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Differentiation Induction of Dendritic Cell Phenotypes from Human Leukemic Cell Lines

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Recent clinical studies have shown that a high proportion of patients with acute promyelocytic leukemia (APL) achieve complete remission after treatment with all-trans retinoic acid (ATRA). However, most patients who receive continuous treatment with ATRA relapse and develop ATRA-resistant leukemia. Dendritic cells (DCs) are important antigen-presenting cells in the development of antileukemic T-cell responses. In this study, we investigated the strategies to overcome ATRA resistance of APL cells by inducing the differentiation of DCs from human leukemic cell lines for the developtment of adoptive immunotherapy. CD83 was used as a mature DC marker in this study and the expression of CD83 mRNA was determined by RT-PCR method. The promyelocytic leukemic cell line HL-60, B lymphoblast cell lines RPMI 7666 and NC-37 could be induced to dendritic cells in vitro. Treatment of HL-60 with phorbol 12-myristate 13-acetate (PMA) resulted in the expression of myeloid-related DC phenotypes, while treatment of RPMI 7666 with fms-like tyrosine kinase 3 ligand (Flt3-ligand, FL) and treatment of NC-37 with PMA and FL led to the expression of lymphoid-related DC phenotypes. In conclusion, myeloid-related DC phenotypes and lymphoid-related DC phenotypes could be generated from HL-60, NC-37 and RPMI 7666 cell lines, respectively. These DC phenotypes can potentially be used to generate antileukemic T cells in vitro for adoptive immunotherapy.

Key Words: Leukemia, Adoptive immunotherapy, Dendritic cells (DCs), HL-60 cells, Myristate acetate, Phorbol

INTRODUCTION

Acute promyelocytic leukemia (APL, AML M3) is distinguished from other types of acute myeloid leukemia by a unique chromosomal translocation t (15;17), distinct morphology, distinct clinical features, and a specific clinical response to all-trans retinoic acid (ATRA) (Borrow et al, 1990; de The et al, 1990). ATRA induces differentiation and inhibits proliferation of HL-60 cells and fresh leukemic cells from patients with APL in vitro (Breitman et al, 1980; Koeffler et al, 1983; Kizaki et al, 1993). Recent clinical studies have shown that a high proportion of patients with APL achieve complete remission after treatment with ATRA (Huang et al, 1988; Castaigne et al, 1990; Warrell et al, 1991; Kanamaru et al, 1995). However, most patients who receive continuous treatment with ATRA relapse and develop ATRA- resistant disease (Warrell et al, 1993). The use of adoptive immunotherapy involving expanded antileukemic, cytotoxic T cells may offer a reasonable approach to eliminate residual disease in these patients (Choudhury et al, 1997). Dendritic cells (DCs) are effective antigen-presenting cells, capable of inducing cytotoxic responses from naive T cells (Steinman et al, 1991; Thomas et al, 1993). Considerable interest has developed in the potential use of DCs for the generation of effective cancer immunotherapy (Choudhury et al, 1999). DCs can be generated in vitro from hematopoietic progenitors when cultured in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF, CSF2), tumor necrosis factor-α (TNF-α) and interleukin-4 (IL-4) (Reid et al, 1992; Romani et al, 1994). Administration of Flt-3 ligand (FL) in vivo

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stimulates the outgrowth of functional DCs in mice (Maraskovsky et al, 1996). Koski et al, described that calcium ionophore-treated myeloid cells acquire many dendritic cell characteristics (Koski et al, 1999). HL-60 can be induced to differentiate terminally in vitro. Treatment with phorbol 12-myristate 13-acetate (PMA) leads to the expression of a monocytic-macrophagic phenotypes (Roverta et al, 1979). A number of genes are known to be regulated during HL-60 differentiation: c-*fms* is induced when cell differentiate along the monocyte-macrophage pathway (Sariban et al, 1985). c-*fos* is rapidly induced by PMA (Mitchell et al, 1985; Muller et al, 1985). c-myc is down-regulated by PMA (Einat et al, 1985). To our knowledge, there was no in vitro culture systems that PMA permits the induction of DC phenotypes from human leukemic cell lines including HL-60. Myeloblastin (mbn) is a serine proteinase involved in the control of growth and differentiation of human leukemic cells (Bories et al, 1989). In HL-60 cells, this proteinase mRNA is down-regulated during retinoic acid (RA)-induced differentiation (Bories et al, 1989). Therefore down-regulated expression of mbn mRNA was used as one of the indicator of differentiation. In this study, we showed that DCs can be derived from the human leukemic cell lines under the influence of the PMA or FL.

