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Cell phenotype analysis using a cell fluid-based microchip with high sensitivity and accurate quantitation

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Abstract

We have assessed a cell fluid chip-based fluorescent cytometric assay that runs on bioanalyzer for fast characterization of small population cell phenotypes characterization. The assay determines the expression of specific cell surface markers on various cell samples. Six samples can be analyzed on each chip in one automated process. Results were in good agreement with conventional flow cytometry in quantitation. Importantly, this procedure used less than 200 cells per sample and produced results consistent with that using 10^5 cells by the conventional staining procedure. The method was also used for screening potential ingredients in herbs. Purpose of this study was to analyze the change of cell subtypes of UCB mononuclear cells in vitro reactivity in herbs. We found that by treatment of the water-soluble extract (F3) of *Ganoderma lucidum*, the presence of $CD56^+$ marker (natural killer cells) significantly increased from 1.1 to 3.2% ($P<0.05$ and P) in UCB mononuclear cells. The results indicated that F3 quantitatively influenced NK cells activities. We suggest this screening method may be useful for a fast phenotypes characterization after extract stimulation utilizing only a small population of cells.

Keywords: Cell phenotype analysis; Cell fluid-based microchip

1. Introduction

Cellular protein expression monitoring is an important procedure for cell sample quality control, cell population characterization and analysis of cellular functional protein. The measurement is usually accomplished by cell surface protein staining with specific fluorescence-labeled antibodies and detected by flow cytometry for quantitation [1].

The Agilent Bioanalyzer has been applied to many molecular assays, including analysis of RNA, DNA and proteins. For cell assays, the movement of cells on the chip is controlled by pressure-driven flow [2–4].

Human primary cells are scarce and are frequently limited in availability and have a limited lifespan. To observe phenotypic changes of living cells after treatment, mononuclear cells (MNCs) isolated from umbilical cord blood (UCB) were used. Recently,

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primitive and mature hematopoietic cells of UCB are increasingly used in allogenic marrow transplantation and treatment of genetic diseases [5–7]. Transplantation of UCB from unrelated donors was also used to treat haematological malignancies [8,9].

In this study, we utilized a cell fluid-based chip on the Bioanalyzer developed by Agilent Technologies. In order to investigate the accuracy of the new tool, a conventional flow cytometry was also used. The results of MNCs phenotypic expression measured by both instruments were compared. Our intention was to develop a fast screening method using small population of cell resources.

Six specific antibodies against cell surface markers were chosen for this experiment. CD3/TCR (T cell receptor) markers are found on mature T cells during thymopoiesis [10]. CD19 is a B cell-specific antigen, which is a critical signal transduction molecule regulating B lymphocyte development, activation, and differentiation [11]. CD14 is a monocyte/macrophage differentiation marker [12]. CD45 is expressed on all nucleated hematopoietic cells [13]. Anti-human CD56 antibodies are used for natural killer (NK) cells identification [14]. CD83 is expressed on dendritic cells and anti-CD83 antibodies can serve as a useful marker for human dendritic cells [15].

MNCs from UCB were treated with F3, extracted from *Ganoderma lucidum* [16]. The cells were cultured for 7 days; then the quantitative changes of MNC subpopulation were investigated. Stained UCB cells were analyzed for their fluorescence intensities using both Bioanalyzer and flow cytometry. In contrast to flow cytometry, the Bioanalyzer does not provide scatter parameters and non-fluorescent cells or particles are not detected. To exclude counting bias of dead cell debris and small particles, a fluorescent dye, Calcein, was used for the assessment of cell viability. Living cells will internalize and metabolize Calcein, and produce a fluorescent product [17]. One of the two-color detection systems in the Bioanalyzer can detect vital cells, the other the presence of surface markers. In each event detected, fluorescence values for the red channel and the blue were assigned and used for data evaluation.

To induce MNC phenotypic change, we used F3, an extract from Chinese fungus, *Ganoderma lucidum* (Reishi). *Ganoderma lucidum* has been believed to be an immuno-tonic for centuries in Asia. It was found that treating with F3 on mice splenocytes in vitro produced several cytokines (e.g., IL-1, IL-2, IFN-γ, tumor necrosis factor) in our previous study [16].

Data obtained by the Bioanalyzer and flow cytometry were analyzed and compared.

2. Material and methods

2.1. Isolation of UCB mononuclear cells

Human UCB from healthy volunteers were drawn into EDTA-coated tubes. Samples were stored at room temperature and processed within 24 h of collection. Mononuclear cells were isolated from cord blood using Ficoll–Hypaque density centrifugation (density 1.077; Pharmacia Biotech, Uppsala, Sweden).

