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Research Article

A Study: On *in Vitro* Maturation of CD56+ NK Cells Using the Human Umbilical Cord Blood, Among the Eleven Natural Nutritional Substances Tested, Wheat Grass Extract Orchestrates and Enforces the Naive Fate of CD56+ NK Cells

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Abstract

Objective: The immuno-modulating activity of natural substances was evaluated to assess their effects on human umbilical cord blood mononuclear cells (MNC). Most of the MNC might have been immunologically primed as they are exposed to immunological stressors, while MNC from the human umbilical cord blood (hUCB) are not. During pregnancy, MNC of hUCB is isolated from bio-stressors by virtue of the trophoblast stratum between the placenta and its neighboring maternal endometrium. Hence, the MNC is well protected from bio-stresses. The trophoblastic layer prevents MNCs of the hUBC from being affected by various factors circulating in the maternal blood, so that the exposure of MNC to those factors is reduced.

Methods: After treatment with eleven different natural substances, including five herbal extracts and six pure compounds, the immunophenotypic expression of MNC subsets was analyzed via fluorescence activated cell sorting (FACS) analysis. Data reported here indicated that the extract wheat grass (*Triticum aestivum* L.) intensified the population of CD56+ natural-killer (NK) cells, whereas the propagation of other MNC was insignificant.

Results: NK cells separated with the positive magnetic bead cell isolation method (MACS) and treated with wheat grass extract showed marked cytotoxicity affecting the cells of Modal karyotype of human leukemia cell line, K562.

Conclusions: This pilot study shows that wheat grass extracts likely orchestrates, enforces, and promotes the in vitro maturation of $CD56^{+}$ NK cells in the hUCB. (Med J South Taiwan 2011;7:79-93).

Keywords: CD56+; Wheat; NK Cells

Introduction

Mononuclear cells (MNCs) of human umbilical cord blood (hUBC) are somewhat unlike adult peripheral MNCs. In peripheral blood MNCs are vulnerable to immunological stressors. Most of the MNCs might have been immunologically primed as they were exposed to bio-stressors while MNCs from cord blood are not. Ac-

cordingly, hUBC MNCs are minimally primed, therefore, they are suitable for investigating the immunomodulating effects after treatment with natural health substances. During pregnancy, MNCs of hUBC are isolated from bio-stressors by virtue of the trophoblast stratum between the placenta and the neighboring maternal endometrium. This trophoblastic layer prevents MNCs of hUBC from being affected by various factors circulating in the maternal blood.

When an immune response is effective, various subsets of immune cells can be distinguished via their protein expression profiles. Through the use of specific fluorescently-labeled monoclonal antibodies and Fluorescent-activated cell sorting can characterize and quantify the differentiation and maturation of MNC subtypes (Figure 1).

We devised a model system (Figure 1) to use it in disclosing the lively and vigorous development of cellular differentiation of MNCs of hUCB after treatment with various

extracts of natural substances. Immunal responses in the profile of immune cell subsets would be significant findings. The differentiation and maturation of MNC subtypes can be observed with flow cytometry using specific fluorescent monoclonal antibody staining [1].

The human immune system is composed largely of B cell-mediated response and T cell-mediated response respectively. While T cells are white blood cells and also known as lymphocytes. The NK cells are a special type of lymphocytes. Its natural cytotoxicity ability diminishes in the peripheral blood of patients who suffer from a variety of cancers, as contrasted to those of healthy controls. Conversely, the cytolytic activity of the purified hUCB NK cells have been reported to be similar to that of the NK cells of the purified adult peripheral blood [2]. The cytolytic activity of purified umblilical cord blood (CB) NK cells was reported to be similar to that of purified adult peripheral blood NK cells [2].

It is known that the mature NK cells express CD56 alone or in combination with CD 16 (CD is the abbreviation of the cluster of differentiation). CD antigens are expressed, as well, on cells of myeloid and lymphoid lineages. As most disease processes involve immune system activation or suppression, these antigens offer unique opportunities for monitoring host responses. Noticeably, the majority of adult peripheral blood NK cells are $CD56^{+}16^{+}$, with a minor population of CD56+16- NK cells. In this study, we also tested the effects of wheat grass extract on the NK cell surface marker expression in MNCs isolated from the UCB of six volunteers.

