Induction of Differentiation of the Human Promyelocytic Cell Line (HL-60) by Conditioned Medium of *Ceathea letifera*-Stimulated Mononuclear Cells *

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Introduction

Studies by some investigators have shown that human leukemic cell lines, primarily of myeloid lineage, retain their ability to differentiate in vitro when exposed to a variety of compounds such as differentiation-inducing factors present in conditioned medium (CM) including retinoic acid [1], phorbol diesters [2], dimethyl sulfoxide, *Clerodendron fragrans* [3], and when cocultured in CMs secreted from lectin-stimulated lymphocytes or Chinese herb-stimulated mononuclear cells [4]. Recently, it became apparent from other studies that interferon-γ (IFN-γ), colony-stimulating factor (CSF), and interleukin-2 (IL-2) have been identified in CM and have been found to express some of their effects by inducing differentiation. However, there is also an unidentified differentiation-inducing activity (DIA) distinct from the above well-known factors that has a similar effect [4].

There are only a few papers reporting CM of Chinese herb-stimulated mononuclear cells to have a capacity to induce differentiation of HL-60 cells. In the present study, we report another CM, called CL-CM, secreted from *Ceathea letifera*-stimulated mononuclear cells, which has the capacity to induce HL-60 cells to differentiate into mature cells.

Material and Methods

Conditioned Media. Human peripheral blood mononuclear cells, separated on a Ficoll-Hypaque gradient, were incubated in RPMI 1640 medium (10^6 cells/ml), either with or without 10% fetal bovine serum (FBS), supplemented with *Ceathea letifera* (1 mg/ml), phytohemagglutinin (PHA; 10 μg/ml), concanavalin A (Con A; 125 μg/ml), and pokeweed mitogen (PWM; 10 μg/ml), for 72 h at 37 °C in 5% CO_2 in air. Cell-free supernatants were collected and used as crude preparations denoted CL-CMF$^+$ (containing 10% FBS), CL-CMF$^-$ (serum-free), PHA-CMF$^+$, PHA-CMF$^-$, ConA-CMF$^+$, ConA-CMF$^-$, PWM-CMF$^+$, and PWM-CMF$^-$. The CL-CMF$^+$ and CL-CMF$^-$ media were shown to have the capacity to induce HL-60 cells to develop into mature monocytes.

Crude Extract of *Ceathea letifera*. The extract was dissolved in 50% ethanol at 40 °C, and concentrated to 1:20 at negative pressure in RPMI 1640. This Chinese herb used as a mitogen was cultured with normal mononuclear cells (10^6 cells/ml) in serum-free RPMI 1640 for 3 days. The resulting CM (called CL-CMF$^-$) was obtained and then cultured with HL-60 cells in RPMI containing 10% FBS for 5 days. The ratio of the volume of CM to

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10% FBS-containing RPMI medium was 3 to 7. In addition, IFN-γ, TNF, and IL-2 (Genzyme, USA) were added to the CL-CM to examine the additive or synergistic effects on differentiation of HL-60 cells. Parameters which identified the differentiation of treated HL-60 cells and analysed the factors in CL-CM included the following:

(a) cell number;
(b) cell viability;
(c) phagocytosis and nitroblue tetrozolium test (NBT) [4];
(d) surface marker study – Mo1 and Mo2, using an indirect immunofluorescence method;
(e) cytochemistry test (POS, PAS, CES, and NES);
(f) the contents of IL-1, IL-2, TNF, and IFN-γ using ELISA kits;
(g) assay for granulocyte-macrophage colony-forming cells (CFU-GM) [5];
(h) sodium dodecylsulphate–polyacylamide gel electrophoresis (SDS-PAGE) [6].

Results

Viability and Cell Number. Table 1 shows that cells cultured in CL-CMF$^-$, IFN-γ, TNF, and IL-2 or in a combination with CL-CMF$^-$ with IFN-γ, TNF, or IL-2 showed no evidence of a decreased cell number in comparison with control on the 3rd and 5th days of culture; in addition, there was a slightly lower cell viability compared with controls on the 3rd and 5th days of culture.

