hexamethylene bisacetamide (HMBA)-induced differentiation was affected only to a little extent by the cell density. Furthermore, the effects of activin A and HMBA were different in sensitivity to the inhibition by dexamethasone; the HMBA-induced differentiation was reduced by about 90% in the presence of dexamethasone, whereas the activin A-induced differentiation was reduced by 40 to 50%. Activin A and HMBA induced the differentiation in a synergistic manner. Especially when the maximal response to activin A was suppressed at high cell density, the simultaneous presence of a suboptimal concentration of HMBA markedly recovered this suppression. This synergism seemed to be generated at the commitment process. Taken together, these results indicate that activin A and HMBA exert their actions, at least in part, through different pathways, and that these pathways interact with each other, resulting in synergistic induction of murine erythroleukemia cell differentiation. Clinical implications of these findings are discussed.

INTRODUCTION

MEL[3] cells are virus-transformed erythroid precursor cells that retain the capacity of self renewal and can be induced to express a program of terminal differentiation by exposure to various chemical agents, among which polar planar compounds such as DMSO and HMBA are representative (1, 2; for reviews, see Refs. 3 and 4). The molecular mechanism of the actions of these compounds has been studied extensively, and it has been revealed that various cellular and molecular reactions occur associated with MEL cell differentiation (3, 4). For example, recent findings have shown that changes of expression of protooncogenes, activation of protein kinase C, and induction of a cytoplasmic *trans*-acting factor may be involved in the action of the chemical inducers (5–10). However, significance of these reactions in the physiological erythroid differentiation remains to be clarified, partly because no physiological agent causing MEL cell differentiation has been identified.

Activin A was first purified from porcine ovarian fluid as a protein with the activity to stimulate secretion of follicle-stimulating hormone from cultured pituitary cells (11, 12; for a review, see Ref. 13). This protein is a homodimer of β_A chains and is highly homologous to transforming growth factor β (11–13). Independently, Eto *et al.* (14) purified from the culture supernatant of a human leukemic cell line (THP-1) a homodimer protein inducing the erythroid differentiation of MEL cells and designated it erythroid differentiation factor. Subsequently, sequence analysis of complementary DNA of the subunit of this factor has revealed that erythroid differentiation factor is identical to activin A (15). Yu *et al.* (16) have reported that activin A not only induces hemoglobin synthesis in K562 cells, a human erythroleukemia cell line, but also enhances the growth of normal erythroid precursor cells. Furthermore, the mRNA of activin A is detected in rat bone marrow (17). These observations suggest that activin A plays an important role as a physiological differentiation factor of normal or transformed hematopoietic cells.

Activin A exerts its action presumably via its cell surface receptor, since receptor molecules of transforming growth factor β and other related proteins have been identified on the cell surface (18, 19). Furthermore, activin A is likely to act as a regulator of hematopoiesis in the bone marrow as described above. These features are unique to activin A among inducers of MEL cell differentiation. Accordingly, study on the mode of action of activin A may provide a new insight into the mechanism of MEL cell differentiation. In the present study, we have investigated the mode of action of activin A in comparison with that of HMBA. Our results indicate that the pathway through which activin A exerts its action is distinct from the pathway through which HMBA exerts its action, and that these pathways interact with each other, resulting in a synergistic effect on MEL cell differentiation.

MATERIALS AND METHODS

Materials. Activin A (erythroid differentiation factor) was prepared as described previously (14). HMBA was purchased from Sigma. DMSO and dexamethasone were from Wako Chemicals (Tokyo, Japan).

Cell Culture. MEL F5-5 cells, established from a DDD mouse by Ikawa *et al.* (20), were cultured in a humidified atmosphere of 5% CO_2 and 95% air in Ham's F-12 medium supplemented with 10% fetal calf serum. The cells were maintained by diluting the cells every 3 days at about 5×10^4 cells/ml in the fresh medium. The present experiments were done with cells that were kept continuously in culture for 3 days.