The buffy coat interface was collected and washed by Dulbecco's phosphate-buffered saline (PBS), pH 7.4, with EDTA (2 m*M*) and then stained by fluorescent antibodies in appropriate buffer described bellow immediately and measured by flow cytometry or the Bioanalyzer.

2.2. Cell culture

UCB mononuclear cells were cultured in RPMI-1640 medium (Gibco-BRL) supplemented with 15% fetal calf serum (FCS) and L-glutamine (1 m*M*), Hepes (10 m*M*), penicillin (100 U/ml), and streptomycin (50 mg/ml, Gibco-BRL).

Cells were plated in 5×10^5 cells/ml density in T75 culture flasks for F3 treatment. The flasks were maintained in 37 °C–5% CO_2 incubator. Three different concentrations of F3 (1, 10, 100 μg/ml) were tested. The *Ganoderma lucidum* extracts were added to the cultures in 1 h of equilibration in the cell culture chambers after seeding. F3 were dissolved in PBS for all experiments. Control cultures were treated with equal volume of PBS without F3. Medium were changed every 2 days. Only half volume of medium was replaced with fresh medium each time in order not to eliminate all the secretory protein induced by the extract. After 7-day cultures, cells were collected for further analysis. Specific cell

types' activities were determined by cell surface expression of activation markers.

2.3. Flow cytometry

PC5-conjugated antibodies were used for fluorescence-activated cell sorter (FACS) analysis: CD3, CD14, CD19, CD45, CD56, and CD83 mAb (Coulter Immunotech, Marseilles, France). Table 1 summarizes the list of antibodies used for monitoring the cell surface proteins expressed from different cell types.

For FACS analysis, cells ($1–2\times10^6$) were pelleted and resuspended in 2 ml of staining buffer (0.2 m*M* EDTA, 2% FCS in PBS. Labeling was performed by adding to the cells of staining buffer (100 μl) containing PC5-conjugated antibodies (5 μl). All samples were incubated at 4 °C for 40 min. The suspension was then centrifuged, and the pellet was washed twice with washing buffer (0.2 m*M* EDTA, 2% FCS in PBS.

Appropriate mouse isotype controls (mouse IgG_{2a} conjugated to PC5) were used as control. Cell surface antigen expression was evaluated by a single immunofluorescence staining, and analysis was performed using BD FACSCalibur with CellQuest software (Becton-Dickinson, San Jose, CA, USA).

Immediately following sample preparation, data acquisition was performed on the flow cytometer. Instrument set-up was instituted for single-color immunofluorescence with adjustment on forward scatter (FSC) and side scatter (SSC) thresholds to exclude debris prior to data collection according to standard protocol (Becton-Dickinson Immunocytometry Systems).

Approximately 10^5 cells in each sample were collected and analyzed using the panel of fluorescence labeled antibodies. Laser excitation was set at 630 nm and the fluorescence of stained cells was measured in the red fluorescence channel (FL3). Win MIDI version 2.8 software was used for flow cytometry data analysis supplied by the manufacturer.

Table 1
Fluorescence Labeled antibodies used to select specific hematopoietic cell types

Cell type	Antibody
Natural killer cell	Anti-CD56-PC5
Monocyte	Anti-CD14-PC5
B lymphocyte	Anti-CD19-PC5
T lymphocyte	Anti-CD3-PC5
Dendritic cell	Anti-CD83-PC5
Nucleated haematopoietic cell	Anti-CD45-PC5

2.4. Bioanalyzer system

Using the Bioanalyzer, cell characterization and quantitation was carried out on mononuclear cells collected from human UCB. The Bioanalyzer and Cell Assay Extension were obtained from Agilent Technologies Deutschland (Waldbronn, Germany). Detection of the antibody-stained cells was performed on the Bioanalyzer in combination with the Cell Fluorescence LabChip® Kit and the Cell Fluorescence software recommended by the manufacturer. The LabChip kit uses a pressure cartridge to drive fluids or molecules within fluids through micro-fluidic channels.

The Bioanalyzer is capable of two-color fluorescence detection. The red laser has an excitation maximum at approximated 630 nm (detection maximum at 680 nm), whereas the excitation maximum of the blue LED is at 470 nm (detection maximum at 525 nm). The two detection channels are far from each other (525 and 680 nm), so that overlap of emission spectra is unlikely. Thus, the instrument's fluorescence color compensation adjustment can be omitted.