METHODS

Materials

Calcium ionophore (CI), phorbol 12-myristate-13-acetate (PMA), all-trans-retinoic acid (ATRA), dimethylsulfoxide (DMSO), cholecalciferol, Ficoll, and Hypaque were obtained from Sigma (St. Louis, Mo. USA), RPMI 1640 media were from Gibco BRL (Grand Island, NY, USA), fetal bovine serum (FBS) were from Trace (Austrailia), RNAZol B were from Tel-test (Friendswood, Tx. USA), reverse transcription kit, and agarose were from Promega (Madison WI. USA), Flt3-ligand (FL) were from R&D systems (Minneapolis, MN. USA), Accupower PCR premix, RT/PCR premix, RT premix, oligo-dT, PCR primers and 100 bp ladder DNA size marker were from Bioneer (Korea). All other chemicals used were of the highest grade available.

Generation of DCs from human leukemic cell lines

To generate DCs, HL-60 cells, K-562 cells, NC-37, and RPMI 7666 cells at 2×10^6 cells/ml were cultured in 75-cm^2 tissue culture flasks in RPMI 1640 supplemented with 10% fetal calf serum, 200 units/ml of penicillin, and 200 ug/ml of streptomycin for 7 days, with various combinations of agents or ligands. CI, FL and PMA were added at a final concentration of 180 ng/ml, 1 ng/ml and 200 ng/ml, respectively. A portion of each cells were harvested and the rest of each cells were exposed to the new RPMI 1640 supplemented with 10% fetal calf serum and 1 ng/ml of Flt3-ligand for 7 more days. RNA was extracted from cells and gene expression of each cells were measured by RT-PCR methods. The morphology of the cells were evaluated from cytospin slide preparations with Wright's staining.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

RNA was extracted from cells and gene expression of each cells were measured by RT-PCR methods. Reverse transcription of 4 μg of RNA was performed with Oligo (dT) 16 primers followed by the addition of a reaction mixture containing 5 X RT buffer (250 mM Tris-HCI (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$, O.1 M DTT), M-MLV Reverse Transcriptase and 10 mM deoxynucleoside triphosphates mix (10 mM each of dATP, dGTP, dCTP and dTTP) in a final volume of 20 μl. The mixture was incubated at 42°C for 1 h followed by termination at 94°C for 3 min. Amplification of myeloblastin (mbn), IL-12, IL-15, CD11a, CD83, CSF2, CD11c and MMP-9 was performed with an automatic thermocycler in a reaction mixture containing 400 mM KCl, 10 mM Tris-HCI (pH 9.0), 1.5 mM $MgCl_2$, 250 μM deoxynucleoside triphosphates mixture, I U of Taq DNA polymerase, 10 pM primers (sense and antisense), cDNA and DRW. Amplification was set at 94°C for 30s, 60°C for 60s, and 72°C for 60s followed by a 2 min extension at 72°C. PCR products were viewed under UV light after 1% agarose gel electrophoresis and staining in ethidium bromide. The myeloblastin (mbn) primer sets were 5'-ACA ACT ACG ACG CGG AGA AC-3' (sense) and 5'-CCA GTC CAC GTA GAG GGC TA-3' (antisense). mbn mRNA expression was used as one of the indicator of differentiation.

The interleukin-12 (IL-12) primer sets were 5'-AAG

GAG GCG AGG TTC TAA GC-3' (sense) and 5'-GTA CTC CCA GCT GAC CTC CA-3' (antisense). IL-12 mRNA expression was used as a marker for differentiation of macrophagic or B lymphocytic phenotypes.

The interleukin-15 (IL-15) primer sets were 5'-ATG GTA TTG GGA ACC ATA GAT TTG-3' (sense) and 5'-TCA AGA AGT GTT GAT GAA C-3' (antisense). IL-15 mRNA expression was used as a marker for differentiation of T lymphocytic phenotypes.

