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Both D factor/LIF and IL-6 inhibit the differentiation of mouse teratocarcinoma F9 cells

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Differentiation-stimulating factor (D factor)/leukemia inhibitory factor (LIF) and IL-6 are reported to be cytokines having multifaced functions including the induction of differentiation in mouse myeloid leukemia M1 cells. We here report that both D factor/LIF and IL-6 inhibit the differentiation of mouse teratocarcinoma F9 cells induced by retinoic acid alone or combined with dibutyryl cAMP. From the microscopic observation as well as Northern blot analysis using cDNA probes encoding several marker proteins for differentiation of F9 cells, we concluded that D factor/LIF and IL-6 are functionally closely related in the induction of differentiation in M1 cells and in the inhibition of F9 differentiation.

Differentiation factor; Leukemia inhibitory factor; IL-6; Differentiation; F9 cell

1. INTRODUCTION

D factor/LIF was originally identified as a differentiation-inducing factor for mouse myeloid leukemia cell line, M1 [1], ans its cDNA was cloned and analyzed [2,3]. M1 can be induced to differentiate into macrophage-like cells by this factor. D factor/LIF is a cytokine with multifaced functions, including the maintenance of the growth of mouse ES cells [4], the stimulation of bone remodeling [5] and of acute-phase protein synthesis in hepatocytes [6], as well as the induction of differentiation in rat sympathetic neurons [7]. IL-6 is also multifunctional cytokine, some functions of which are similar to those of D factor/LIF, including the induction of differentiation in M1 cells. It has not been reported, however, whether IL-6 maintains the growth of undifferentiated mouse ES cells.

The mouse teratocarcinoma cell line, F9, was induced to differentiate into visceral endoderm-like cells by retinoic acid, and to parietal endoderm-like cells with retinoic acid in combination with dbcAMP [8]. In this study, we examined whether D factor/LIF and IL-6 have any effect on the differentiation of F9 cells.

2. MATERIALS AND METHODS

Mouse teratocarcinoma F9 cells were maintained in Dulbecco's modified Eagle's medium containing 10% foetal bovine serum (MA Bioproducts, Walkersville, MD), penicillin (50 units/ml), and streptomycin (50 μg/ml), under humidified 5% CO_2 and 95% air. Subcultures were performed every other day using 0.25% trypsin and 1 mM EDTA. For differentiation study, F9 cells were plated at a density of 2×10^4 cells/3.5 cm plastic dish and incubated with 10^{-6} M retinoic acid (Sigma, St. Louis, MO) alone or combined with 10^{-3} M dBcAMP (Wako Chemical Co. Ltd., Osaka). Recombinant human D factor/LIF was added at concentrations of 5, 50 and 500 units/ml, and recombinant human IL-6 at concentrations of 6, 60 and 600 units/ml. D factor/LIF was purified to homogeneity from conditioned medium of CHO cells transfected with the plasmid containing cDNA encoding human D factor/LIF [3], its specific activity was determined as previously reported [1] and was 100 u/ng protein. Recombinant human IL-6 was produced and purified in the laboratory of Tosoh Corp. (Kanagawa) and the specific activity was assessed in terms of B cell stimulatory activities utilizing the B cell line SKW6-CL4 as previously reported [9].

To prepare RNA, 4×10^5 cells/10 cm plastic dish were incubated for 24 h, then treated with retinoic acid alone or combined with dbcAMP in the presence or absence of D factor/LIF or IL-6 for another 48 h. Total RNAs were isolated according to the method of Chirgwin et al. [10]. Northern blot analysis was performed according to the method previously described [11]. Mouse osteonectin (*Pst*I fragment of pSE48; a kind gift from Dr Kitagawa, Nagoya University), laminin (*Pst*I fragment of pSE38; from Dr Kitagawa), alkaline phosphatase (*Pst*I-*Kpn*I fragment of pKUT1AP; kindly donated by Dr Shigesada, Institute for Virus Research, Kyoto University), type IV collagen (*Pst*I fragment of pH221; a generous gift from Dr Sakai, Saga Medical College) and mouse β-actin (*Eco*RI fragment of pMAβ3'UT; a kind gift from Dr Sakiyama, Chiba Cancer Center Research Institute) were used as probes for Northern blot analysis. Probes were labeled with [α-^{32}P]dCTP (spec. act. 3000 Ci/mol) by the method of Feinberg et al. [12].