In the standard protocol, stained cells (10 μl) are loaded onto the chip. The recommended cell concentration is 2 million cells/ml requiring only 20 000 cells per sample.

The same aliquot of UCB cells was analyzed individually for their fluorescence intensities. In contrast to the flow cytometer, the Bioanalyzer did not acquire scatter parameters and therefore non-fluorescent cells or particles are not detected. To exclude cell debris and small particles, a fluorescent dye (Calcein) was used to assess cell viability. Vital cells can internalize and metabolize Calcein to produce a fluorescent product in the cytoplasm. Cells were tested both for viability and the presence of surface markers. Fluorescent values in the red and blue channel were measured and used for data evaluation. After data acquisition was finished, the evaluation was carried out by presentation against

fluorescence values in the semi-logarithmic scale against cell numbers.

Cell sample preparation and staining procedure were the same as previously described. After washing steps, cells were resuspended in staining buffer and stained by 10 μl of Calcein AM (1 mg/ml solution in dry DMSO) (Molecular Probes, Eugene, OR, USA) for 30 min at 4 °C. Membrane-permeant calcein AM is cleaved by esterases in live cells to yield cytoplasmic green fluorescence. Finally, the stained cells were washed and resuspended in an isobuoyant cell buffer at $1–2\times10^6$ cells/ml, 10 μl of sample were pipetted into each of the six sample wells, and data acquisition was then performed (Fig. 1).

The prepared chips were used within 5 min, and up to six cell samples were analyzed automatically in less than 30 min. One hundred, 300, 1000 and 3000 cell number were set by the Bioanalyzer and measured for linearity.

2.5. Statistical methods

All parameters under study were examined and compared by SAS 8.2 software PROC GLM procedure.

The GLM procedure uses the method of least squares to fit general linear models. PROC GLM was used here to perform the analysis of variance due to our unbalanced data.

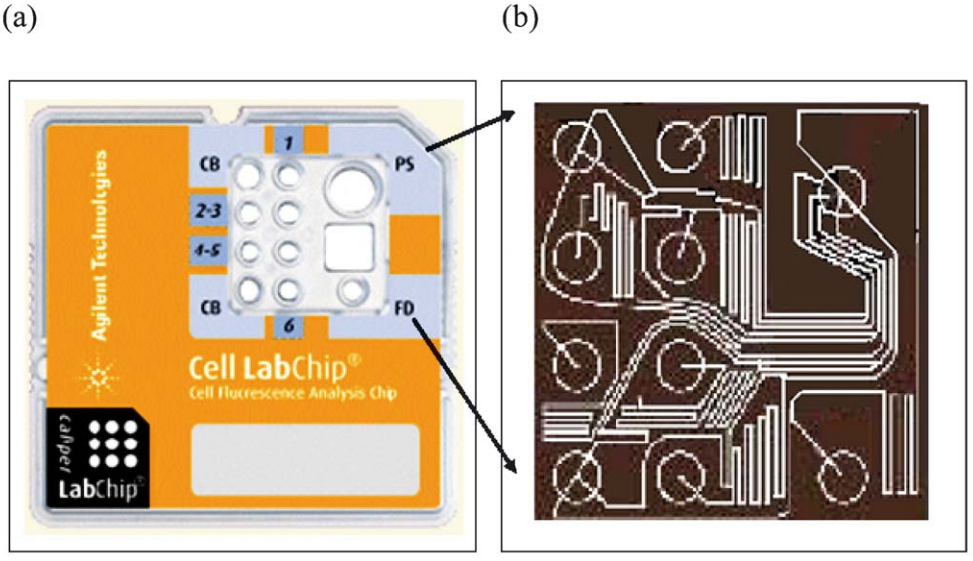

Fig. 1. Cell fluorescence LabChip used in the Agilent 2100 Bioanalyzer. (a) Top side of the chip showing layout of marker and sample wells. The large priming well (PS) was initially added with the chip priming solution (10 μl); the micro-fluidic channels of the cell chip were then filled with low viscosity priming solution by capillary action. Focusing dye solution (10 μl) was added into the FD well, with the help of a dye solution, the Bioanalyzer optics was aligned with a dedicated dye channel. Focusing dye was used to adjust the Bioanalyzer optics for each individual chip. Cell buffer (30 μl) was loaded into each of the two cell buffer wells (CB) before sample loading. Each chip is capable of detecting six samples, sample wells were designated as 1–6. (b) The chip performs controlled cell fluid movement by pressure-driven flow inside the interconnected networks of microfluidic channels. Cells are hydrodynamically focused in these channels before passing the fluorescence detector in single file. Each chip accommodates up to six samples and data acquisition of all samples is fully automated while data analysis allows for user-specific settings.