The CD11a primer sets were 5'-GAA CTG TGG GGA GGA CAA GA-3' (sense) and 5'-GAT GGG GAT GAT GGT AGT GG-3' (antisense). CD11a mRNA expression was used as a marker for differentiation of leukocytes phenotypes.

The CD83 primer sets were 5'-GGT GGT GAA GAG AGG ATG G-3' (sense) and 5'-CTT GTG AGG AGT CAC TAG CCC-3' (antisense). CD83 mRNA expression was used as a DC phenotypic marker.

The CSF2 primer sets were 5'-GCT GCT CTT GGG CAC TGT GG-3' (sense) and CAG CAG TCA AAG GGG ATG AC-3' (antisense). CSF2 mRNA expression was used as a marker for differentiation of T lymphocytic phenotypes.

The GAPD primer sets were 5'-GGT GAA GGT CGG AGT CAA CGG-3' (sense) and 5'-GGG GTG CTA AGC AGT TGG TGG-3' (antisense).

The CD11c primer sets were 5'-GAG CTT CAC CTG GTC TGG AG-3' (sense) and 5'-GAA CAG CAT CAC ACC ACC AC-3' (antisense). CD11c mRNA expression was used as a marker for differentiation of granulocytic phenotypes.

The matrix metalloproteinase-9 (MMP-9, gelatinase B) primer sets were 5'-ACC GCT ATG GTT ACA CTC GG-3' (sense) and 5'-AGG GAC CAC AAC TCG TCA TC-3' (antisense). MMP-9 mRNA expression was used as a marker for differentiation of macrophagic phenotypes.

Morphology study

The morphology of the cells were evaluated from

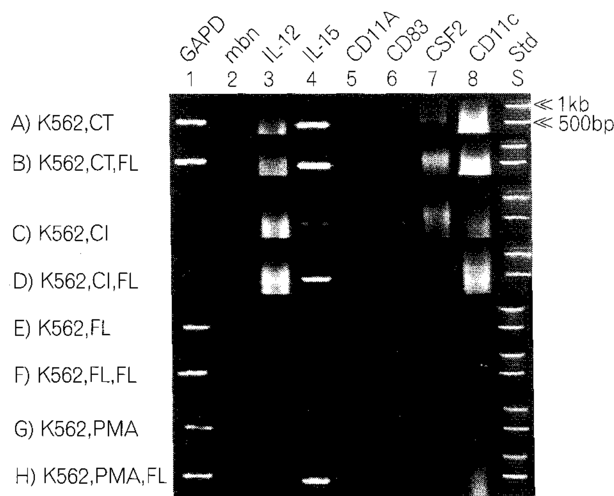

Fig. 1. Differential gene expression of CI-, FL- and PMA-treated K-562 cells. RNA was extracted from K-562 (panels A-H) cells following exposure to culture media only (panel A), CI (panel C), FL (panel E), PMA (panel G) for 7 days. Parts of the each (panels A, C, E, G) cells were exposed to FL for 7 more days and RNA was extracted (panels B, D, F, H), respectively. The gene expression of cells was measured by RT-PCR with the following primers: lane 1: GAPD; lane 2: mbn; lane 3: IL-12; lane4: IL-15; lane5: CD11a: lane 6: CD83; lane 7: CSF2; lane8: CD11c. lane S: 100 bp DNA ladder; CT: control; CI: calcium ionophore; FL: Flt-3 ligand; PMA: phorbol 12-myristate-13-acetate.