Differentiation Effects. Following treatment with CL-CMF$^-$ for 5 days, about 50% of the HL-60 cells underwent a morphological change from promyelocytes to mature monocytoid cells. Cytochemical studies revealed that these cells were 22% positive for NES stain but negative for CES, POS, and PAS stains. These cells became phagocytic (38% $\pm$ 8.3%) (Table 2) but only 5% $\pm$ 25% positive for NBT. Correspondingly, 28% $\pm$ 6.4% and 10% $\pm$ 1.5% of the treated cells were positive for Mo1 and Mo2 antibodies, respectively (Table 2).

The biological response modifiers IFN-γ, TNF, and IL-2 (Genzyme, USA) were added to the CL-CMF$^-$ to examine the additive or synergistic effects on differentiation. Our results showed that a combination of CL-CMF$^-$ with TNF, IFN-γ, or IL-2 has an enhanced differentiation-inducing effect on HL-60 cells according to surface marker studies (Table 2).

TNF, IFN-γ, IL-1, and IL-2 Contents. ELISA test kits were used to detect the concentrations of TNF, IFN-γ, IL-1 and

Table 1. Cellularity (cell) and viability (viab) of HL-60 cells treated by various compounds

	Day 1		Day 3		Day 5	
	cell	viab	cell	viab	cell	viab
Control	53 $\pm$ 8	98 $\pm$ 2	90 $\pm$ 8	96 $\pm$ 1	172 $\pm$ 12	94 $\pm$ 1
CL–CM	45 $\pm$ 5	97 $\pm$ 2	82 $\pm$ 8	95 $\pm$ 3	138 $\pm$ 10	93 $\pm$ 2
TNF	45 $\pm$ 7	94 $\pm$ 1	99 $\pm$ 4	89 $\pm$ 2	124 $\pm$ 11	84 $\pm$ 2
IFN-γ	51 $\pm$ 6	93 $\pm$ 3	76 $\pm$ 10	90 $\pm$ 4	127 $\pm$ 13	88 $\pm$ 3
IL-2	50 $\pm$ 5	96 $\pm$ 2	93 $\pm$ 8	95 $\pm$ 2	166 $\pm$ 20	90 $\pm$ 1
CL–CM + TNF	43 $\pm$ 4	92 $\pm$ 2	92 $\pm$ 6	87 $\pm$ 3	112 $\pm$ 15	84 $\pm$ 4
CL–CM + IFN-γ	40 $\pm$ 3	95 $\pm$ 2	74 $\pm$ 7	88 $\pm$ 4	110 $\pm$ 14	82 $\pm$ 5
CL–CM + IL-2	51 $\pm$ 5	95 $\pm$ 2	83 $\pm$ 2	93 $\pm$ 3	115 $\pm$ 17	90 $\pm$ 4

Values are (10^4 cells/ml) expressed as mean $\pm$SD, $n = 5$.
CL–CM: Ceathea letifera-conditioned medium.

Table 2. Surface markers Mo1 and Mo2 and phagocytosis of HL-60 cells treated by various compounds

	Day 1			Day 3			Day 5		
	Mo1	Mo2	Phago	Mo1	Mo2	Phago	Mo1	Mo2	Phago
Control	0±0	0±0	2±1	2±1	1±1	3±1	3±1	1±1	4±1
CL−CM	12±3	3±1	20±7	22±5	7±2	25±4	28±6	10±1	38±8
TNF	10±2	2±1	17±3	17±2	7±2	24±6	25±4	12±3	52±8
INF-γ	12±2	7±2	18±4	22±7	13±4	43±7	31±6	20±3	62±5
IL-2	5±1	2±1	6±1	6±3	3±1	13±2	8±2	4±1	24±6
CL−CM+TNF	14±4	5±8	25±5	28±6	12±2	36±1	36±4	17±4	51±7
CL−CM+IFN-γ	16±3	10±3	20±4	27±6	16±4	48±1	44±8	23±6	65±1
CL−CM+IL-2	14±4	4±1	24±6	21±5	10±3	29±7	27±8	15±3	40±6

Values are expressed as percentages, mean ± SD, $n = 5$.
Phago: Phagocytosis.