The decrease in levels of natural cytotoxicity in peripheral blood of patients with various types of cancers is more evident than that in healthy adults [3]. Polysaccharides such as what has been isolated from Ganoderma lucidum, a fucose- containing glycoprotein fraction (F3), enhances the cytotoxic activity of NK cells [4]. F3 effects the immunophenotypic expression in MNCs. When hUCB MNCs were treated with F3 (10-100 mg/mL) for 7 days, the population of CD14+CD26+ monocyte/macrophage, CD83 + CDla+ dendritic cells and CD 16+CD56+NK cells were 2.9, 2.3 and 1.5 times respectively higher than those of the untreated controls. The B-cell population had no significant change. The T cell growth was, however, slightly inhibited and its CD3 marker expression decreased about 20% in the presence of higher concentrations of F3 (100 ig/iml). F3 is not harmful to human cells in vitro, and after F3 treatment, NK cell- mediated cytotoxicity was significantly enhanced by 31.7% at effector/target cell ratio (Effector cells/Target cells, E/T) 20:1, but was not altered at E/T 5:1. In one of the studies [5] a fructose-containing glycoprotein fraction (F3), isolated from the water-soluble extracts of *G. lucidum*, is shown to stimulate mice spleen cell proliferation and cytokine expression.

Figure 1: Model of systemic approach from UCB cells to stimulated cells.

Flow cytometry uses the principles of light scattering, light excitation, and emission of fluorochrome molecules to generate specific multi-parameter data from particles and cells in the size range of 0.5um to 40um diameter. Cells are hydro- dynamically focused in a sheath of PBS before intercepting an optimally focused light source.

Lasers are most often used as a light source in flow cytometry. For example, an analysis of a marine sample of photosynthetic picoplankton by flow cytometry is showing three different populations (Prochlorococcus, Synechococcus, and picoeukaryotes) (Figure 2).

Figure 2: Analysis example of marine sample of photosynthetic picoplankton by flow cytometry showing three different population (Prochlorococcus, Synechococcus, and picoeukaryotes) Wikipedia accessed 5 July 2011.

The natural health substances (NHS) that have been used in this study include Ganoderma lucidum, quercertein dehydrate, astaxanthin, CK-1 (ginsenoside compound- K), TCS-W (cordyceps sinensis), caffeic acid, bilobalide, eugenol, rutin hydrate, GSPE (grape seed proanthocyanidin extract) and wheat grass extracts. They are considered to be antioxidants. Wheat grass is used as a general health tonic and is reported to be effective against several medical disorders, although detailed literature is not available. Besides drug therapy, for example, a number of medicinal plants are effective in treating hyperlipidemia. Among those studies, the recent one by Sethi J, Yadav M, Dahiya K, Sood S, Singh V, Bhattacharya SB. from Department of Physiology, Pt.B.D.Sharma University of Health Sciences, Rohtak, Haryana, India, researchers have studied and rececently published in Methods Find Exp Clin Pharmacol. 2010 May; 32(4):233-5. They researched and reported antioxidant effect of *Triticum aestivium* (wheat grass) in high-fat diet-induced oxidative stress in rabbits.

Wheat grass extract is produced by sprouting and planting the seeds of the common wheat plant, *Triticum aestivum*. Young leaves of this plant are crushed to create a juice or dried to make a powder. The unprocessed plant contains high levels of cellulose, which makes it indigestible. It possesses chlorophyll, dietary fiber, amino acids, minerals, vitamins, and enzymes. The chlorophyll molecule is similar in structure to hemoglobin, hence leads some to believe that wheat grass helps blood flow, digestion and general detoxification of the body. These claims have neither been proven nor disproven. Conversely, crested wheat grass, Agropyron desertorum, with a deep and fibrous root system is used for drought resistance. The use of wheat grass extracts for the treatment of various gastrointestinal disorders, especially for ulcerative colitis, and other conditions had been suggested by its proponents for more than 30 years. This extract demonstrates a prominent tracing in cyclic voltammeter methodology, presumably corresponding to compounds that exhibit anti-oxidative properties. Under optimal reaction conditions, wheat grass peroxidase catalyzes the oxidation of certain aromatic amine substrate. Wheat grass, alfalfa and barley grass is green plants that many people also believed that they are holistically beneficial. Although, they are all a good source of chlorophyll that has some antibacterial effects in general, there is no evidence to support most of the claims made by these products. Among the rest of ten NHS, GSPE (grape seed proanthocyanidin extract) contains 5,000 ppm of Resveratrol and is said to facilitate oxidant-induced vascular endothelial growth factor (VEGF) expression in keratinocytes as well as to be able to up-regulate hydrogen peroxide. On the other hand, *G. lucidum* has a fuctose- containing glycoprotein fraction as forestated (Figure A).

Figure a: Eleven nature substances used in this study.

Materials and Methods

This study was approved by the Internal Review Board of the Hospital where the babies were delivered and conformed to the Declara-

tion of Helsinki. The authors report JDLO conflict of interest. Each subject whoever participated in this study gave a written informed consent.