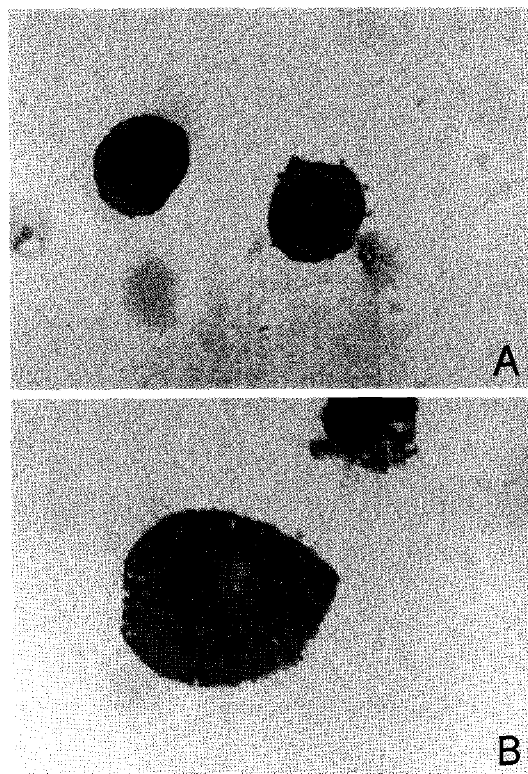

Fig. 2. Morphology of promegakaryocyte phenotype induced from K-562 cells by CI and FL. K-562 cells were cultured with either 180 ng/ml CI and 1 ng/ml FL (B) or culture media alone (A) for 14 days, and slides were prepared and stained with Wright's staining solution. Original magnification $\times$1,000.

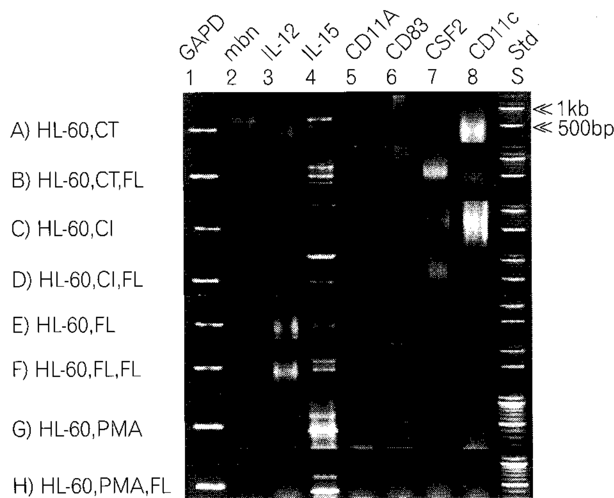

Fig. 3. Differential gene expression in CI-, FL- and PMA-treated HL-60 cells. RNA was extracted from HL-60 (panels A-H) cells following exposure to culture media only (panel A), CI (panel C), FL (panel E), PMA (panel G) for 7 days. Parts of the each (panels A, C, E, G) cells were exposed to FL for 7 more days and RNA was extracted (panels B, D, F, H), respectively. The gene expression of cells was measured by RT-PCR with the following primers: lane 1: GAPD; lane 2: mbn; lane 3: IL-12; lane4: IL-15; lane5: CD11a: lane 6: CD83; lane 7: CSF2; lane8: CD11c. lane S: 100 bp DNA ladder; CT: control; CI: calcium ionophore; FL: Flt-3 ligand; PMA: phorbol 12-myristate-13-acetate.

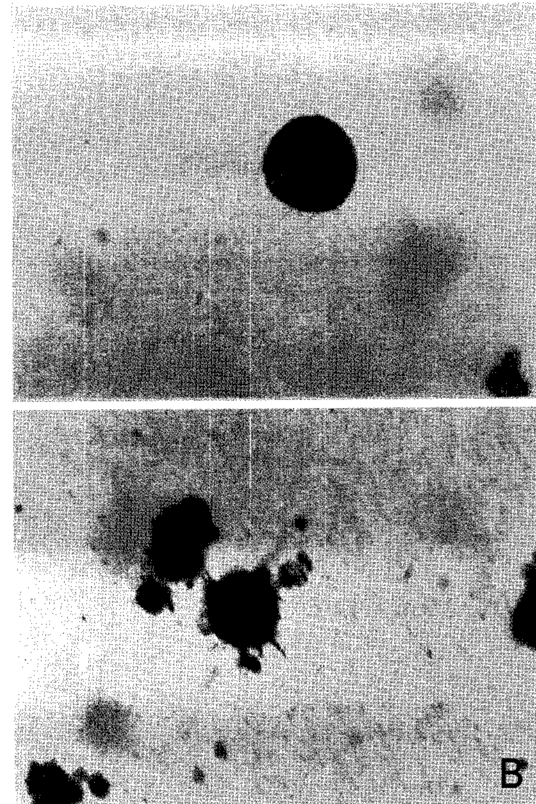

Fig. 4. Morphology of dendritic cell phenotype induced from HL-60 cells by PMA. HL-60 cells were cultured with either 200 ng/ml PMA (B) or culture media alone (A) for 3 days, and slides were prepared and stained with Wright's staining solution. Original magnification $\times$1,000.

cytospin slide preparations with Wright's staining.