IL-2 in CL-CM, PHA-CM, ConA-CM, and PWM-CM. The results are shown in Table 3. In the CL-CMF$^+$ which contained 10% FBS in CM, IL-1 at 1320 pg/ml, TNF at 300 mg/mm, and IL-2 at 0.05 L/ml were detected, but no IFN-γ was found. In contrast, in CL-CMF$^-$ which did not contain FBS in CM, only IL-1 at 110 pg/mg was detectable.

Effect of CL-CMF$^-$ on GM-CSF. Table 4 shows the average of three separate experiments in which the effect of CL-CMF$^-$ on granulocyte-macrophage colony formation was tested. On day 7, only a few colony and cluster formations were induced by CL-CMF$^-$ or in the control group, whereas the formation of many colonies was noted when placental CM was added as a colony-stimulating factor.

The Results of SDS-PAGE. Figure 1 shows the 10% SDS-PAGE profile of serum-free CMs using the reducing method. CL-CMF$^-$ (lane 1), RPMI

Fig. 1. 10% SDS-PAGE profile of serum-free CMs using reducing method. *1*, CL-CMF$^-$; *2*, RPMI media; *3*, *Ceathea letifera*

Table 3. Components in the conditioned media with different stimulant

		IFN-γ (U/ml)	TNF (pg/ml)	IL-2 (U/ml)	IL-1 (pg/ml)
PHA	+	0	300	0.05	ND
	−	7	0	0	ND
ConA	+	30	970	0.05	ND
	−	38	710	0	ND
PWN	+	32	750	0	ND
	−	65	225	0	ND
CL	+	0	300	0.05	1320
	−	0	0	0	110

ND: not detectable.

Table 4. Colony-stimulating activity

	CSF (placenta)	CL−CMF$^-$
No. of CFU−GM	86/167	0/24

Data are expressed as colonies versus clusters.
CSF: colony-stimulating factor.

media (lane 2), and *Ceathea letifera* (lane 3), were prepared and analyzed on SDS-PAGE. Lane 1 shows a wide homogeneous band, mainly at 50−60 kDa. There are no such bands in lanes 2 and 3.

Discussion

Traditionally, the Chinese herb *Ceathea letifera* was widely used for treatment of hemorrhage, infection, and diarrhea and also as an antidote for some poisons. The main components have been analyzed and consist of adianton, aspidinol, fernene, filicin, hopene-diploptene, hopanol-29, neritoiol, and tannic acid. To our knowledge, this is the first report describing CM of *Ceathera letifera*-stimulated mononuclear cells to have the capacity to induce differentiation of HL-60 cells into mature cells.

Based on the cytochemical and surface marker studies described here, we can conclude that CL-CM can promote the differentiation of the HL-60 cell line along the monocytic pathway.

We were also interested in the differentiation-inducing activity of CL-CMF$^-$. These data show that components of CL-CMF$^-$ are distinct from well-known differentiation-inducing factors described by others [7–9]. Firstly, no IFN-γ, TNF, IL-2, or CSF was found using ELISA test kits and GM-CSF assay in CL-CM; secondly, combination of CL-CMF$^-$ with TNF, IFN-γ or IL-2 has an enhanced differentiation-inducing effect on HL-60 cells by Mo1 and Mo2 surface marker study; and thirdly, the protein has a molecular weight of 50−60 kDa, as determined using SDS-PAGE, which is different from the CMs of other mitogens such as PHA, ConA-stimulated mononuclear cells, which contained TNF, IFN-γ, and IL-2 (Table 3) [7–9]. Although the nature of this protein remains to be determined, CL-CMF$^-$ is obviously an important candidate for further studies.

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