Table 2
Comparative quantitation of different cell markers in UCB using the Agilent 2100 Bioanalyzer and flow cytometry

Cell markers	Percent of total cells (mean±SD, range)		Statistics between two instruments $P_r^b > F$
	Agilent 2100 Bioanalyzer ($n=4$[a])	Flow cytometry ($n=3$)	
CD 3	21.0±2.1 (18.5–23.4)	19.3±0.7 (18.8–20.2)	0.2605
CD 14	9.6±2.6 (7.8–13.5)	10.9±0.7 (10.9–11.6)	0.4645
CD 19	4.4±0.8 (3.5–5.3)	4.6±0.1 (4.4–4.8)	0.6844
CD 45	71.4±2.3 (68.2–73.6)	69.3±4.0 (66.8–74.0)	0.4183
CD 56	6.4±3.1 (3.6–10.9)	4.3±0.4 (4.0–4.7)	0.2926
CD 83	6.2±1.4 (4.7–7.6)	6.6±0.3 (6.3–6.8)	0.6782

[a] Number of samples.
[b] Type I error rate (the null hypothesis is $U_a = U_f$).

3. Results

We found that stained cells detected by the Bioanalyzer set at 100, 300, 1000, 3000 cell numbers produced histograms highly similar to that of the flow cytometry with 10^5 cells. Table 2 reveals that different subpopulations of UCB cells detected by the Bioanalyzer had no significant difference compared with the results obtained from the flow cytometry. When the cell number increased, the Bioanalyzer histogram profile became smoother and was more similar to that from flow cytometry. The actual cell events counted by the detector were in fact slightly higher than the setting events in the Bioanalyzer. In this test, while the cell number in the lowest setting events was 100, the actual events might be as high as 140 (Table 3). This might be caused by the capillary action of microchip.

Results shown in Fig. 2 indicate that the setting number as low as 100 cells per assay was sufficient to establish accuracy.

The proportions of specific cell types in this report were comparable with other reported data [18–20]. Replicate analysis using different chip sets reproduced similar results (all data presented were triplicates).

The Bioanalyzer seems suitable for natural products' functional assay using living cells. Cell phenotypic changes were detected and the results are shown in Fig. 3. The data shows $CD56^+$ NK cells of UCB increased from 1.1 to 3.2% after treatment with *Ganoderma lucidum* extract (100 μg/ml). There were no significant changes in $CD14^+$ and $CD19^+$ MNCs. $CD83^+$ dendritic cell proportion decreased after 1 μg/ml F3 treatment compared to the control. $CD3^+$ T cells also decreased from 19 to 15%. However, data for $CD3^+$ cells had large standard deviations compared with other data sets in the same experiment (Fig. 4). This might be caused by nonlinearity of the fluorescent detection on the CD3 standard curve. Since this is the first report using the cell fluid chip-based analyzer to measure cell phenotypic changes on natural product treatment, optimized parameters of screening procedures needs further study.

4. Discussion

In this study, we report a new method to measure cell phenotypic change profiles. In contrast to conventional flow cytometry, the Bioanalyzer requires only small population of cells. When cell resources

Table 3
Serial diluted cell number test of the Agilent 2100 Bioanalyzer (mean±SD, $n=5$)

	Setting events[a]			
	100	300	1000	3000
Actual events[b]	140.2±13.8	335±14.2	1041.7±17.8	3528.5±373.8
Data range	(119–155)	(315–347)	(1020–1073)	(3036–4041)

[a] Setting number of cells for detection.
[b] Actual cell events measured by the Bioanalyzer.