Assay for Erythroid Differentiation. After the cells were treated with differentiation-inducing agents for 4 days, hemoglobinized cells were scored by benzidine staining according to the method of Orkin *et al.* (21). At least 600 cells were examined under a microscope at each assay.

Assay of Colony-forming Ability of MEL Cells. MEL cells were treated with differentiation inducers for 48 h, washed with phosphate-buffered saline, and plated on a semisolid medium (Ham's F-12 with 10% fetal calf serum and 0.3% agar) at 100 cells per well in 96-well plates. After incubation for 2 wk, the number of colonies greater than 50 μm in diameter was counted.

RESULTS

Activin A induced the erythroid differentiation of MEL cells in a dose-dependent manner, as shown in Fig. 1*A*. The 50%

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[3] The abbreviations used are: MEL, murine erythroleukemia; DMSO, dimethyl sulfoxide; HMBA, hexamethylene bisacetamide.

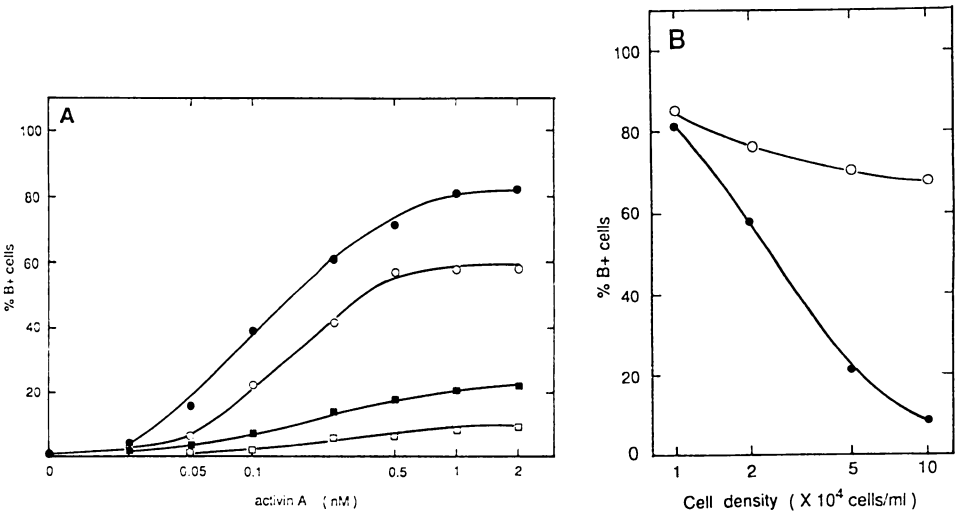

Fig. 1. Effect of activin A on induction of MEL cells to differentiation. *A*, dose-response curve of activin A for MEL cell differentiation. MEL cells were inoculated at cell densities of 1×10^4 (●), 2×10^4 (○), 5×10^4 (■), and 1×10^5 (□) cells/ml, and cultured with various concentrations of activin A. *B*, effect of the initial cell density on activin A- and HMBA-induced differentiation. MEL cells were inoculated at various cell densities and cultured with 1 nM activin A (●) or 5 mM HMBA (○). The percentage of benzidine-positive (*B*+) cells was determined as described in "Materials and Methods." Each value is the mean of triplicate determinations.

effective concentration was about 130 pmol, consistent with the previous report (14). The degree of differentiation depended also on the initial cell density, as clearly illustrated by Fig. 1*B*. When the cells were inoculated at a low density (1×10^4 cells/ml) and cultured with 1 nM activin A, about 80% were benzidine positive. When the cells were seeded at higher cell densities (2×10^4, 5×10^4, and 1×10^5 cells/ml), maximal responses of benzidine-positive cells were markedly suppressed (about 60%, 20%, and 10%, respectively). This suppression was not recovered even when the concentration of activin A was increased up to 10 nmol. In contrast, the percentages of benzidine-positive cells induced by 5 mM HMBA at initial cell densities of 1×10^4 to 1×10^5 cells/ml were about 80 to 70% and affected only to a small extent by the cell density.

