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A VINCRISTINE-RESISTANT MURINE ERYTHROLEUKEMIA CELL LINE SECRETES A DIFFERENTIATION ENHANCING FACTOR

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A clone of vincristine resistant murine erythroleukemia cells V3.17[44], characterized by high sensitivity to terminal erythroid differentiation induced by hexamethylene bisacetamide, secretes into the extracellular medium a protein factor which partially reduces the latent period before commitment and accelerates the expression of the terminal differentiated phenotype in a slow responding murine erythroleukemia N23 cell variant. This differentiation enhancing factor increases the rate of protein kinase C down-regulation which occurs at slower rate during cell differentiation. The activity of the factor is detected either by co-culture of the two cell line variants or by addition of conditioned medium from V3.17[44] cells to a culture of N23 cells in the presence of the inducer. In addition to being secreted by V3.17[44] cells, this factor can also be detected in the cytoplasm of both V3.17[44] and N23 cells, associated with a particulate fraction from which it can be released by sonication.

Hexamethylene bisacetamide (HMBA) induced murine erythroleukemia (MEL) cell differentiation (1,2) is a multistep process (3,4), characterized by an early latent period of commitment (5,6), followed by a period during which the characteristics of terminal differentiation are expressed. In MEL cell lines, developed for low levels of vincristine (VC) resistance, an increased sensitivity to HMBA, as well as the suppression of the latent period has been observed (7). These VC-resistant clones were found to be enriched in protein kinase C (PKC) activity, in particular that of PKCβ isotype, the amount of which correlated with the acceleration of the rate of HMBA induced differentiation (8). Moreover, introduction of the homologous PKCβ isoform resulted in the increase in the rate of differentiation in a slow-responding MEL cell clone (9). In this paper we provide evidence for an additional component of the multistep differentiating process, a MEL cell factor which promotes an increase in the rate of HMBA-mediated induction of MEL cell differentiation. This differentiation enhancing factor (DEF) is present in the cytoplasm of the two cell lines studied and can also be released into the extra-

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cellular medium but only by the VC-resistant variant which rapidly responds to induction by HMBA. Co-culture of N23 cells with the VC-resistant V3.17[44] variant or the addition of V3.17 [44] conditioned medium, as well as that of a purified preparation of the factor, to cultures of the slowly inducing N23 cell line, accelerates HMBA-mediated induction of N23 cells with a partial reduction of the latent period before commitment.

Materials and Methods

Cell culture. N23 and the VC-resistant V3.17[44] MEL cell clones were obtained from DS-19 line, as previously described (8,10). In the standard condition the cell culture was started with an innoculum of 10E5 cells/ml in flasks containing αminimal essential medium supplemented with 10 % (v:v) fetal calf serum. Cultures in the Transwell plate (Costar, U.S.A.), equipped with a 0.4 μm pore size membrane, were started with an innoculum of 10E5 cells/ml, 2.5 ml, in the lower compartment and the same number of cells in the same volume in the upper compartment.
Assay of MEL cell commitment. Commitment was assayed as described (6).
Preparation of subcellular fractions. The MEL cell culture, 10 ml containing 10E7 cells, was collected, the cells washed with 10 ml of 0.25 M sucrose and then lysed by sonication (4 strokes of 10 sec each) at 5°C. The lysate was centrifuged at 150,000xg for 10 min; the supernatant (soluble fraction) was collected and the pellet (membrane-particulate fraction) was suspended in 1 ml of 0.25 M sucrose and dispersed by sonication (two strokes). Alternatively, the same aliquot of cells in 0.25 M sucrose was lysed with 70 strokes in a Teflon-glass homogenizer at 5 °C and centrifuged at 800xg for 15 min. The supernatant was collected and centrifuged at 10,000xg for 20 min. The supernatant was then removed and centrifuged at 150,000xg for 10 min and the resulting clear solution was collected and defined as cytosolic fraction. The pellets obtained from the second and third centrifugation were pooled, suspended in 1 ml of 0.25 M sucrose, sonicated with 4 strokes of 10 sec each at 5 °C to disrupt any subcellular organelle (particulate fraction).
Assay of the activity of the differentiation enhancing factor (DEF). N23 MEL cells (10E5 cells/ml), were incubated for 72 hrs in standard conditions with 5 mM HMBA in the absence or presence of DEF. The differentiated cells were identified by staining with benzidine (6). One unit of DEF activity is defined as the amount which doubles the proportion of benzidine reactive cells in the specified conditions.
Purification of DEF. The conditioned medium (11) was collected when V3.17[44] MEL cells were at a density of 10E6 cells/ml. The cells were discarded by centrifugation and the supernatant was treated with 50 % saturated ammonium sulfate at 5° C. The precipitated material, collected by centrifugation at 10,000xg for 15 min, was suspended in 20 ml of 20 mM sodium-phosphate, pH 7.0 (buffer A) and dialyzed overnight against the same buffer. The solution was then loaded onto a phosphocellulose column (1.5x8 cm) previously equilibrated with buffer A. The adsorbed proteins were eluted with a linear gradient of NaCl from 0 to 200 mM. The flow rate was 0.25 ml/min and fractions of 2 ml were collected. The fractions containing the peak of DEF activity, eluted between 80 and 100 mM NaCl, were pooled, concentrated by ultrafiltration to 4 ml on an Amicon YM10 membrane and dialyzed against buffer A. The resulting DEF preparation showed a specific activity of 454 units/mg. The partially purified material was submitted to hydrophobic chromatography on a column of butyl agarose (1x3 cm) previously equilibrated with buffer A. DEF activity was not retained by the column and totally recovered in the washing fractions. The specific activity of this DEF preparation was 3,700 units/mg. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (11).