RESULTS

Differentiation induction of promegakaryocytes from K-562 cells

There was no expression of CD83, which is considered as a mature DC marker (Zhou et al, 1996), and there was no DC phenotypes on morphology study. IL-12 and CD11c mRNAs were expressed in a portion that was exposed to CI for 7 days and to FL for 7 more days (Fig. 1D). Morphology of the fraction was promegakaryocytes with two nuclei, granular blue cytoplasm and marginal bubbly cytoplasm structure (Fig. 2B) (Diggs et al, 1984).

Differentiation induction of DC from HL-60 cells

HL-60 cells appeared as spherical cells with a smooth surface morphology (Fig. 4A). There was CD83 mRNA expression in a portion that was exposed to PMA for 7 days (Fig. 3G). After 3 days of culture, the cells of this fraction appeared larger and short projection emerging from the surface (Fig. 4B). On day seven, cells with large-cell bodies and long dendritic projections were prominent. The viability and dendritic morphology of these cells could be maintained for up to 21 days.

Differentiation induction of DC from NC-37 cells

NC-37 cells appeared as spherical cells with lymphoblast-like morphology (Fig. 6A). Expression of CD83 mRNA was observed in a portion that was exposed to PMA for 7 days and to FL for 7 more days (Fig. 5H). After 3 days of culture, the cells of this fraction appeared large-cell bodies with veiled cell morphology (Fig. 6B). The viability and veiled

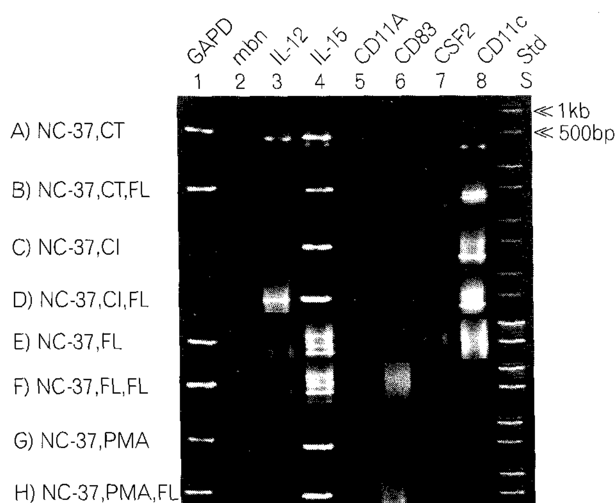

Fig. 5. Differential gene expression in CI-, FL- and PMA-treated NC-37 cells. RNA was extracted from NC-37 (panels A-H) cells following exposure to culture media only (panel A), CI (panel C), FL (panel E), PMA (panel G) for 7 days. Parts of the each (panels A, C, E, G) cells were exposed to FL for 7 more days and RNA was extracted (panels B, D, F, H), respectively. The gene expression of cells was measured by RT-PCR with the following primers: lane 1: GAPD; lane 2: mbn; lane 3: IL-12; lane4: IL-15; lane5: CD11a: lane 6: CD83; lane 7: CSF2; lane8: CD11c. lane S: 100 bp DNA ladder; CT: control; CI: calcium ionophore; FL: Flt-3 ligand; PMA: phorbol 12-myristate-13-acetate.

