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Original article

Differentiation-stimulating potency of differentiated HL60 cells after drug treatment

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ABSTRACT

Differentiation therapy in the treatment of leukemia is often hampered by limitations on using certain pharmaceutical regents or on the required doses due to various reasons, such as drug-resistance and retinoic acid syndrome. To circumvent these problems, a strategy might be developed on the basis of the ability of drug-differentiated cells to stimulate differentiation in leukemia cells. Using the promyelocytic leukemia cell line HL60 as a cell model, we assessed the differentiation-stimulating potency of differentiated granulocytes and monocytes/macrophages after treatments with all-trans retinoic acid (ATRA) and 12-O-tetradecanoylphorbol-13-acetate (TPA), respectively. ATRA- and TPA-differentiated cells were able to stimulate differentiation in fresh HL60 cells, accompanied by inhibition on cell growth to various extents. The differentiated cells of the second generation, especially those originated from TPA treatment, were as potent as the drugs themselves in stimulating differentiation in fresh HL60 cells. On the basis of "differentiation induced by differentiated cells", we explored the feasibility of ex vivo therapy.

1. Introduction

Now that leukemia is characterized by the blockade of differentiation in cellular maturation, differentiation therapy [1,2] would be a reasonable choice in clinical practice. Indeed, since 1980s, differentiation therapy has achieved great success in the treatment of acute promyelocytic leukemia (APL). This specific type of leukemia accounts for 10% to 15% of new cases of adults with acute myeloid leukemia (AML) per year in the United States [3]. In the treatment of APL, all-trans retinoic acid (ATRA) is most widely used as a differentiation inducer, providing "the best proof of principle for differentiation therapy" [4]. Through binding to retinoic acid receptor, ATRA induces a profound change in the phenotype of APL blasts, making them quickly shift from immature promyelocytes to short-lived, terminally differentiated, granulocytes, either ex vivo or in vivo [5,6]. In combination with chemotherapy, the ATRA-based differentiation therapy has made APL the most curable subtype of all the AML cases [3,4]. However, ATRA by itself only rarely yields prolonged remissions in clinical practice [7]. Besides, the development of retinoic acid syndrome (RAS; or more generally, differentiation syndrome, DS [8]) ranges from 2% to 27% in clinical trials and case reports, which has been recognized as a distinct complication and a potential life-threatening adverse reaction [9]. Since the emerging extramedullary relapse was not observed frequently prior to the ATRA treatment era, this unique phenomenon may be related to the development of the RAS [10].

Another efficient differentiation inducer is 12-O-tetradecanoylphorbol-13-acetate (TPA). TPA has a broad range of cellular and pharmacological effects. By mimicking the second messenger diacylglycerol to activate protein kinase C (PKC) and to modulate cell-signaling pathways, TPA affects a variety of cellular responses including cell death, cell survival, cell-cycle progression, and differentiation [11]. It has been shown that the TPA-evoked PKC activation leads to the differentiation of several myelocytic leukemia cell lines along the monocyte/macrophage pathway [12], including the human promyelocytic leukemia cell line HL60. TPA can lead HL60 cells to differentiate into macrophage-like phenotype [13,14], a distinct pathway from the ATRA-led granulocytic phenotype. Besides, TPA exhibits cytotoxicity by modulating the ERK signaling pathway in primary AML cells [15]. Importantly, clinical studies have shown that TPA is likely to have pharmacological activity at doses that are well tolerated without irreversible adverse effects [16–18]. The reduction in leukemic blasts and the improvement in blood counts have been seen in a pilot trial of TPA for patients with myeloid malignancies [19].

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Nevertheless, since only a minority of leukemia samples underwent apoptosis after TPA treatment, experts have called for more extensive investigation before a broader use of TPA in clinical trials [20].

ATRA and TPA represent two typical drugs in the treatment of leukemia: one is currently used with some drawbacks (such as RAS and drug-resistance), and the other, not having been authorized for clinical use though, demonstrates pharmacological potency. It would be desirable to develop a strategy by which therapists could overcome or circumvent the negative effects while, at the same time, allowing these drugs to play their roles to the full extent. In this regard, the differentiation-stimulating potency of drug-differentiated cells might be exploited. Reportedly, mononuclear blood cells are able to produce differentiation-inducing factor(s) upon stimulation with various mitogens [21]. The conditioned media from some drug-differentiated HL60 cells and normal blood mononuclear cells have been shown to make HL60 cells undergo macrophage differentiation [22–24]. The TPA-differentiated macrophages might likewise be capable of inducing differentiation in leukemic cells. As for ATRA-differentiated granulocytes, the production of inflammatory and hematopoietic cytokines [25] has been reported, of which some may facilitate cell differentiation. The aim of the present study was to assess the differentiation-stimulating potency, as well as its inheritability, of ATRA- and TPA-differentiated cells. And further, on the basis of "differentiation induced by differentiated cells", we intended to explore the feasibility of ex vivo therapy. Since HL60 cells undergo differentiation along the granulocytic and monocytic/macrophage pathways upon ATRA and TPA treatments, respectively, it may serve as a suitable cell model for the investigation.