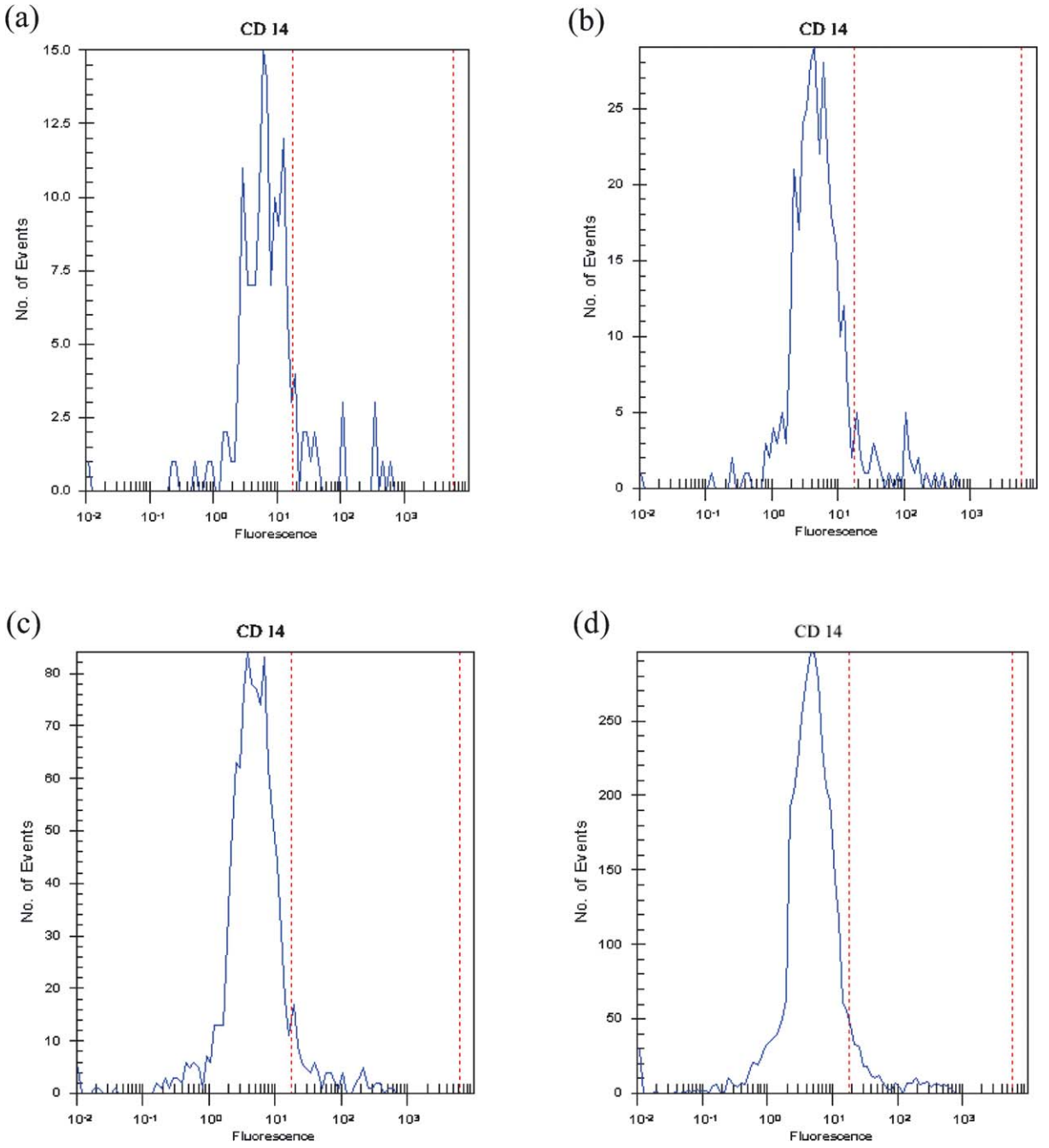

Fig. 2. Fluorescent intensity versus cell number profiles of UCB mononuclear cells stained with anti-CD14-PC5. Its an example to illustrate that under four different cell number settings: (a) 100, (b) 300, (c) 1000, (d) 3000. The Bioanalyzer produced consistent results with flow cytometry. Higher cell amounts measured by the Bioanalyzer produced more similar histograms with 10^5 cells measured by flow cytometry.