3. RESULTS AND DISCUSSION

A significant characteristic of F9 cell differentiation was the morphological changes. We analyzed the effect of D factor/LIF on the differentiation of F9 cells by

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microscopic observation. Inhibition of the differentiation in F9 cells induced by retinoic acid or retinoic acid and dbcAMP was observed when D factor/LIF was added to the culture (Fig. 1). This inhibition by D factor/LIF was dose dependent (data not shown), and the high concentrations of D factor/LIF were required to inhibit the differentiation induced with retinoic acid than that with retinoic acid plus dbcAMP. Also, the inhibitory effect of the D factor/LIF in terms of differentiation induction was higher in the cells treated with retinoic acid alone than in those cells treated with retinoic acid and dbcAMP (data not shown).

We quantitated the inhibition of differentiation by the Northern blot analysis. The differentiation markers, osteonectin [13] and type IV collagen [8], were expressed in the differentiated F9 cells induced with retinoic acid and with retinoic acid plus dbcAMP. Laminin was solely expressed in the parietal endoderm-

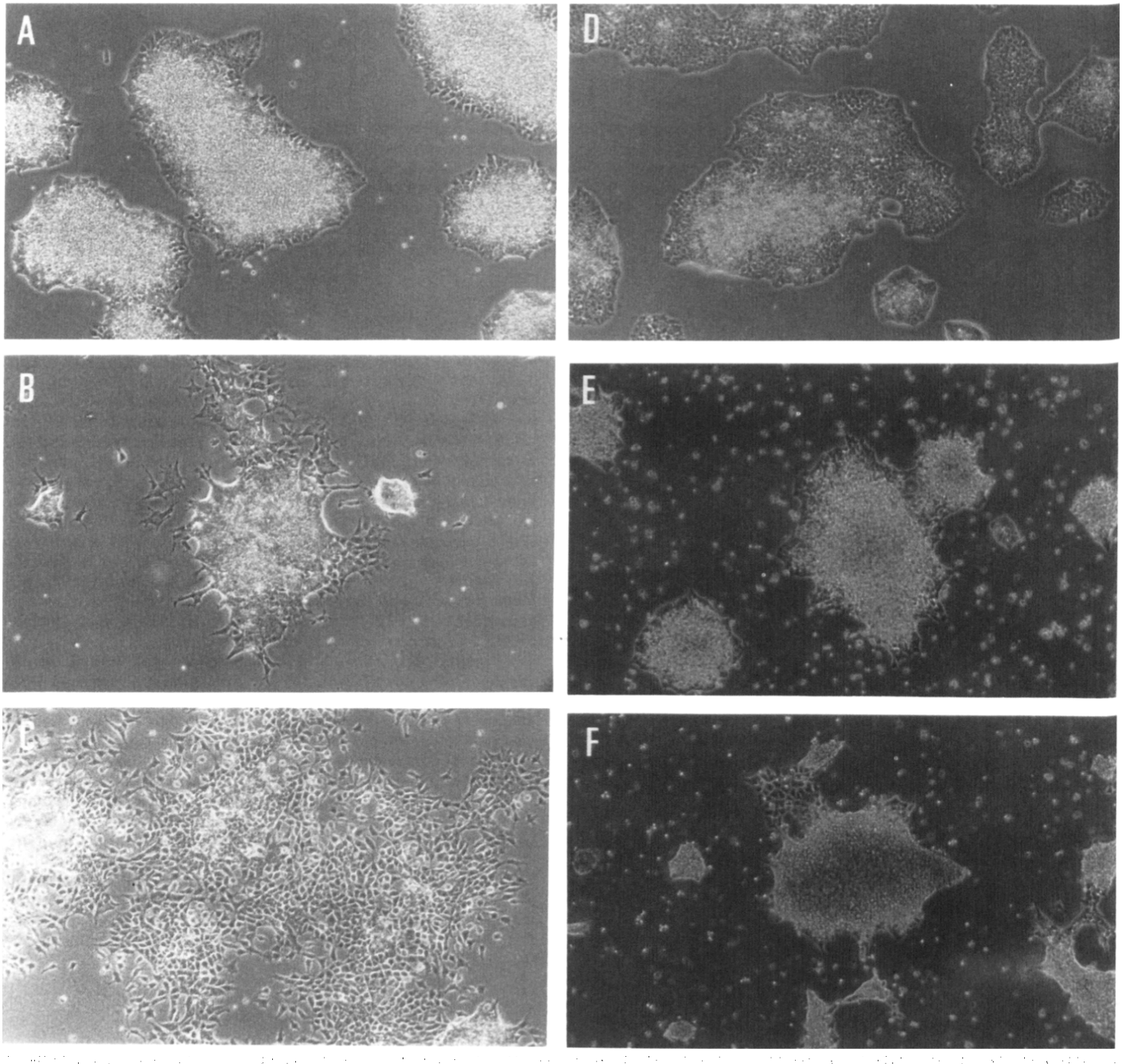

Fig. 1. Morphological changes in F9 cells observed by phase contrast microscopy. (A and D) Undifferentiated F9 cells; (B and E) F9 cells treated with 10^{-6} M retinoic acid for 3 days; and (C and F) F9 cells treated with retinoic acid (10^{-6} M) and dbcAMP (10^{-3} M) for 3 days. D factor/LIF (500 units/ml) was absent (A, B and C) or present (D, E and F) throughout the incubation period.

