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PARTIAL PURIFICATION OF NOVEL DIFFERENTIATION-INDUCING SUBSTANCE(S) FROM HOT WATER EXTRACT OF JAPANESE PINE CONE

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The effects of hot-water extract of pine cone (PCE) of *Pinus parviflora* Sieb. et Zucc. on the growth and differentiation of ML-1 cells, derived from a patient with human myeloblastic leukemia, were investigated. Growth of ML-1 cells was slightly inhibited at 3% (v/v) PCE, and a cytotoxic effect appeared at >10%. Growth inhibition was accompanied by conversion to morphologically macrophage-like cells with α-naphthyl acetate esterase activity. In contrast, PCE dose-dependently increased the Fc receptor and nitroblue tetrazolium (NBT)-reducing activity up to 3%; above 3% its effect declined. Most of the cytotoxic activity was extracted from PCE with ethanol, and separated from the insoluble pellet, which contained the differentiation-inducing activity. The differentiation-inducing activity was eluted near the void volume on Sephadex G-200 gel filtration, with a 260-fold increase in the specific activity.

Key words: Differentiation — Cytotoxicity — Human leukemic cells — Pine cone extract — Partial purification

About 20–30 years ago, oral administration of hot water extract of pine cone (PCE) of *Pinus parviflora* Sieb. et Zucc. significantly improved the condition of patients who had stomach cancer, and many of them recovered from the disease (Mr. T. Mori, personal communication). Although this story has not been officially documented, administration of this extract has been popular since then, especially in the Kyushu district. However, no investigator has yet reported biochemical analysis of the substance(s) that might be responsible for these effects. Therefore, we initiated a study to identify various components of PCE that might be helpful in cancer therapy. As a part of this study, we report here the partial purification from PCE of novel differentiation-inducing substances that induce the differentiation of a human myeloblastic leukemic cell line, ML-1, into macrophage-like cells.

Materials and Methods

Cell Culture ML-1 cells were cultured in RPMI1640 medium (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO), as described previously.[1]

Partial Purification of Differentiation-inducing Substances Fifty-three pine cones of *Pinus parviflora* Sieb. et Zucc. (235 g) were boiled in 4,000 ml of distilled water. The volume was reduced to 2,000 ml by continuous heating, then the supernatant (PCE) was obtained by centrifugation at 10,000*g* for 30 min at 20°. The osmolarity of the PCE was 38.5 mOsm; this was adjusted to 290 mOsm by adding solid NaCl for some experiments. The PCE (50 ml) was evaporated under vacuum in a rotary evaporator (Tokyo Rikakikai Co.) and the residue was suspended in ethanol (Wako Co.) and stirred overnight in a cold room. After centrifugation (1,000*g*, 10 min), the supernatant (ethanol-soluble fraction) was saved. The pellet (ethanol-insoluble materials) was dissolved in 50 ml of distilled water and filtered through a CF25 ultrafiltration membrane cone (Amicon, USA). The materials, concentrated to 2.5 ml by means of the membrane cone (MW> 25,000), were applied to a column of Sephadex G-200 superfine (Pharmacia, Uppsala) (2.5×32 cm), equilibrated with 0.9% NaCl, and each fraction (80 drops) was collected.

The materials that passed through the CF25 membrane cone (MW <25,000) were further filtered through a UK-10 ultrafilter (Toyo Roshi Co.). The materials retained on the filter (25,000> MW>10,000) were suspended in 50 ml of distilled water.

Assay of Cell Growth and Differentiation-associated Characteristics Cell growth was assayed with a hemocytometer, and viability was estimated by evaluating trypan blue dye exclusion.

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Using this criterion, control cultures were routinely found to contain >95% viable cells.

Changes in morphology were assessed by preparing smear slides followed by staining with May-Grunwald-Giemsa (Merck, Darmstadt). At least 200 cells in each preparation were examined with a light microscope.[1] Intermediate and mature monocyte-macrophages were classified together as maturing cells.

Naphthol AS-D chloroacetate esterase and α-naphthyl acetate esterase activities were examined in smear preparations according to the procedure specified in Sigma Technical Bulletin No. 90.