2. Materials and methods

2.1. Chemicals and reagents

RPMI-1640 medium, certified fetal bovine serum, antibiotics (penicillin, streptomycin), trypsin-EDTA solution, and phosphate buffer solution (PBS) were products of Corning Co. (Corning, NY, USA). ATRA and TPA were purchased from Sigma Chemical Co. (St. Louis, MO, USA). PE mouse anti-human CD11b/Mac1 and PE mouse IgG1 isotype control were from BD Biosciences (San Jose, CA, USA). PE mouse anti-human CD38, PE mouse anti-human CD14 and PE mouse IgG1 isotype control were purchased from Biolegend (San Diego, CA, USA). Other reagents used were commercially available products of the highest purity.

ATRA and TPA were dissolved in dimethyl sulfoxide (DMSO) at concentration of 100 mM and 200 μM, respectively, and stored at –20 °C in the dark. The working solutions were made by dilution with RPMI-1640 medium.

2.2. Cell line

The promyelocytic leukemic cell line HL60 was acquired from Cell Resource Center of Peking Union Medical College and kept in low passage (< 2 months). HL60 cells were maintained in RPMI-1640 growth medium supplemented with 10% heat-inactivated fetal bovine serum, containing 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C, 5% CO_2 and humidity. In bulk culture, cells were in continuous logarithmic growth (less than 10^6 cells/mL).

2.3. Treatment of HL60 cells with drugs

HL60 cells were cultured in six-well plates (Costar, Cambridge, MA, USA) with complete growth medium and treated with various doses of ATRA (0.1, 1 μM) and TPA (10, 100 nM), respectively, for indicated period of time. In the treatment with ATRA and TPA, the amount of DMSO in cell culture medium never exceeded a final concentration of 0.1%.

2.4. Assessment of the differentiation-stimulating potency of drug-treated cells

Cocultures were prepared using Millicell inserts (Millipore Corporation, Billerica, MA, USA) and six-well culture plates. As shown in Fig. 1, fresh HL60 cells were seeded in the lower part of a chamber at the density of 4×10^5 cells in 2 mL culture (middle of Fig. 1), and the drug-differentiated cells (left of Fig. 1), after removal of the excessive drug by washing twice with PBS and centrifugation, were added to the upper part (4×10^5 cells in 2 mL). The coculture was thereafter incubated for indicated period of time.

2.5. Assessment of the inheritability of the differentiation-stimulating potency of drug-treated cells

After being cocultured with drug-differentiated cells (middle of Fig. 1), the HL60 cells in the lower part of the chamber, which had undergone differentiation, were cocultured with equal number of fresh HL60 cells in the upper part for indicated period of time (right of Fig. 1).

2.6. Analysis of cell proliferation

To examine the effect of a drug or differentiated cells on cell proliferation, cells were collected and counted using a hemocytometer after treatment or coculture.

2.7. Determination of cell differentiation

Differentiation was assessed by measuring the cell surface antigen CD11b, CD38, and CD14 with flow cytometric analysis. CD11b is a cell surface marker for differentiation into either monocytes or granulocytes [26], while CD38 and CD14 are expressed in the differentiated granulocytes [27] and monocytes/macrophages [28] respectively. After treatment with a drug or differentiated cells, samples of 10^6 cells were washed twice with PBS, and suspended in 100 μL of RPMI-1640 medium containing 2% fetal bovine serum. Then 10 μL of PE mouse IgG1 isotype control was added to the cell suspension, for setting threshold parameters. Cells of no less than 10^4 were examined on FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA). Data were analyzed with Cell Quest Pro.

2.8. Statistical analysis

Results were presented as mean ± SD of three independent experiments conducted in triplicate. Means were compared by the single factor analysis of variance (ANOVA) test. Statistical significant

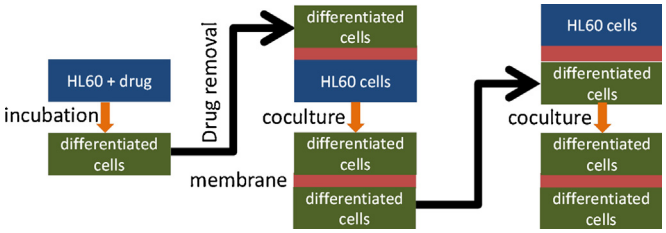

Fig. 1. Schematic of the coculturing experiments for the assessment of differentiation-stimulating potency of differentiated cells in fresh HL60 cells.