like cells [14]. Alkaline phosphatase was only expressed in undifferentiated F9 cells [8]. Fig. 2 shows the inhibition of the differentiation of F9 cells by the addition of D factor/LIF. Syntheses of mRNA of osteonectin, laminin and type IV collagen increased in F9 cells treated with retinoic acid alone or combined with dbcAMP. D factor/LIF inhibited increase in syntheses of their mRNA (Fig. 2a, b and c). The inhibition of the F9 cell differentiation by D factor/LIF was also confirmed by the increased level of alkaline phosphatase mRNA in the cells treated simultaneously both with inducers and with D factor/LIF (Fig. 2d). Northern blot analysis using osteonectin cDNA (the most sensitive differentiation marker in our system) as a probe revealed the dose-dependent effect of the D factor/LIF on the inhibition of F9 differentiation (Fig. 3A). Using D factor/LIF at the concentration of 500 units/ml, F9 cell differentiation was inhibited even after 4 days of incubation with differentiation inducers (Fig. 3B).

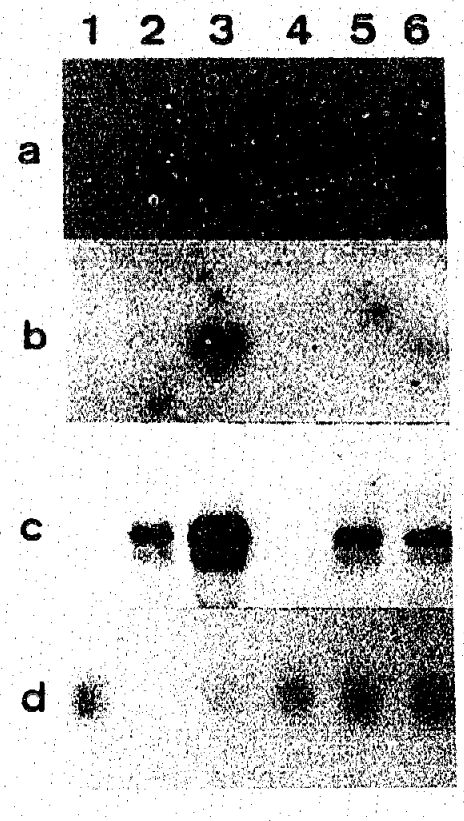

Fig. 2. Effect of the presence of D factor/LIF on the accumulation of mRNAs encoding the marker proteins for the differentiation of F9 cells. RNAs from undifferentiated F9 cells (lanes 1 and 4), F9 cells treated with 10^{-6} M retinoic acid (lanes 2 and 5) and F9 cells treated with retinoic acid plus 10^{-3} M dbcAMP (lanes 3 and 6). D factor/LIF (500 units/ml) was absent (lanes 1, 2 and 3) or present (lanes 4, 5 and 6). After separation on 1% agarose gels, mRNAs were analysed by Northern blot hybridization using several cDNA probes including osteonectin (a), laminin (b), type IV collagen (c) and alkaline phosphatase (d). Filter was hybridized with type IV collagen, rehybridized with laminin and then with osteonectin.

IL-6 at the concentration of 600 units/ml also inhibited the differentiation of F9 cells (Fig. 4). Dose-dependent inhibition by IL-6 at 60–600 units/ml was observed by Northern blot analysis as observed for D factor/LIF (data not shown). Observations by phase contrast microscopy also confirmed the inhibitory effect of IL-6 on the induction of F9 cell differentiation (data not shown).