Assay of the appearance of Fc receptor was performed using standard techniques for erythrocyte-antibody rosette formation, as described previously.[2] Viable cells with at least 10 attached erythrocytes were scored as Fc receptor-positive.

NBT-reducing ability was measured as described previously.[1] One unit of differentiation-inducing activity was defined as the dry weight (mg/ml) necessary to cause 50% (midpoint of the ordinate of the titration curve) of maximal differentiation, assayed with the NBT reagent. The dry weight of each sample was measured in a weighing bottle after complete evaporation of water in a desiccator.

Results

Effect of PCE on the Growth and Differentiation of ML-1 Cells When ML-1 cells were treated with increasing amounts of PCE, their growth was dose-dependently inhibited. Slight growth inhibition was observed up to 3% (v/v) PCE (0.258 mg dry weight/ml) and a cytotoxic effect appeared above 10% (0.86 mg dry weight/ml) (Fig. 1). At 30% (2.58 mg dry weight/ml), about 50% of the cell suspensions were stained with trypan blue within 24 hr. Control ML-1 cells were predominantly typical myeloblasts with large round nuclei, each containing prominent nucleoli and few cytoplasmic azurophilic granules. The nuclear/cytoplasmic ratio was relatively high (Fig. 2A). PCE-treated cells exhibited the following changes: smaller nuclear size, decreased nuclear/cytoplasmic ra-

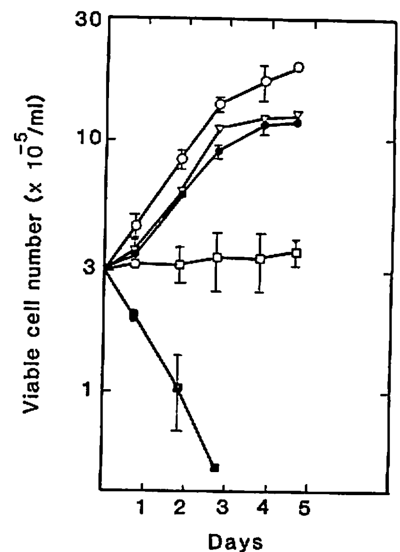


Fig. 1. Effect of concentration of PCE on the growth of ML-1 cells. ML-1 cells (3×10^5/ml) were incubated with 0 (○), 1 (▽), 3 (●), 10 (□) or 30% (■) PCE for the indicated periods after isotonization of PCE with solid NaCl, and the viable cell number was determined. Each point represents the mean ± SE of three independent experiments.

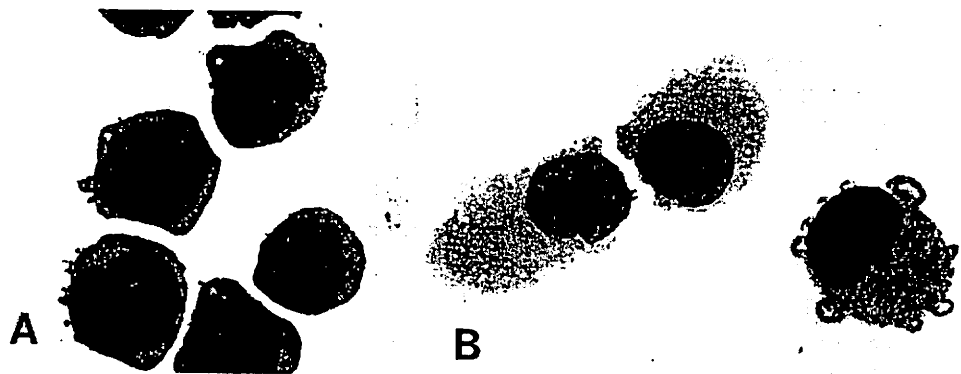


Fig. 2. Morphology of control and differentiated ML-1 cells. (A) Control ML-1 cells. (B) ML-1 cells treated for 5 days with 10% PCE. May-Grunwald-Giemsa. × 1,000.