In the next set of experiments, we examined the interaction of HMBA and activin A in the induction of erythroid differentiation of MEL cells under several culture conditions. Fig. 2 shows the effect of suboptimal concentrations of activin A on the HMBA-induced differentiation at initial cell densities of 1

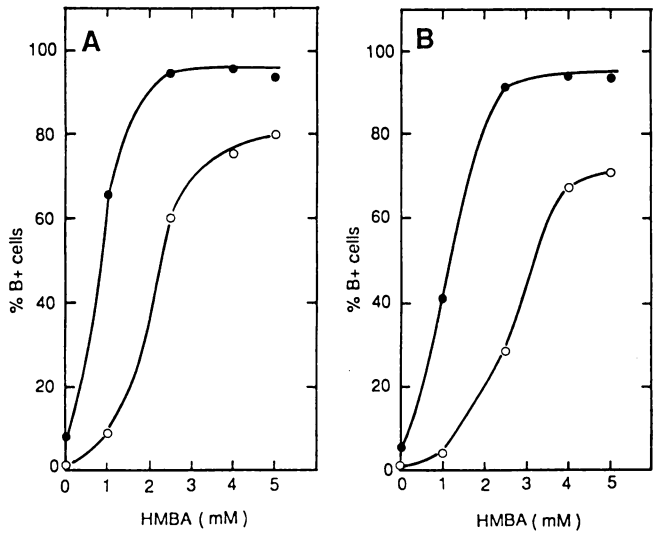

Fig. 2. Effect of activin A on the HMBA-induced differentiation of MEL cells. *A*, MEL cells were inoculated at a cell density of 1×10^4 cells/ml and cultured with various concentrations of HMBA in the presence (●) or absence (○) of 50 pM activin A. *B*, MEL cells were inoculated at a cell density of 1×10^5 cells/ml and cultured with various concentrations of HMBA in the presence (●) or absence (○) of 500 pM activin A. The percentage of benzidine-positive (*B*+) cells was determined as described in "Materials and Methods." Each value is the mean of triplicate determinations.

$\times 10^4$ and 1×10^5 cells/ml, respectively. At both cell densities, the addition of activin A at suboptimal doses (50 and 500 pmol, respectively), where it induced less than 10% benzidine-positive cells by itself, enhanced the HMBA-induced differentiation in a synergistic manner, especially at lower concentrations of HMBA. Fig. 3*A* shows the synergistic enhancement by a suboptimal concentration (1 mM) of HMBA of the activin A-induced differentiation at initial cell densities of 1×10^4 and 1×10^5 cells/ml. At the lower cell density, the dose of activin A required for the differentiation was markedly reduced, and the maximal response was also increased in the presence of 1 mM HMBA. At the higher cell density, the maximal response of benzidine-positive cells induced by activin A was markedly enhanced. This finding is more clearly illustrated by Fig. 3*B*. With regard to DMSO, similar synergism with activin A was observed (data not shown).

To examine whether the synergistic effect of activin A and HMBA is generated at the commitment process of MEL cells to differentiation, the colony-forming ability of the cells treated for 48 h by these inducers was evaluated (Table 1). Activin A markedly suppressed the colony formation when the cells were seeded at the low cell density (1×10^4 cells/ml), implying that the committed cells cannot proliferate in this assay condition, as described in the previous report (14). The colony formation was also suppressed when the cells were treated by 5 mM HMBA at the cell density of 1×10^5 cells/ml. Under the same condition, neither 1 mM HMBA nor 1 nM activin A reduced the number of colonies, whereas simultaneous treatment with the two agents markedly inhibited the colony formation. These results indicate that the synergistic effect of activin A and HMBA was evident in the early period during the treatment with these inducers corresponding to the commitment process.