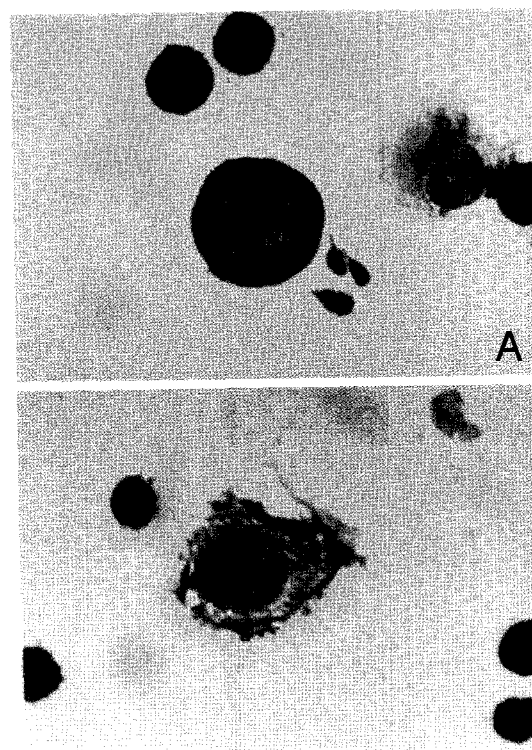

Fig. 6. Morphology of dendritic cell phenotype induced from NC-37 cells by PMA. NC-37 cells were cultured with either 200 ng/ml PMA (B) or culture media alone (A) for 3 days, and slides were prepared and stained with Wright's staining solution. Original magnification $\times$1,000.

cell morphology of these cells could be maintained for up to 21 days.

Differentiation induction of DC from RPMI 7666 cells

RPMI 7666 cells appeared as spherical cells with a smooth surface morphology (Fig. 8A). CD83 mRNA was induced in a portion that was exposed to FL for 7 to 14 days (Fig. 7E, 7F). After three days of culture, the cells of this fraction appeared larger and were associated in adherent grape-like clusters with short projection emerging from the surface (Fig. 8B). On day seven, cells with large-cell bodies and long dendritic projections were prominent. The viability and dendritic morphology of these cells could be maintained for up to 21 days.

DISCUSSION

DCs are important antigen-presenting cells that can enhance generation of helper T-cell responses and cytotoxic cells from naive T cells in vitro (Inaba et al, 1987; Mehta-Damani et al, 1994; Porgador et al, 1995). In this study, The promyelocytic leukemic cell line HL-60, B lymphoblast cell lines RPMI 7666 and NC-37 could be induced to dendritic cells in vitro. Treatment of HL-60 with PMA results in the expression of myeloid-related DC phenotypes, while treatment of RPMI 7666 with FL and treatment of NC-37 with PMA and FL led to the expression of lymphoid-related DC phenotypes.

Santiago-Schwarz (Santiago-Schwarz et al, 1994) and colleagues described the generation of DC-like cells from the peripheral blood mononuclear cells (PBMNC) of a single acute myelogenous leukemia (AML) patient. In their studies, the terminal differentiation of an adherent DC-like population was achieved using a combination of GM-CSF, TNF-α, and IL-6.

Choudhury et al reported that DCs can be generated ex vivo from myelomonocytic precursors in chronic

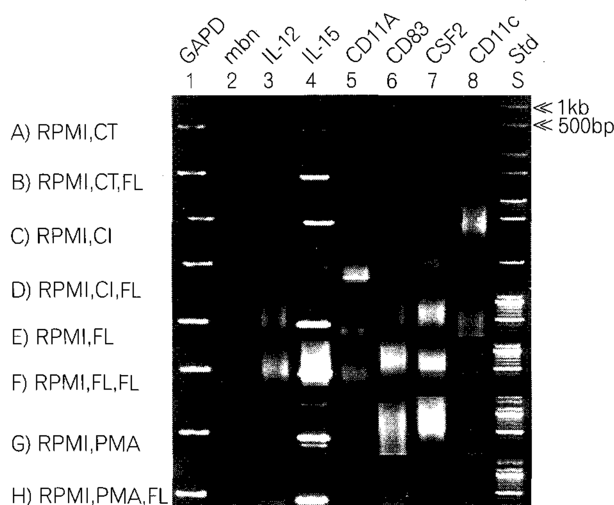

Fig. 7. Differential gene expression in CI-, FL- and PMA-treated RPMI 7666 cells. RNA was extracted from RPMI 7666 (panels A-H) cells following exposure to culture media only (panel A), CI (panel C), FL (panel E), PMA (panel G) for 7 days. Parts of the each (panels A, C, E, G) cells were exposed to FL for 7 more days and RNA was extracted (panels B, D, F, H), respectively. The gene expression of cells was measured by RT-PCR with the following primers: lane 1: GAPD; lane 2: mbn; lane 3: IL-12; lane4: IL-15; lane5: CD11a: lane 6: CD83; lane 7: CSF2; lane8: CD11c. lane S: 100 bp DNA ladder; CT: control; CI: calcium ionophore; FL: Flt-3 ligand; PMA: phorbol 12-myristate-13-acetate.