The source of cord blood and its cell preparation

Human UCB from six healthy volunteers was drawn into ethylenediamine tetraacetic acid (EDTA) coated tubes. The blood was collected after the full-term baby was delivered, and before the placenta was separated from the uterus. Using aseptic procedures, an 18-gauge needle was inserted into the umbilical vein and umbilical cord blood drawn for tests. The UCB (50 -100mL) was processed using density gradient centrifugation with Ficoll-Paque (density 1.077; Pharmacia Biotech; Uppsala, Sweden). The buffy coat interface was retrieved and washed with Dulbecco's phosphate-buffered saline (PBS, pH 7.4) and EDTA (0.2 mM). It was resuspended in a complete culture medium in RPMI- 1640, consisting of L-glutamine and antibiotics (Gibco BRL), and was then supplemented with 20% fetal bovine serum (FBS). MNCs isolated through these procedures were prepared at a final concentration of 1 x 106 cells/mL.

Cells were cultured for 7 days after being treated with extracts of natural substances. Cells (1 - 2x106) were pelleted and resuspended in staining buffer (0.2 mM EDTA, 2% FBS in PBS). Staining buffer solution (l00iL) containing 1 0iL of fluorescence-conjugated antibody was joined to the cell suspension for labeling. After incubation all samples were centrifuged and the pellets with rinsed buffer.

The preparation of K562 as target cells for the cytotoxicity assays

K562 (CCL-243, ATCC), a human erythroleukemia cell line, was used as a NK- sensitive target for the cytotoxicity assays. NK cells can usually and customarily kill quite a few NK-sensitive cells. For mice, their target cells have the seal of YAC-1 and EL-4, while for the human cells, the appearance of seals such as K562, U937, and MOLT4 are prone to become the target attacked by N K cells. Hence, it is very reasonable to use K562 cells as target cells in this study. These cells were cultured in RPMI-1640 medium (Gibco Laboratories) containing FBS and antibiotics (Gibco) in culture flasks (Falcon). On the day of testing, cells were rinsed with PBS and resuspended.

Cytotoxicity analysis

It was done at the effector/target cells' ratios of 5:1, 20:1, and 80:1. Flow cytometry, with cellular fixation in this study, was performed with a FACSclibur cytometer (Becton Dickinson). The instrument was set for two-colour analysis using FACScomp software and was calibrated using Calibrite beads (Beckton Dickinson). Data were collected, and analyses were performed using CellQuest software version 3.1f (Becton Dickinson). To proceed for flow cytometry, cells were pelleted and re-suspended in 2mL of staining buffer (0.2mM EDTA, 2% FBS in phosphate buffered saline [PBS]). Staining buffer (1001L) containing 101L of fluorescence- conjugated antibody was added to the cell suspension for labeling. After incubation at 4 C for 40min, all samples were then centrifuged at 1500rpm for 5 more minutes followed by washing of the pellets twice with washing buffer (0.2mM EDTA, 2% FBS in phosphate buffered saline [PBS]).

The separation of CD56+ NK'cells

The separation of CD56+ NK-cells from other UCB MNCs was performed by a positive magnetic-bead cell separation method (MACS, Miltenyi Biotec). MNCs were segregated from the buffy coat of hUCB with Ficoll-Paque®. Cells were then passed through 30 im nylon mesh (Milipore). Filtered cells were rinsed with buffer PBS.

The cell pellets were placed in 500 iL of the same buffer with the addition of 200 iL of FcR Blocking Reagent (Miltenyi Biotec) and incubated on ice to block FcR. Two hundred iL of CD56+ microbeads per 108 total cells were added and followed by supplementary incubation on ice. The cells were re-perched in the buffer. The magnetically bound cells were applied to two MACSy RSI separation columns (Miltenyi Biotec). These columns had been equilibrated with the buffer in the magnetic field of the Vario MACSy separator according to the method of Gritzapis., *et al*. [6].

Cell staining

Aliquots of the cells were stained by PC5-labeled anti-CD56+ monoclonal antibody (Coulter Immunotech, USA) to analyze the purity of CD56+ NK cell, which was at the level of 95%. All monoclonal antibodies to surface at antigens, CD 14, CD 16, CD26, and CD56 (FITC; Serotec) were obtained from Coulter Immunotech, USA.

Cell counting

Cell counting was observed using light microscopy, based on the ability of living cells to exclude trypan blue. Cell proliferation was evaluated by their reducing activity on sodium (2,3)-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H- tetrazolium-5-carboxanilide, inner salt (XTT) [7,8].