It was reported that dexamethasone markedly inhibited the differentiation of MEL cells induced by the polar planar compounds, DMSO and HMBA (22, 23). The notion that the pathways through which HMBA and activin A exert their actions may be distinguished by the sensitivity to the inhibition by dexamethasone led us to the examination of the effects of this agent on the differentiation induced by maximum doses of activin A and HMBA. As shown in Table 2, the proportion of benzidine-positive cells induced by 5 mM HMBA was reduced by about 90% in the presence of 1 μM dexamethasone, consistent with the previous reports (22, 23). On the other hand, the inhibition of the activin A-induced differentiation by dexameth-

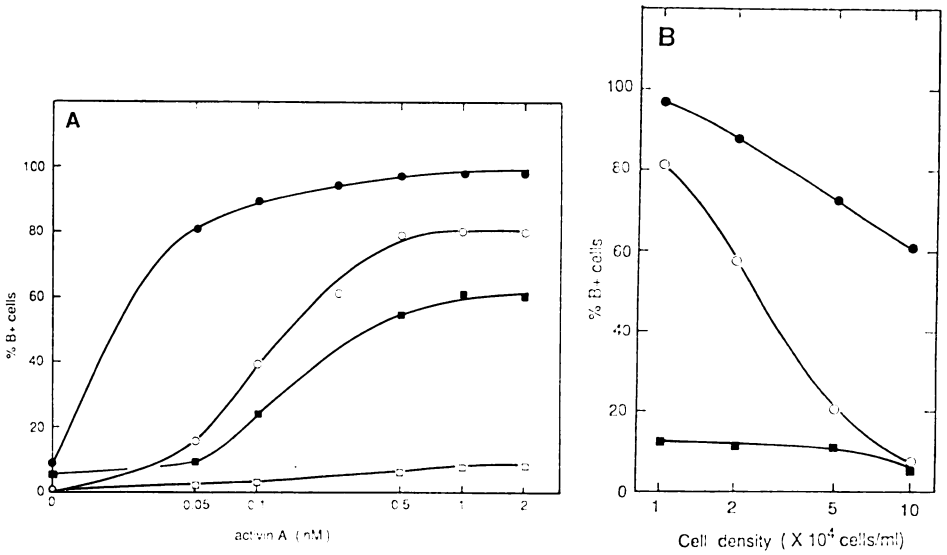

Fig. 3. Effect of HMBA on the activin A-induced differentiation of MEL cells. *A*, MEL cells were inoculated at a cell density of 1×10^4 cells/ml (○, ●) and 1×10^5 cells/ml (□, ■) and treated with various concentrations of activin A in the presence (●, ■) or absence (○, □) of 1 mM HMBA. *B*, MEL cells were inoculated at cell densities of 1×10^4, 2×10^4, 5×10^4, and 1×10^5 cells/ml and cultured with 1 nM activin A (○), 1 mM HMBA (■), or 1 nM activin A plus 1 mM HMBA (●). The percentage of benzidine-positive (*B*+) cells was determined as described in "Materials and Methods." Each value is the mean of triplicate determinations.

Table 1 *Colony-forming ability of MEL cells treated by activin A and HMBA*

MEL cells were cultured under various conditions specified in the table for 48 h, and the colony-forming ability of these cells was examined as described in "Materials and Methods." Each value is the mean of triplicate determinations.

Culture condition			
Inducer	Cell density (cells/ml)	Colony-forming efficiency (%)	% of control
None	1×10^5	72	100
1 nM activin A	1×10^5	73	101
1 mM HMBA	1×10^5	83	115
1 nM activin A + 1 mM HMBA	1×10^5	21	29
5 mM HMBA	1×10^5	19	26
1 nM activin A	1×10^4	33	45

Table 2 *Effect of dexamethasone on HMBA- and activin A-induced differentiation of MEL cells*

MEL cells were cultured with 5 mM HMBA at an initial cell density of 1×10^5 cells/ml and with 1 nM activin A at an initial cell density of 1×10^4 cells/ml in the absence or presence of 1 μM dexamethasone. After 4 days, the percentage of benzidine-positive cells was determined as described in "Materials and Methods." Each value is the mean of triplicate determinations.

