Inhibition of development of Na^+-dependent hexose transport in renal epithelial LLC-PK1 cells by differentiation-stimulating factor for myeloid leukemic cells/leukemia inhibitory factor

Mikio Tomida[1], Yuri Yamamoto-Yamaguchi[1], Motoo Hozumi[1], William Holmes[2], David G. Lowe[2] and David V. Goeddel[2]

[1]*Department of Chemotherapy, Saitama Cancer Center Research Institute, Ina-machi, Kitaadachi-gun, Saitama-Ken 362, Japan* and [2]*Department of Molecular Biology, Genentech, Inc., 460 Pt. San Bruno Boulevard, South San Francisco, CA 94080, USA*

Received 27 April 1990; revised version received 2 June 1990

Differentiation-stimulating factor (D-factor)/leukemia inhibitory factor is a cytokine inducing differentiation of mouse myeloid leukemic Ml-T22 cells. The effect of recombinant human D-factor on growth and differentiation of pig kidney LLC-PK_1 cells was examined. LLC-PK_1 cells did not concentrate α-methylglucoside during their early growth in culture but developed the capacity to concentrate this hexose as they reached confluence and their growth rate decreased. Purified D-factor caused dose-dependent inhibition of the development of this concentrative capacity. It did not affect the growth rate of the cells, but inhibited the formation of multicellular domes in confluent cultures. LLC-PK_1 cells were found to have high-affinity binding sites (831 per cell) for D-factor with a dissociation constant of 197 pM.

Pig kidney cell; Sugar transport; Differentiation-stimulating factor; Leukemia inhibitory factor; Differentiation-inhibiting activity

1. INTRODUCTION

Previously, we purified a cytokine inducing differentiation of mouse myeloid leukemic M1-T22 cells into macrophages [1,2] and designated this cytokine as D-factor. We also characterized D-factor receptors on M1 cells [3] and isolated complementary DNA encoding human D-factor [4]. Metcalf and coworker also purified the same cytokine [5], isolated complementary DNA for the cytokine [6] and named the cytokine leukemia inhibitory factor (LIF). D-factor/LIF was found to be identical to human interleukin that supported proliferation of murine leukemia DA-la cells [7] and differentiation inhibiting activity that inhibited differentiation of pluripotent mouse embryonal stem cell lines [8,9]. In addition, D-factor/LIF stimulated bone-resorbing activity in mouse fetal calvaria [10], synthesis of acute phase plasma proteins in hepatoma cells [11] and differentiation of rat sympathetic neurons [12]. Thus D-factor, like interleukin 6, affects the growth and differentiation of a wide variety of cells. We screened various cell lines for D-factor receptors, and found that some kidney cell lines have relatively higher numbers of D-factor receptors. In the present work, we examined the effects of D-factor on the growth and differentiation of LLC-PK_1 cells, a dome-forming cell line derived from juvenile pig kidney [13]. The dome is a fluid-filled hemicyst and is considered to be, at least in part, a manifestation of transepithelial ion and water transport [14]. In addition, LLC-PK_1 cells show Na^+-dependent hexose transport activity [15-18]. This activity is normally found only in terminally differentiated epithelia such as those of the kidney and intestine.

Correspondence address: M. Tomida, Department of Chemotherapy, Saitama Cancer Center Research Institute, Ina-machi, Kitaadachi-gun, Saitama-Ken 362, Japan

Abbreviations: D-factor, differentiation-stimulating factor; LIF, leukemia inhibitory factor; α-meG, methyl α-D-glucopyranoside; cAMP, adenosine 3′,5′-cyclic monophosphate; HBSS, Hank's balanced salt solution; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid

2. MATERIALS AND METHODS

2.1. *Cells and cell culture*

The pig kidney epithelial cell line LLC-PK_1 (ATCC CRL1392) was obtained from Flow Laboratories (Tokyo, Japan) at passage 190, and cultured in a 50:50 mixture of Dulbecco's modified Eagle's medium and Ham's F12 (Flow Laboratories) supplemented with 10% fetal calf serum (Gibco, Grand Island, NY) and 100 μg/ml of streptomycin in an atmosphere of 5% CO_2 in air at 37°C. Cells in confluent monolayers were separated with 0.25% trypsin and 0.02% EDTA and subcultured at a split ratio 1:10. Cultures reached confluence on day 3-4 after subculture.