Results

Effect of V3.17[44] conditioned medium on the inducibility of N23 MEL cells. Two MEL cell clones, one characterized by a low rate of induced differentiation (N23) and one with a high rate of induced differentiation (V3.17[44]), were co-cultured in the presence of 5 mM HMBA in Transwell plates (see Methods), which permit free exchange of extracellular medium without direct contact betweeen the two variant cell lines. In these conditions, as shown in Fig.1A, the rate of differentiation, measured following the accumulation of benzidine reactive cells, of the slow responding variant N23 is accelerated 2 to 3 fold. The stimulatory efficiency is independent of the position of the two variants in the upper or lower compartment of the Transwell culture, and the induction of N23 cells is not influenced by co-culture with homologous N23 cells. As shown in Fig.1B an additional effect, which results from the co-culture of the two clones, is the decrease in N23 cells of the latent period prior to the onset of commitment to terminal differentiation from 12 hrs to about 8-9 hrs. Similar effects on the kinetics of N23 cell induced differentiation have been observed following the addition of 0.1 ml aliquots of the V3.17[44] conditioned medium to 1 ml of a N23 cell culture, in the presence of the inducer. The addition results in a more rapid accumulation of differentiated cells, as compared with cells cultured in fresh medium only (Fig.1C). After 72 hours of incubation N23 cells induced in standard conditions show approximately 9-11% benzidine-reactive cells, whereas N23 induced in the presence of the V3.17[44] cells conditioned medium show 26-30% benzidine-reactive cells. Similar differences in the rate of differentiation are observed at longer period of incubation. As observed in co-culture experiments (see Fig.1B), also in these conditions the onset of commitment of N23 MEL cells is reduced from 12 to about 8 hrs. These results suggest that V3.17[44] MEL cell clone secretes in the extracellular medium a differentiation enhancing factor, defined DEF, which stimulates HMBA-induced differentiation of the slow responsive clone N23. To better define this effect of V3.17[44] conditioned medium, we have evaluated the accumulation of DEF activity during the growth of this MEL cell clone. As shown in Fig.2, maximum DEF activity is detected in the V3.17[44] medium collected between