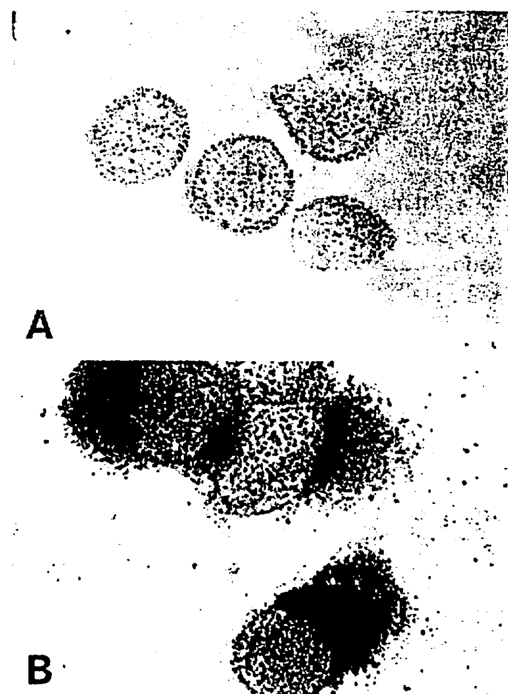


Fig. 3. Induction of α-naphthyl acetate esterase-positive cells by PCE treatment. (A) Control ML-1 cells. (B) ML-1 cells treated for 5 days with 10% PCE. Smear preparations were stained in α-naphthyl acetate solution as described in "Materials and Methods." Note the appearance of cytoplasmic α-naphthyl acetate esterase in PCE-treated cells. $\times$1000.

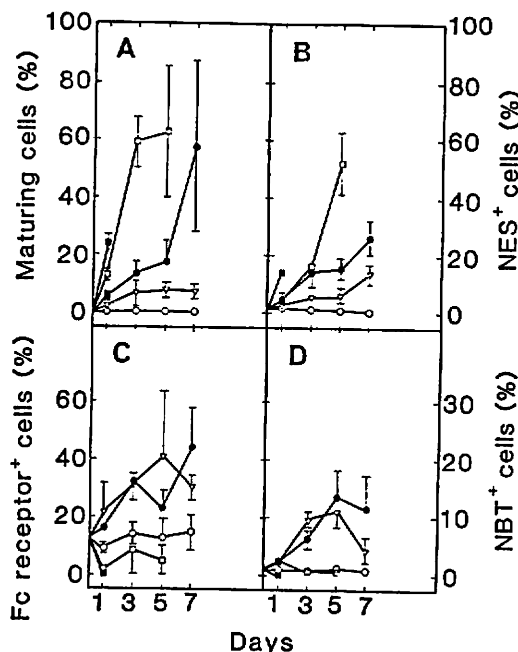
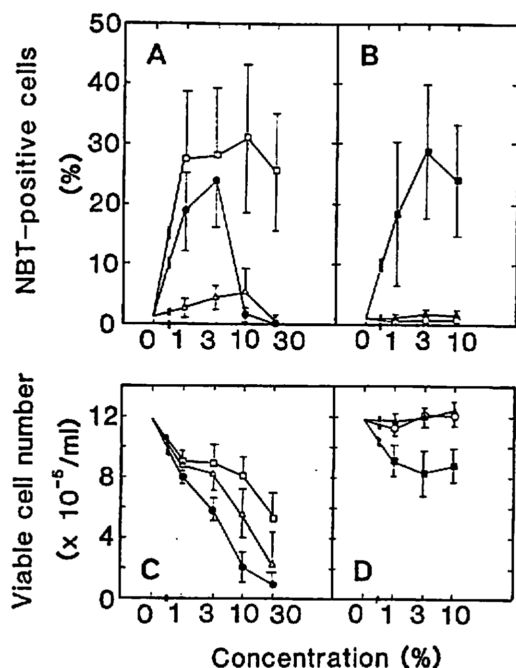