2.2. *Sugar uptake*

Sugar uptake was measured by the method of Amsler and Cook [15]. The cells were plated in Costar 6-well dishes (35 mm diameter), at 1.5×10^5 cells per well, and the medium was changed every 2 days. D-Factor was added at the time of the first medium change 2 days after plating. For assay of uptake, the medium was poured off, and

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

the monolayers were rinsed twice with 2 ml of Hank's balanced salt solution (HBSS) buffered at pH 7.3 with 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes). The uptake assay was initiated by addition of 1 ml of HBSS containing [^{14}C]methyl-α-D-glucopyranoside (α-meG, Amersham) at a final concentration of 100 μM and 0.2 μCi/ml. After incubation at 37°C for 60 min, the medium was removed, and the monolayers were washed three times with 2 ml of ice-cold HBSS. Then they were solubilized in 1 ml of 1% sodium dodecyl sulfate, and radioactivity was measured using ACS III (Amersham) as scintillation liquid. For determination of cell protein, the monolayers were solubilized in 0.25 N NaOH, and protein was determined by the dye fixation method (Bio-Rad Laboratories) with bovine serum albumin as a standard.

2.3. *D-factor*

D-factor was purified to homogeneity from conditioned medium of CHO cells transfected with the plasmid containing cDNA encoding human D-factor [4]. Recombinant human D-factor and native mouse D-factor induced differentiation of M1-T22 cells at similar concentrations. Purified recombinant human D-factor (0.5 ng/ml) induced 50% phagocytic cells in M1-T22 cell cultures.

2.4. *Cellular binding of ^{125}I-D-factor*

D-factor was iodinated with chloramine-T in the presence of 12.5% dimethyl sulfoxide as described previously [3]. The specific radioactivity of the ^{125}I-D-factor preparation was 1.6×10^5 cpm/ng protein.

For assay of binding of ^{125}I-D-factor, LLC-PK$_1$ cells were plated in 12-well plates. The confluent cells (8.88×10^5/well) obtained by culture for 3 days were washed twice with phosphate-buffered saline and incubated in 1 ml of culture medium containing ^{125}I-D-factor and 25 mM Hepes with or without 30-fold excess of unlabelled D-factor for 3 h at 21°C as described [3]. After incubation, the medium was removed and the cells were washed three times with cold phosphate-buffered saline. Then they were solubilized in 1 ml of 1 N NaOH, and their radioactivity was measured in a gamma-counter. Specific binding was determined by subtracting the binding in the presence of 30-fold excess of unlabelled D-factor.

2.5. *Enzyme activities*

Cultures were rinsed 3 times with ice-cold 0.9% NaCl. Cells were harvested in 1 ml of 0.25 M sucrose with a rubber policeman and sonicated in an ice-bath by 3 × 15 s bursts at intervals 30 s. Alkaline phosphatase (EC 3.1.3.1) was determined by a reported method [19]. The reaction mixture (0.5 ml) contained 8 mM *p*-nitrophenylphosphate, 0.5 M 2-amino-2-methyl-1-propanol (pH 10.3) and 25 μl of cell lysate. After incubation at 37°C for 10 min, the reaction was stopped with 1.5 ml of 0.25 N NaOH. The absorbance of the samples at 410 nm was compared with that of a standard solution of *p*-nitrophenol.

γ-Glutamyl transpeptidase (EC 2.3.2.2) was determined as in [18]. The reaction mixture (1 ml) contained 2.2 mM γ-glutamyl-*p*-nitroanilide, 20 mM glycylglycine, 0.1 M Tris-HCl, pH 8.2, and 100 μl of cell lysate. After incubation at 37°C for 10 min, the reaction was stopped with 2 ml of 10% acetic acid. The absorbance of the samples at 410 nm was compared with that of a standard solution of *p*-nitroaniline. Enzyme activities are expressed in μmol·mg protein^{-1}·h^{-1}.