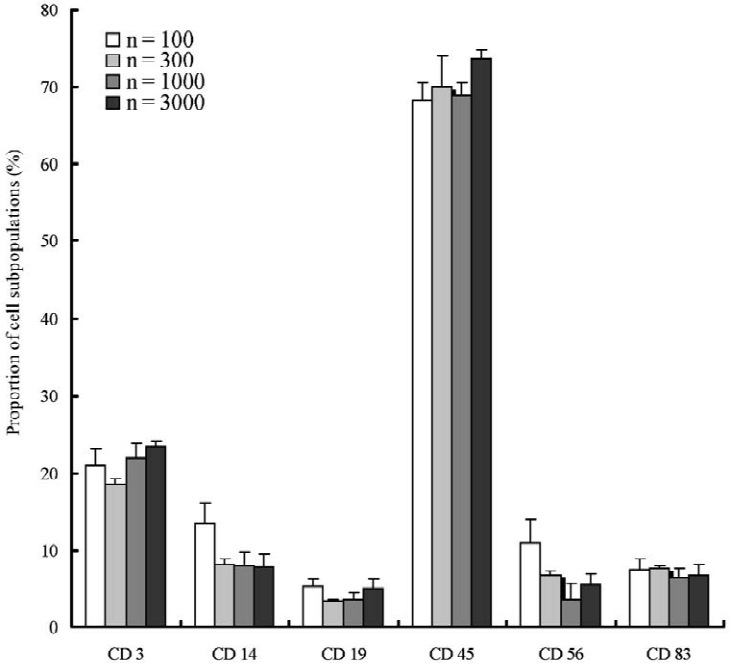

Fig. 3. Comparison of the results measured by the Agilent Bioanalyzer 2100 in different cell numbers (with standard devariation).

are very limited, a good method using only small number of cell is accordingly crucial.

Another advantage of cell fluid-based microchip was the short analysis time, with one complete chip run takes less than 24 min, and data collection within 4 min/sample. Using disposable cell assay chips also minimizes sample contamination in clinical application.

Six fluorescent monoclonal antibodies were used to measure the different subsets of lymphocytes in this study. We found the data produced by the Bioanalyzer had good correlation with the conventional flow cytometry.

The plasticity of progenitor cells and enrichment in mononuclear cells in UCB makes this method a potential tool to screen for potent ingredients in the herbs. The Chinese herb *Ganoderma lucidum* extract was used for treating MNCs. Changes of cell phenotypic expression were detected. We demonstrated in this experiment that F3 alone could enhance NK cell surface marker CD56 expression. Knowledge of the functional attributes of F3 and its interaction with other cytokines might help us to explore new drug candidates that enhance antitumour activity of human NK cells.

The drawback of the Bioanalyzer is the lack of multicolor staining function when cells required more than one antibody typing. However, it can be used as a complimentary method to the convention flow cytometric analysis.

We concluded that the Bioanalyzer is a versatile instrument which was originally design for DNA and protein analysis. With no modification required, we have developed a high sensitive and fast method for cell phenotype study. We viewed that the cell fluid-based microchip could be a relatively less expansive, easier to operate and space-saving tool than flow cytometry system.

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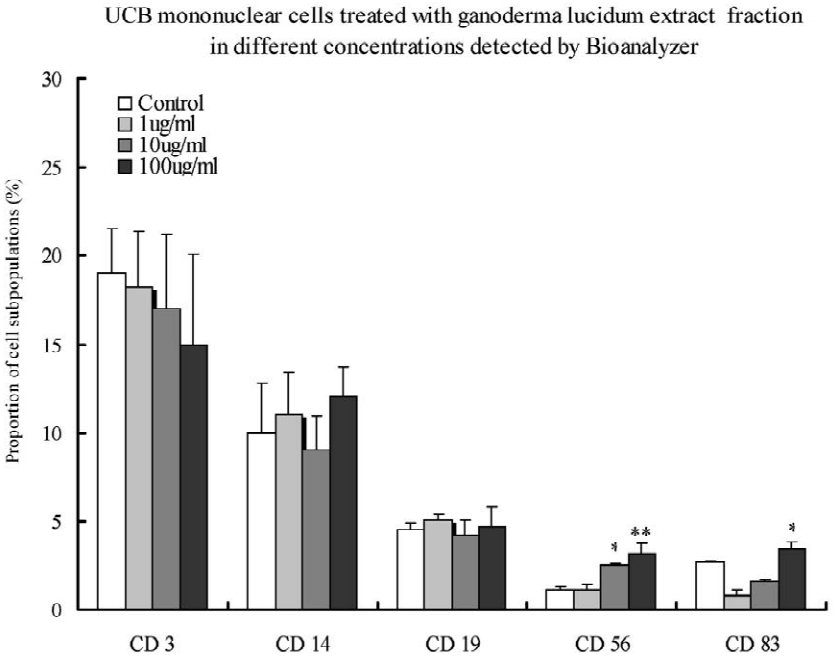

(*, p < 0.05; **, p< 0.01 as compared with the control group without F3 treatment.)

Fig. 4. Comparison of the percentage of $CD3^+$, $CD14^+$, $CD19^+$, $CD56^+$ and $CD83^+$ cells in UCB mononuclear cells after treated with 1, 10 and 100 μg/ml *Ganoderma lucidum* extracts. The results are presented as mean±SD of three experiments.

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