Fig. 4. Effects of PCE on expression of differentiation-associated markers in ML-1 cells. ML-1 cells (3×10^5/ml) were incubated with 0 (○), 1 (▽), 3 (●), 10 (□), or 30% (■) PCE for the indicated periods after isotonization of PCE with solid NaCl, and the percentages of morphologically maturing macrophage-like cells (A), α-naphthyl acetate esterase (NES)-positive cells (B), Fc receptor-positive cells (C) and NBT-positive cells (D) were then determined. Each point represents the mean$\pm$SE of three independent experiments.

tio, and marked reduction of nucleoli (Fig. 2B). Differentiation toward the monocyte/macrophage stage was confirmed by the appearance of α-naphthyl acetate esterase-positive cells (Fig. 3) without induction of naphthol AS-D chloroacetate esterase activity (data not shown). Morphological maturation and the appearance of α-naphthyl acetate esterase-positive cells increased with increasing concentration of PCE, as did the growth inhibition (Figs. 1 and 4A, B). Low doses of PCE (up to 3%) also increased Fc receptor and NBT-reducing activity after a prolonged incubation time (Fig. 4C, D). However, the expression of these activities was inhibited dramatically by higher PCE concentrations.

Removal of Cytotoxic Substances by Ethanol Extraction We thought that the inhibitory effect of high doses of PCE on Fc receptor and NBT-reducing activity might be due to the action of contaminating cytotoxic substance(s). As expected, most of the cytotoxic activity, which had little differentiation-inducing activity, was recovered from an ethanol-extractable fraction (2.72 mg dry weight/ml PCE), whereas most of the NBT-reducing activity was recovered from the residual fraction (4.80 mg dry weight/ml PCE) (Fig. 5A, C). After removal of cytotoxic substances, the NBT reducing activity in the residual fraction remained at the maximum level at concentrations up to 30% (1.44 mg/ml) (Fig. 5A).

Other organic solvents, such as n-butanol, acetone, and chloroform, also efficiently extracted the cytotoxic substances from PCE and minor impurities present in these sol-



vents had no effect on the growth or differentiation of control ML-1 cells (data not shown). No significant amount of cytotoxic substance was extracted by ethyl ether.

Fig. 5. Separation of differentiation-inducing substance(s) by ethanol extraction and centrifugation through two different filters. Cytotoxic substances were removed from 50 ml of PCE (●) with absolute ethanol (△) (A, C) and the residual pellet, containing differentiation-inducing activity (□), was further fractionated by filtration through the CF25 membrane cone and UK10 filter into three fractions: I, MW<10,000 (○); II, 10,000<MW<25,000 (▲); III, MW>25,000 (■); as described in "Materials and Methods" (B, D). The volume of fractions was adjusted to 50 ml after isotonization with solid NaCl. ML-1 cells (3×10^5/ml) were incubated for 3 days with the indicated concentrations (v/v) of fractions, and the percentage of NBT-positive cells (A, B) and numbers of viable cells (C, D) were then determined. Each point represents the mean±SE of three independent experiments.