One hundred iL of 2x105 cells/mL was incubated with different concentrations of the wheat extracts for 48 hours. Absorbance was measured with a spectrophotometer, using test and reference wavelengths of 450 and 650 nm, respectively.

The preparation of the control group

Such a group was treated with PBS. The CD56+ NK-cell suspensions were cultured in a medium supplemented with RPMI-1640 with FBC for 24h (37°C, 10% C02). NK-cells were then treated with the eleven NHSs. Among them, a couple of NHSs are to be introduced here about their preparation as follows.

NHSs — Wheat as the first example

For the first example, wheat was planted in our campus experimental farm; wheat grass extract was used in this study at seven different concentrations ranging from 10 to 500 ig/mL for 7 days, prepared by serial dilutions. Wheat (*Triticum aestivum* L.) seeds were planted individually in 4 cm diameter x 20 cm high containers. These 2 Current Proteomics, 2008, Vol. 5, No. 4 Hsieh and Chen seeds were grown in chambers with 24:18°C day: night temperature cycle and 14 h photoperiod for 10 days. Wheat leaves were harvested, milled by a laboratory-scale milling machine and filtered through filter paper. The extract was then salted out with solid AS and collected at 0-40, 40-60, 60-70, 70-80, 80- 100 % (w/v) saturation of AS, respectively. Each fraction was collected by centrifugation (12,000g for 40 min, 4°C) and dialyzed extensively against phosphate buffer (50 mM, pH 7.5) at 4°C for 24 h. The precipitated proteins were suspended in a chilled (-20°C) solution containing 10% TCA, 90% acetone with 0.07% -Me. The mixture was incubated at -20°C for 4 h, and then centrifuged at 12,000 xg for 40 min. The pellet was washed three times with 5 ml of chilled (-20°C) acetone with 0.07% -ME centrifuging at 12,000 xg for 40 min between rinses.

G. lucidum

For the second NHSs example, Crude Reishi extracts (prepared via alkaline extraction (0.1N NaOH), neutralization and ethanol precipitation) was obtained from Pharmanex Co. (CA, USA). Immobiline Dry Strip [pH 3-10NL (nonlinear), 18 cm] and IPG buffer (pH 3-10NL) were purchased from Amershan Pharmacia Biotech (Uppsala, Sweden). CHAPS, Tris buffer, agarose, iodoacetamide and a-cyano-4- hydroxycinnamic acid were from Sigma Co. (St. Louis, MO, USA); dithioerythreitol (DTE) was from Merck Co. (Darmstast, Germany); acrylamide, ammonium persulfate (APS) and TEMED were from Bio-Rad (Hercules, CA, USA); sodium dodecyl sulfate (SDS) and glycine was from Fluka (Buchs, Switzerland); sequencing grade trypsin was from Promega (Madison, Wl, USA). Purification of Reishi extracts ♦Twenty-eight mg of the crude extract were dissolved in 2mL of Tris buffer (pH 7.0, 0.1N) and centrifuged to remove the insoluble materials (7 mg). The supernatant was purified by gel filtration chromatography using a Sephacryl S-500 column (100 - 1.6 cm) with 0. IN Tris buffer (pH 7.0) as the eluent. The flow rate was set at 0.5 mL/min, and 7.5mL per tube was collected. After the chromatography, each fraction was subjected to anthrone analysis to detect sugar components. Five fractions were collected (fractions 1-5), each dialyzed to remove excessive salt and lyophilized to give 1.0, 6.2, 5.3, 2.1, and less than 1 mg, respective.

Positive control

Positive control was respectively added to cell suspensions for preincubation treatment prior to the subsequent cytotoxicity assay. A positive control was prepared by using a prototype tumor promoter 12-O-tetradecanoyl phorbol-13 acetate (TPA), 10 and 1 iMol respectively. The results of wheat grass extract on CD56+NK-cell cytotoxicity were expressed as ratios of survival of K562 cells of such NHS-treated groups versus the control groups.

Purified CD56+ NK'cells using magnetic beads

The CD56+ NK" cells were purified using magnetic beads, conjugated with anti- CD56+ monoclonal antibodies, and cultured target cells (K562cells) at different effector/target cell ratios for cytolytic comparison.

Co-cultured cells

Different concentrations of activated effector cells, that is, NHS-treated CD56+ NK'cells, and target cells, that is, K562cells, were co-cultured in six-well plates (Falcon) in triplicate. The effector to target cell (E:T) ratios were 5:1, 20:1, and 80:1, respectively. Note that the cytolytic activity of purified cord blood NK'cells was noticeably reported be similar [2] to that of purified adult peripheral blood NK cells [data not presented here].