3. RESULTS

3.1. *Binding of ^{125}I-D-factor to LLC-PK$_1$ cells*

We examined the bindings of ^{125}I-D-factor to various kidney cell lines. ^{125}I-D-factor bound to only 2 of the 8 cell lines tested. These two cell lines did not produce D-factor, whereas the other 6 cell lines produced significant amounts of D-factor. Thus even if the cells did not bind ^{125}I-D-factor, they might have receptors, because autocrine D-factor might block the binding of ^{125}I-D-factor added exogenously.

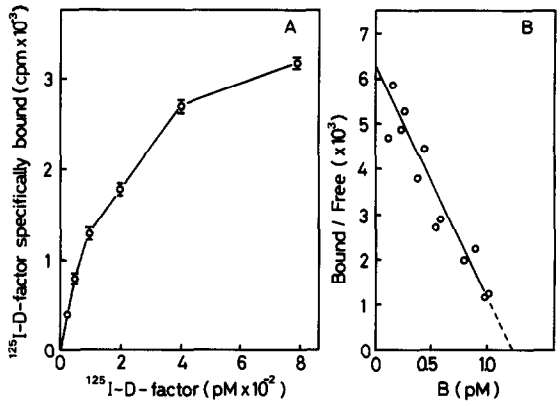

Fig. 1. Binding of ^{125}I-D-factor to LLC-PK$_1$ cells. (A) Specific binding was determined by subtracting the binding in the presence of 30-fold excess of unlabelled D-factor. Points and bars are means ± SE for duplicate determinations. (B) Data plotted by Scatchard's method. B = bound ^{125}I-D-factor concentration.

We analyzed the binding of D-factor to LLC-PK$_1$ cells as described previously [3]. Scatchard analysis revealed that the cells expressed 831 high affinity D-factor receptors per cell with an apparent dissociation constant of approximately 197 pM (Fig. 1).

3.2. *Inhibition of development of Na$^+$-dependent hexose transport by D-factor*

We examined the effects of D-factor on growth and differentiation of LLC-PK$_1$ cells. D-factor did not inhibit growth of the cells inoculated at low density, and

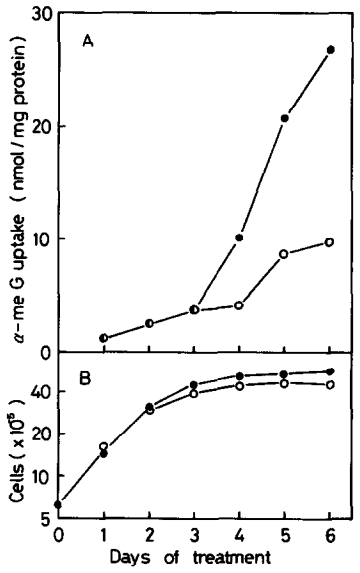

Fig. 2. Effects of D-factor on development of activity for uptake of α-methylglucoside (A) and on growth of the cells (B). Cells were incubated with (○) or without (●) 20 ng/ml of D-factor from 2 days after plating. Values are means for duplicate determinations.

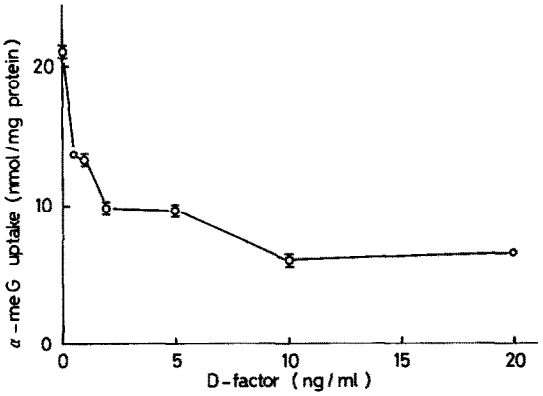

Fig. 3. Inhibition by D-factor of development of activity for uptake of α-methylglucoside. Cells were treated with various amounts of D-factor from 2 days after plating, and sugar uptake was determined after treatment with D-factor for 5 days. Points and bars are means ± SE for duplicate determinations.