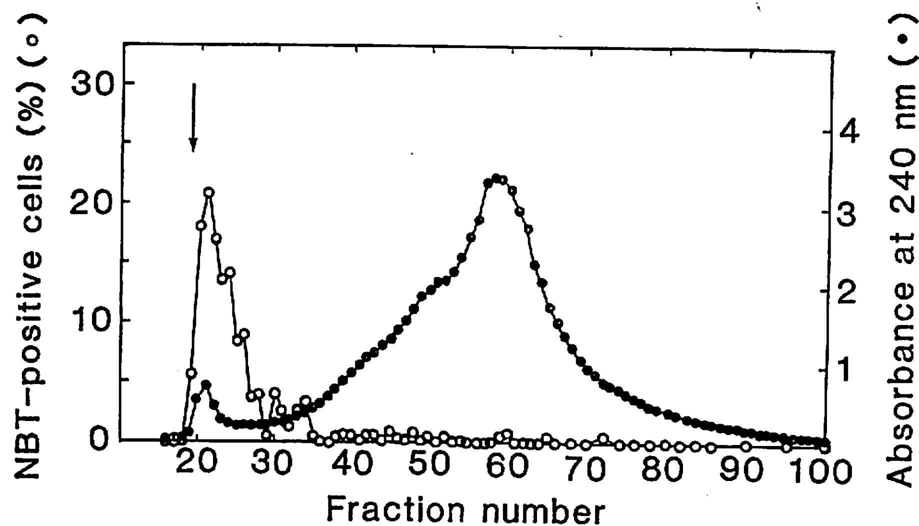


Fig. 6. Partial purification of differentiation-inducing substance(s) by Sephadex G-200 gel filtration chromatography. The active fraction (which increased the percentage of NBT-positive cells) was concentrated to 2.5 ml by filtration through a CF25 membrane cone and applied to Sephadex G-200 as described in "Materials and Methods." ML-1 cells (3×10^5/ml) were treated for 3 days with 10% (v/v) of each fraction, and the percentage of NBT-positive cells (○) was then determined. (●) Absorbance at 240 nm. The arrow indicates the void volume determined by using blue dextran 2,000. Similar elution patterns were obtained in two other independent experiments.

Table I. Purification of Differentiation-inducing Substance(s) from PCE

Purification step	Dry weight (mg)	Total activity (units)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
Pine cone	5877				
1. PCE (water extraction)	430	3125	7.3	1	100
2. Ethanol precipitation	240[a]	4347	18.1	2.5	139
3. CF25 filtration	63[a]	5868	93.1	12.8	188
4. Sephadex G-200	2.4[b]	4522	1884.2	258.1	145

Fifty ml of PCE was used as starting material for small scale purification. One unit was defined as the dry weight (mg/ml) necessary to produce 50% (midpoint of the ordinate of the titration curve) of maximal differentiation; assayed with NBT reagent. Specific activity at each purification step was determined at the time of using cells from the same suspension.

a) Measured after resuspension in distilled water.

b) Measured after removal of NaCl through a CF25 membrane cone and resuspension of the product in distilled water.

Partial Purification of Differentiation-inducing Substance(s) The residual (ethanol-insoluble) fraction was further fractionated by successive filtration through a CF25 membrane cone and a UK-10 ultrafilter into the following three fractions (I (MW<10,000), II (10,000<MW<25,000), III (MW>25,000)). Fig. 5B, D shows that the differentiation-inducing substances, which induced NBT-reducing activity and inhibited ML-1 cell growth, had a molecular weight exceeding 25,000.

The substance retained on the CF25 membrane cone (MW>25,000) was subjected to Sephadex G-200 gel filtration chromatography. As shown in Fig. 6, the differentiation-inducing activity was eluted near the void volume (fr. 20–28). This fraction (as little as 1.0 μg/ml) had the ability to induce α-naphthyl acetate esterase activity and to accelerate cell-to-cell aggregation induced by phorbol esters (data not shown). The biological activity of this fraction did not diminish after heat treatment (100°, 6 hr). Table I shows that the differentiation-inducing activity was purified approximately 260-fold after ethanol extraction and gel filtration.

Discussion

The present study revealed that PCE contains both differentiation-inducing and cytotoxic substances, which could be separated from each other by ethanol extraction. The presence of these substances was confirmed in the PCEs of another two pine cones, which had been harvested on different occasions. The differentiation-inducing substance(s) described here was heat-stable, ethanol-insoluble and had extremely high molecular weight. These properties discriminate the substances from previously reported differentiation-inducing factors (purified from an organism,[3] conditioned medium of an organ[4] and cultured normal and leukemic cells[5–8]), which have lower molecular weights (ranging from 270 to 62,000) and are sensitive to heat treatment, as well as from mitogens (concanavalin A and pokeweed mitogen), which have little or no differentiation-inducing activity against ML-1 cells (data not shown), and from phorbol esters, which are soluble in organic solvents.

Repeated experiments revealed that the yield of differentiation-inducing activity increased from 1.4- to 10-fold after ethanol extraction. This suggests that some cytotoxic substance(s) might be present in the ethanol-soluble fraction. It would be interesting to test whether this fraction contains antitumor activity similar to that isolated from other plants by methanol extraction.[9–11] Further purification and characterization of the differentiation-inducing substance(s) as well as the cytotoxic substance(s) described in this paper are in progress in our laboratory.

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