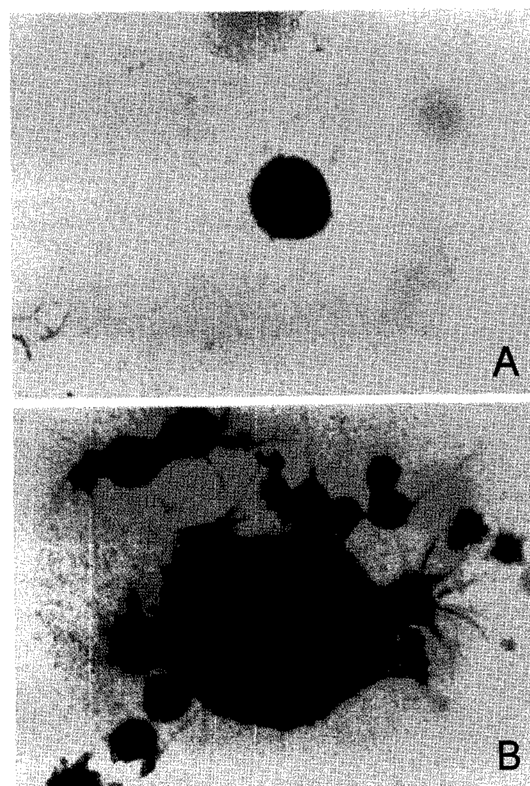

Fig. 8. Morphology of dendritic cell phenotype induced from RPMI 7666 cells by FL. RPMI 7666 cells were cultured with either 1 ng/ml FL (B) or culture media alone (A) for 3 days, and slides were prepared and stained with Wright's staining solution. Original magnification $\times$1,000.

myelogenous leukemia (CML) (Choudhury et al, 1997) and from AML cells (Choudhury et al, 1999) and their potent ability to stimulate leukemia-specific cytolytic activity in autologous lymphocytes. DCs were generated in vitro using GM-CSF+IL-4 in combination with either TNF-α or CD40 ligand (CD40L). However, all those cytokines are recombinant proteins and are too expensive to use in clinical trial. In their study, it took about 10 to 14 days to generate DCs from leukemic cells. That method, therefore, may result in a significantly higher risk of genetic alteration of leukemic cells for a prolonged time. In this study, it took only 3 days to generate DCs from human leukemic cell lines and the induction procedure is very simple.

Koski et al (1999) reported that calcium ionophore-treated myeloid cells acquire many dendritic cell characteristics including CD83 expression. In this study, however, treatment of leukemic cell lines with CI did not result in the expression of DC phenotypes. The significance and reasons behind the observed differences in our results and those of Koski are not clear. We speculate that differences in results may arise from differences in culture conditions and intrinsic variations in ability and default pathways of differentiation of leukemic cells.

The use of DCs for immunization has focused on the generation of cytotoxic T lymphocyte (CTL) responses to tumor or viral antigens (Young et al, 1996; Schuler et al, 1997). Successful, protective CTL responses have been developed in mice immunized with DCs pulsed with antigenic peptides and intact soluble proteins (Paglia et al, 1996). Transfection of DCs with relevant genes is thought to bypass MHC restriction. Adenoviral and retroviral vectors are also shown to be effective for immunizing tumor-bearing mice (Song et al, 1997; Specht et al, 1997). Development of monoclonal antibodies recognizing MHC-peptide complexes may help optimize and evaluate antigen-loading procedures for DC. Autologous lymphocytes

cocultured with acute myelogenous leukemia-derived DCs lyse autologous leukemia targets with little cytotoxicity noted against normal cells from patients in remission (Choudhury et al, 1999). DC immunotherapy in vivo and in vitro has shown promising results for cancer treatment and prevention.

Adoptive immunotherapy with autologous antileukemic T cells may be potentially useful in the context of autologous bone marrow transplantation for leukemia. Using this methodology, it may be feasible to generate sufficiently a large number of cells for cellular immunotherapy (Choudhury et al, 1997; Choudhury et al, 1999). We speculated that this approach to the development of antileukemic immunoreactivity might be extended to other micrometastatic diseases.

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