did not stimulate growth of cells inoculated into medium without serum. However, the saturation density of the cells treated with D-factor was slightly lower than that of untreated cells (Fig. 2B). Moreover, although cells treated with D-factor formed confluent monolayers, their formation of multicellular domes was suppressed. In control cultures, domes appeared on day 3 (5 days after plating), and on day 5 there were 97 domes/cm^2: 65 small domes and 32 large domes. On the other hand, in cultures treated with D-factor, there were only small domes (29 domes/cm^2). In addition, a few round cells were released into the medium from confluent monolayer treated with D-factor, and although the medium was changed every 2 days, it became more acidic than that of control cultures.

In control cultures Na^+-dependent α-methylglucoside transport started on day 4 (Fig. 2A). D-factor inhibited its appearance, causing appreciable inhibition at concentrations of more than 0.5 ng/ml and maximum inhibition at concentrations of more than 10 ng/ml (Fig. 3).

Next, we assayed the activities of the apical marker enzymes, alkaline phosphatase and γ-glutamyl transpeptidase [19]. The activities of these enzymes were low in sparse cultures, but increased after the cells reached a confluent density (Table I). D-Factor did not inhibit increase in these enzyme activities, and slightly stimulated increase in alkaline phosphatase.

4. DISCUSSION

At confluence, LLC-PK$_1$ cells simultaneously develop the differentiated properties of apical Na^+-hexose cotransport, apical marker enzyme activities and transepithelial fluid transport. In this work, we showed that recombinant human D-factor inhibited development of Na^+-dependent hexose transport in pig kidney cells. It also inhibited dome formation, but did not inhibit increase in the activities of alkaline phosphatase and γ-glutamyl transpeptidase. Van Den Bosh et al. [17] isolated several subclones which differed with respect to development of Na^+-dependent hexose uptake from LLC-PK$_1$ cells and showed that the expressions of Na^+-dependent hexose transport and the activities of alkaline phosphatase and γ-glutamyl transpeptidase were not correlated. These results suggest that the expressions of several differentiated characteristics are regulated independently. Na^+-dependent hexose transport activity is a typical characteristic of renal proximal tubules. Its expression is influenced by the time after confluency and by the D-glucose concentration in the growth medium [16]. The Friend cell inducer hexamethylene bisacetamide and phosphodiesterase inhibitors, including dibutyryl cAMP, theophylline and 1-methyl-3-isobutylxanthine, accelerated the development of hexose concentrating capacity [15]. On the other hand, the tumor promotor 12-*O*-tetradecanoylphorbol-13-acetate inhibited the development of the concentrative capacity. The cAMP levels in the cells were determined, but were found not to explain the mechanism of inhibition of 12-*O*-tetradecanoyl phorbol-13-acetate [15].

Various cytokines that affect the growth and differentiation of myeloid cells and lymphocytes have been identified recently. Many of these cytokines were found to be active on a wide variety of other cells besides the blood cells. However, the effects of these cytokines on the differentiation of LLC-PK$_1$ cells have not yet been examined. To our knowledge, this is the first report that

Table I

Effects of D-factor on inductions of marker enzymes in LLC-PK$_1$ cells

Cells		Alkaline phosphatase ($\mu mol \cdot mg^{-1} \cdot h^{-1}$)	γ-Glutamyl transpeptidase ($\mu mol \cdot mg^{-1} h^{-1}$)
Day	D-factor		
1	–	11.0 ± 0.2[b]	9.6 ± 0.2
5	–	53.2 ± 2.8	42.2 ± 3.5
5	+[a]	70.9 ± 4.3	44.1 ± 2.3

[a] Cells (3×10^5) were incubated into 60 mm dishes. D-factor (20 ng/ml was added at the time of the first medium change 2 days after plating, and the cells were harvested after treatment with D-factor for 5 days

[b] Mean ± SE of determinations in 4 dishes.

a well characterized cytokine, D-factor, inhibits the development of Na^+-dependent hexose transport in these cells.