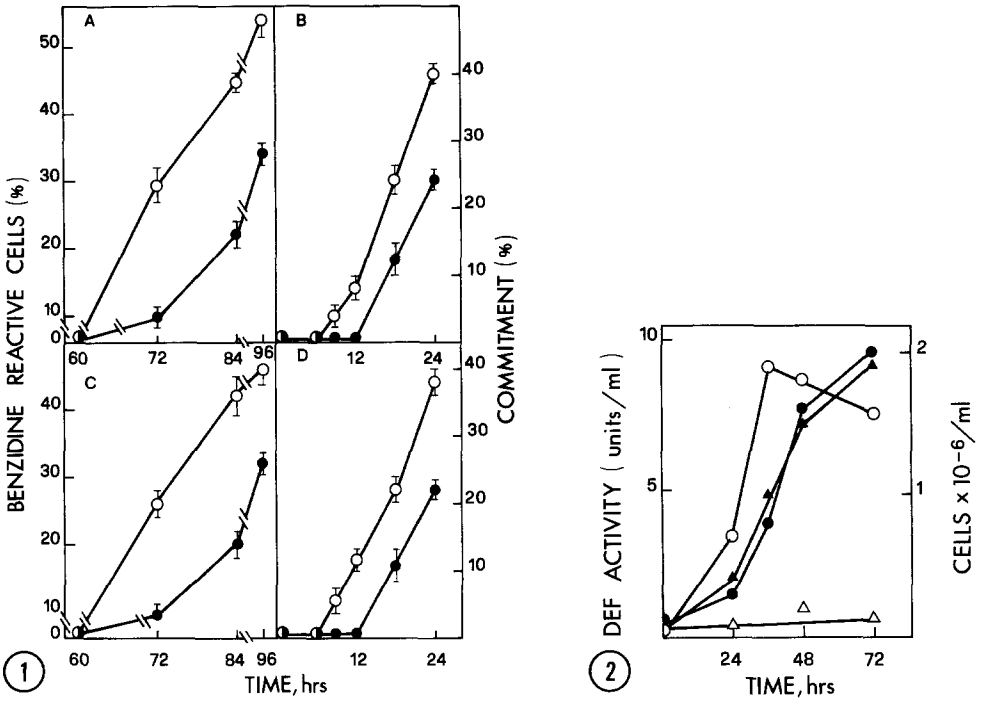

Fig.1. Inducibility of N23 cells cultured in the presence of V3.17[44] MEL cells or V3.17[44] conditioned medium. A, N23 and V3.17[44] cells were cultured in the two compartments of a Transwell plate as described under Methods. HMBA was immediately added to both compartments at a final concentration of 5 mM and, at the times indicated, samples of 0.1 ml of N23 cell suspension were removed and the cells stained with benzidine as reported under Methods (○). As control, the rate of induced differentiation was measured for N23 cells cultured in both compartments of a Transwell plate (●). B, The commitment of N23 MEL cells, cultured as in A, was evaluated as described in Methods, either for N23 cultured in Transwell with V3.17[44] cells (○), or N23 cultured in both compartments of the Transwell plate (●). C, N23 MEL cells were placed, at a cell density of 10E5 cells/ml, in a culture flask containing 5 mM HMBA and 0.1 ml/ml of conditioned medium obtained from a culture of V3.17[44] MEL cells which had reached a density of 10E6 cells/ml. At the times indicated 0.1ml of the cell suspension were collected and the cells stained with benzidine (○). As control N23 cells were cultured in the absence of V3.17[44] conditioned medium (●). D, N23 cells were cultured as in C. At the times indicated the cell commitment was evaluated as described in Methods. (○), N23 cells cultured in the presence of V3.17 [44] conditioned medium; (●), N23 cells controls. Each experiment was done in duplicate. The values given are the arithmetical mean.

Fig.2. DEF activity in the conditioned medium from V3.17[44] and N23 MEL cells. V3.17 [44] and N23 MEL cells were inoculated in culture flasks at a density of 10E5 cells/ml. At the times indicated the cell density was determined either for N23 (▲), or V3.17[44] (●) MEL cells. At the same times samples (0.1ml) of the conditioned medium from the two cell cultures were assayed for DEF activity : (○), V3.17[44] cells; (△), N23 cells.

30-40 hrs of culture, when a maximal rate of cell growth is also observed. The conditioned medium from N23 cell culture shows little or no DEF activity.

Molecular properties of DEF. DEF has been purified (approximately 1,000 fold), see Methods, from the conditioned medium of V3.17[44] MEL cells, and aliquots submitted to SDS-PAGE. As shown in Fig.3, the preparation contains a major protein band

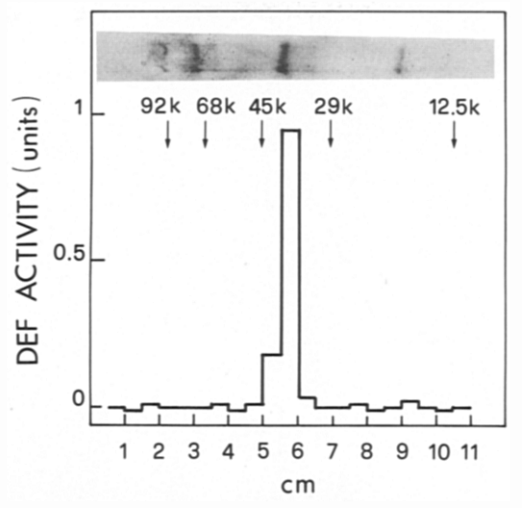

Fig.3. SDS-PAGE of DEF purified from the conditioned medium of V3.17[44] MEL cells. A sample (10 μg) of DEF obtained from chromatography on butyl agarose (see Methods) was loaded onto a 8% SDS-slab gel. The electrophoretic run was 4 hrs at 100 mA. Then the gel was immersed in buffer A, containing 20% methanol, to remove the excess of SDS. After 20 min the gel was transferred in buffer A and gently stirred for additional 20 min. Then it was cutted into 0.4 cm slices. Each slice was homogenized in 0.3 ml of buffer A. The gel was removed by centrifugation while the supernatant was assayed, with 0.05 ml,for DEF activity .

which, following its elution from the gel, expresses DEF activity. Two minor contaminating protein bands contain no detectable DEF activity. The molecular size of DEF, in these denaturing conditions, is approximately 35-38 Kd and its protein nature has been established by its inactivation following exposure to trypsin or pronase (data not shown).