	% of benzidine-positive cells		
Inducers	In the absence of dexamethasone	In the presence of dexamethasone	% of inhibition
HMBA	87	10	89
Activin A	79	42	47

asone was 40 to 50%, which was about half of the inhibition of the HMBA-induced differentiation.

DISCUSSION

In the present study, we have investigated several aspects of the action of activin A in the induction of differentiation of MEL cells in comparison with that of HMBA, a representative chemical inducer (2–4). The most prominent difference between the actions of these two agents is seen in the dependency of their inducing capabilities on the cell density in the initial inoculum. While the HMBA-induced differentiation was affected only to a little extent by the initial cell density, the activin A-induced differentiation was markedly suppressed at an increasing cell density. These results suggest that some step(s) in the process during the action of activin A, from receptor binding to expression of the erythroid phenotype, is inhibited at the high cell density, whereas this cell density-sensitive step is not involved in the action of HMBA. Moreover, a difference between the modes of action of activin A and HMBA was observed also in the sensitivity to the inhibition of dexamethasone. Taken together, these results suggest that activin A and HMBA exert their actions, at least in part, through different pathways.

The mechanism of the cell density-dependent suppression of the activin A-induced differentiation is unknown at present. It is possible that this inhibition is secondary to a change of growth state, since previous reports showed that the differentiation was closely related to the cell division cycle in the case of other cells (24, 25). It was also reported that the HMBA-induced commitment of MEL cells occurred in the early S phase of the cell cycle, which causes the slight suppression of HMBA-induced differentiation at high cell density (26). Similarly, some cell cycle-associated event(s) may be necessary for the action of activin A. An alternative attractive hypothesis is that an unidentified autocrine factor secreted from MEL cells specifically inhibits the action of activin A. The possibility that an activin A-binding protein secreted from MEL cells inactivates this agonist, as reported with transforming growth factor β (27, 28), is unlikely because the high concentration of activin A did not overcome this inhibition.

We have shown that activin A and HMBA synergistically induce the differentiation of MEL cells under several kinds of culture conditions. Of note, the cell density-dependent suppression of the activin A-induced differentiation was largely circumvented by a suboptimal dose of HMBA. Moreover, our results suggest that the synergistic effect is generated at the commitment process. These observations, taken together with the notion that activin A and HMBA induce MEL cell differentiation, at least in part, through different pathways, suggest that some of the signals mediating the action of HMBA bypass the pathway through which activin A exerts its action and interact with the signals mediating the action of activin A, resulting in the synergistic induction of commitment of MEL cells to differentiation. However, the molecular mechanism for this interaction is unknown at present.

Recently, great interest has been focused on "differentiation therapy," control of the malignancy of tumor cells by inducing terminal differentiation (29). A wide variety of agents, physiological or nonphysiological, have been shown to induce differentiation of leukemic cells *in vitro* (30, 31). However, the clinical effectiveness of these agents in the therapy of leukemia has not been established. We demonstrate that MEL cells are resistant to activin A, when the cells are inoculated at high cell density. Interestingly, Jimenez and Yunis (32) reported a sim-

ilar observation that differentiation of murine myeloid leukemic cells induced by a differentiation factor derived from various conditioned media was markedly suppressed when the cells were inoculated at an increasing cell density and attributed this suppression to a deficiency in the amount of differentiation factor (32). By contrast, our results indicate that MEL cells can be resistant to a sufficient amount of activin A under a similar condition, and that this resistance can be overcome by the addition of a suboptimal concentration of polar planar compounds. Thus, combined use of different classes of differentiation-inducing factors may be a useful approach to differentiation therapy of malignancy.

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