We found that LLC-PK_1 cells have specific receptors for D-factor and that the affinity and number of these receptors are similar to those on myeloid leukemic M1 cells [3] and mouse embryonal stem cells [8,9]. The structure of the D-factor receptor and mechanism of action of D-factor on these cells remain to be examined.

Acknowledgements: This work was partly supported by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture of Japan, and a grant from the Ministry of Health and Welfare for a Comprehensive 10-Year Strategy of Cancer Control, Japan and by Genentech, Inc., USA.

REFERENCES

[1] Tomida, M., Yamamoto-Yamaguchi, Y. and Hozumi, M. (1984) J. Biol. Chem. 259, 10978-10982.
[2] Tomida, M., Yamamoto-Yamaguchi, Y. and Hozumi, M. (1984) FEBS Lett. 178, 291-296.
[3] Yamamoto-Yamaguchi, Y., Tomida, M. and Hozumi, M. (1986) Exp. Cell Res. 164, 97-102.
[4] Lowe, D.G., Nunes, W., Bombara, M., McCabe, S., Ranges, G.E., Henzel, W., Tomida, M., Yamamoto-Yamaguchi, Y., Hozumi, M. and Goeddel, D.V. (1989) DNA 8, 351-359.
[5] Hilton, D.J., Nicola, N.A., Gough, N.M. and Metcalf, D. (1988) J. Biol. Chem. 263, 9238-9243.
[6] Gearing, D.P., Gough, N.M., King, J.A., Hilton, D.J., Nicola, N.A., Simpson, R.J., Nice, E.C., Kelso, A. and Metcalf, D. (1987) EMBO J. 6, 3995-4002.
[7] Moreau, J.-F., Donaldson, D.D., Bennett, F., Witek-Giannotti, J., Clark, S.C. and Wong, G.G. (1988) Nature 336, 690-692.
[8] Williams, R.L., Hilton, D.J., Pease, S., Willson, T.A., Stewart, C.L., Metcalf, D., Nicola, N.A. and Gough, N.M. (1988) Nature 336, 684-687.
[9] Smith, A.G., Heath, J.K., Donaldson, D.D., Wong, G.G., Moreau, J., Stahl, M. and Rogers, D. (1988) Nature 336, 688-690.
[10] Abe, E., Tanaka, H., Ishimi, Y., Miyaura, C., Hayashi, T., Nakagawa, H., Tomida, M., Yamaguchi, Y., Hozumi, M. and Suda, T. (1986) Proc. Natl. Acad. Sci. USA 83, 5958-5962.
[11] Baumann, H. and Wong, G.G. (1989) J. Biol. Chem. 143, 1163-1167.
[12] Yamamori, T., Fukuda, K., Aebersold, R., Korsching, S., Fann M. and Patterson, P.H. (1989) Science 246, 1412-1416.
[13] Hull, R.N., Cherry, W.R., and Weaver, G.W. (1976) In Vitro 12, 670-677.
[14] Lever, J.E. (1979) Proc. Natl. Acad. Sci. USA 76, 1323-1327.
[15] Amsler, K. and Cook, J.S. (1982) Am. J. Physiol. 242, C94-C101.
[16] Handler, J.S. (1986) Kidney Int. 30, 208-215.
[17] Van Den Bosch, L., De Smedt, H. and Borghraef (1989) J. Cell. Physiol. 141, 483-489.
[18] Miller, J.H. and Heath, L.N. (1989) J. Cell. Physiol. 139, 538-549.
[19] Rabito, C.A., Kreisberg, J.I. and Wight, D. (1984) J. Biol. Chem. 259, 574-582.