IL-6 has similar functions to D factor/LIF, for example, the induction of differentiation in mouse myeloid leukemia M1 cells [15,16] and PC12 cells [17], and the stimulation of synthesis of acute phase plasma proteins [6]. Although D factor/LIF inhibits the induction of differentiation in mouse ES cells [4], it was not yet known whether IL-6 also did so. In this study, we

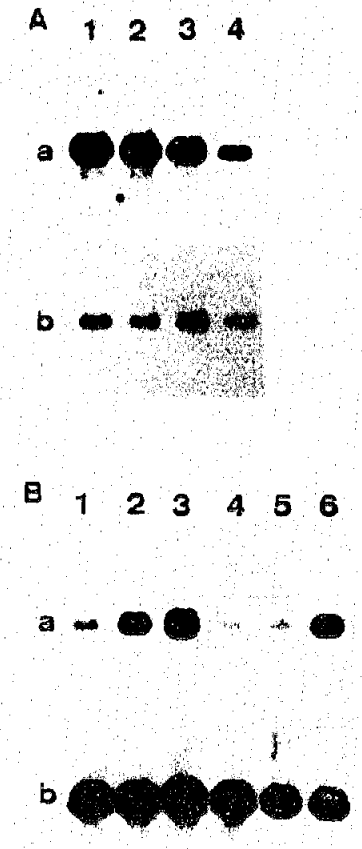

Fig. 3. Effect of the concentration of D factor/LIF on the inhibition of differentiation of F9 cells (A) and the time course of the differentiation in the presence or absence of D factor/LIF (B). (A) F9 cells were incubated with 10^{-6} M retinoic acid and 10^{-3} M dbcAMP (lanes 1–4) in the presence of 5 (lane 2), 50 (lane 3), and 500 units/ml (lane 4) of D factor/LIF. Lane 1 shows the control without the addition of D factor/LIF. (B) F9 cells were incubated with 10^{-6} M retinoic acid and 10^{-3} M dbcAMP in the presence (lanes 4–6) or absence (lanes 1–3) of 500 units/ml D factor/LIF. RNAs were isolated from the cells at days 2 (lanes 1 and 4), 3 (lanes 2 and 5), and 4 (lanes 3 and 6). Northern analyses were performed using cDNA encoding osteonectin in both A (a) and B (a), and cDNA encoding β-actin A (b) and B (b) as an internal control.

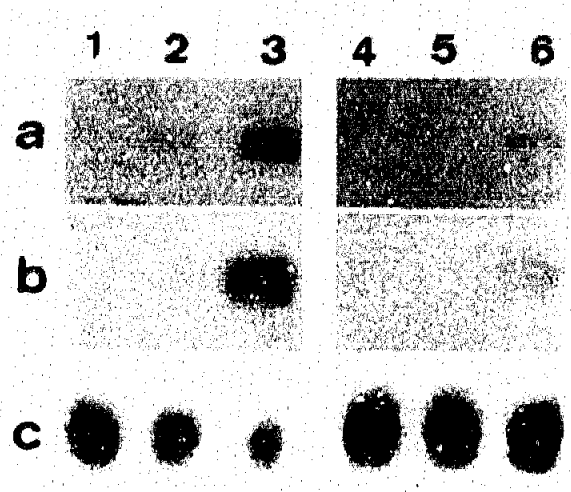

Fig. 4. Effect of IL-6 on the differentiation of F9 cells. Northern blot analyses were performed as described in Fig. 2 using probes for osteonectin (a), laminin (b), and alkaline phosphatase (c). Lanes 1 and 4, undifferentiated F9 cells; lanes 2 and 5, F9 cells treated with 10^{-6} M retinoic acid for 3 days; lanes 3 and 6, F9 cells treated with 10^{-6} M retinoic acid and 10^{-3} M dbcAMP for 3 days. IL-6 (600 units/ml) was absent (lanes 1, 2 and 3) or present (lanes 4, 5 and 6) for 3 days.

showed that both D factor/LIF and IL-6 inhibit the differentiation of F9 cells, which adds a new evidence of similar nature of D factor/LIF to IL-6.

A cDNA clone encoding the IL-6 receptor has been isolated [18]. The molecular size of the IL-6 receptor is 80 kDa in the mature form, which is processed from a 50 kDa precursor protein [19]. When the IL-6 receptor is immunoprecipitated using anti-IL-6 receptor antibody after the cells are treated with IL-6, a 130 kDa glycoprotein (gp130) is also precipitated with the 80 kDa IL-6 receptor. The following observations suggest that the IL-6 receptor provides the ligand binding sites and that gp130 associated with IL-6 receptor on the cell surface is a signal transducer. The IL-6 receptor has a small intracellular domain without any protein kinase activity which is not necessary for signal transduction, and soluble IL-6 receptor without an intracellular domain stimulates the growth inhibition by IL-6 in M1 cells [19]. Although a D factor/LIF receptor has not been identified, it is different from that of IL-6 since D factor/LIF did not compete for the binding of radiolabeled IL-6 to its receptors (our unpublished observations using M1 cells), and likewise IL-6 did not compete the binding of D factor/LIF to its receptors [20]. It remains to be clarified why the biological functions of D factor/LIF and IL-6 overlap each other in some cases as described above, and especially whether D factor/LIF and IL-6 utilize a common signal transducer such as gp130.

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