Table I

Intracellular localization of DEF activity

Clone	Cell lysis with	Subcellular fraction	DEF Activity (units/10E6 cells)
V3.17[44]	sonicator	soluble	70.0±10.4
		membrane-particulate	n.d.
	Teflon-glass homogenizer	cytosolic	1.8±0.4
		particulate	65.5±8.2
N23	sonicator	soluble	25.8±3.0
		membrane-particulate	n.d.
	Teflon-glass homogenizer	cytosolic	1.0±0.20
		particulate	23.0±3.0

n.d.= not detectable.
The cells were lysed and the subcellular fractions were isolated as described in Methods.

Intracellular distribution of DEF. When V3.17[44] cells are lysed by sonication, DEF activity is completely recovered in the soluble fraction, none being found in the membrane-particulate fraction (Table I). To better define the localization of DEF, V3.17[44] cells were lysed with a Teflon-glass homogenizer in isotonic medium, to prevent disruption of intracellular organelles. In these conditions, see Table I, DEF activity is recovered in the particulate fraction, containing all the cell organelles, while less than 3% is found in the soluble fraction. These results indicate that DEF activity is probably segregated into intracellular vesicles, which are disrupted by sonication. Also N23 cells contain, in an identical intracellular localization, DEF activity in amounts however corresponding to only 30% of that present in the V3.17[44] cells. No DEF activity could be detected in the conditioned medium or in the subcellular fractions of another malignant murine cell line, P3 myeloma cells (data not shown).

Effect of DEF on down regulation of protein kinase C. We have previously (8) shown that in MEL cells, in the course of HMBA induced differentiation, a progressive decrease in total protein kinase C activity occurs and that the rate of this down-regulation process is correlated to that of cell differentiation. We have now investigated if the addition of DEF to N23 differentiating cells had some effect on the rate of down-regulation of PKC. As shown in Fig.4, addition of purified DEF

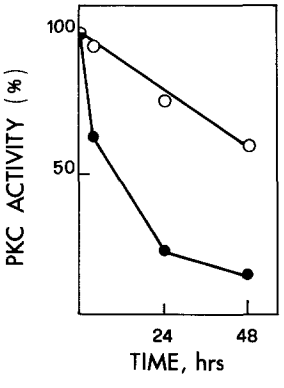

Fig.4. Effect of DEF on down-regulation of PKC in N23 cells induced with HMBA. N23 cells were cultured (starting from a cell density of 2X10E5 cells/ml) in the presence of 5 mM HMBA and 0.01 ml/ml of purified DEF. At the times indicated, 4X10E7 cells were collected and cytosolic PKC activity was determined by DEAE-chromatography (8) (●). For control the same cell culture was performed in the absence of DEF and PKC activity was determined as above (○).

to N23 MEL cells exposed to HMBA produces an acceleration in the rate of down-regulation of PKC activity as well as a decrease in total kinase activity that are four times higher than that observed in the absence of the factor.

Discussion

A protein factor has been identified which promotes an acceleration in the rate of terminal differentiation of MEL cells accompanied by a partial reduction in the latent period which preceeds commitment. The factor (DEF) was found to be present in association with the particulate fraction of all MEL cell clones, although in greater amount in a VC-resistant clone, the only one in which DEF is secreted in the extracellular medium. In the presence of phorbol 12-myristate 13-acetate, known to act as an activator of PKC (12-18) and of the cell secretory mechanisms (19-24), DEF is recovered in the extracellular medium of all MEL cell clones (data not shown). DEF promotes when added to low responding MEL cells, an accelerated rate of PKC down- regulation, which occurs, although at much slower rate, when these cells are exposed to the inducer (8). This observation suggest an involvement of DEF in the modulation of intracellular PKC activity. This suggestion is strongly supported by a recent report concerning the identification of a protein factor, secreted by V3.17[44] MEL cell clone, found to be a potent activator of PKC activity and specifically of the β isoform (25). This PKC activator as well as DEF share common properties including copurification in all steps throughout the purification procedures suggesting that the same protein molecule may act as an enhancer of MEL cell differentiation and as an activator of PKC.

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