The preparation of MCNs isolated from the 6 hUCB specimens

The MCNs isolated from the 6 hUCB specimens were placed in six T75 culture flasks at 5x10^ cells/mL density in preparation for treatment. After seeding cells, the flasks were maintained in a

37°C, 5% C02 incubator for one hour to equilibrate before 100 ig/mL of natural substances were added to each culture. The extracts of natural substances, including wheat grass were dissolved in PBS for all experiments.

The evaluation of cell proliferation

Cell proliferation was evaluated by their reducing activity on WST-1 (4-3-4- lodophenyl-2-(4-nitrophenyl)-2H-5- tetrazolio-1,3-benzene disulfonate). Basically, l00il of 4x103 cells/well were incubated for 48 hours at 37°C and 5% C02- WST-1 reagent (Roche) was added at a level of lOil/well and incubated for a further 4 hours at 37°C and 5% C02.

The number of lyses of target cells was revealed by the Alamar Blue assay[l]. Alamar Blue indicator was added to the culture wells at a ratio of 200 iL of an indicator to 2 mL of a medium at twelve hours after the co-culture of effector and target cells. The cultured plates were incubated for 4 hours at 37°C. Absorbance of color was measured on an ELISA reader at wavelengths of 570 nm and 595 nm. Controls, which contain only medium and Alamar Blue reagent that had been incubated for 4 hours were measured at the same wavelengths. Alamar Blue, a colorimetric indicator, which changes its colors from oxidized (nonfluorescent, blue) to a reduced (fluorescent, red) after being taken up by cells, was used for detecting target cell survival after treatments with effector cells and/or positive controls.

The MCNs isolated from the 6 hUCB specimens were placed in various culture flasks at 5x10^ cells/mL density in preparation for treatment. After seeding cells the flasks were maintained in a 37°C - 5% C02 incubator for one hour in order to equilibrate before the addition of 100 ig/mL of each natural substance to each culture. The extracts of natural substances were dissolved in PBS for all experiments. Cells were seeded by using pipettes and were cultured for 24 hours before adding the 11 nature substances to a final concentration of the natural substances of 100ig/ml. The duration that the cells were exposed to the substances was 7 days. The concentrations of the solution before cells were harvested was 1,000,000/ml.

The ratio of sample % vs. control % of cell gated of 1.50 is considered as being remarkable while values of 2.0 are regarded as highly remarkable. Remarkable and highly remarkable are used in this article for easy referral by the readers. Their usage purposefully is to be distinguished from what has been usually and customarily referred to as statistically significant and highly significant.

The natural health substances used in this study include: quercetin dehydrate, astaxanthin, caffeic acid, bilobalide, eugenol, rutin hydrate, wheat grass extracts, GSPE, *G. lucidum*, andCK-1 (Figure a).

Statistical analysis

Data were analyzed with statistical software (SPSS 10.0). A two-tailed p value < 0.05 was recognized as significant. This study has 11 treatments with 1 sample of CD56 cells per treatment with one of each of the 11 natural health substances (one single number as an outcome per treatment), and same for each of the rests of 4 CDs. Unfortunately, because of a shortage of umbilical cord blood we do not have multiple measures within treatment variance, but merely one outcome per treatment. Hence, we could not do detailed statistics on the differences in effects between treatments, but to merely express the finding in a purely descriptive manner. All statistical analysis was done by the first author, namely, Dr. Tang.

Results

Flow chart of CD immunocharacters

The flow cytometric analysis of different lymphocytes cell subtypes of uUCB among 55 biomeasures treated with extract of eleven different natural substances was determined.

There were 55 measures for 11 nature health substances. Each measure represents the ratio of sample % vs. control %. The mean ± sd of the total samples is 0.963 ± 0.4306. The 95% confidence interval for Estimated Mean of Population was 0.845 to 0.117. The standard error of samples means were 0.904 to 1.0211 in the ratios of survival of K562 cells of the treated groups versus the controls. For CD56+ NK cells, the ratio of samples % vs. controls % of wheat grass extract 2.23 was the highest.

Treatments with 11 different natural health substances in cord blood mononuclear cells significantly alter the phenotypic expression of CD3+ T lymphocytes. Quercerin and GSPE significantly depress the expression of CD3+ T lymphocytes/ macrophage cell (Figure 3a). Treatments with different NHSs except *G. lucidum* (its ratio of sample % vs. control % is greater than 1.5) in cord MNCs do not significantly alter the phenotypic expression of CD 19+ B

lymphocytes (Figure 3b). Treatments with different natural substance in cord blood mononuclear cells do not significantly alter the phenotypic expression of CD 19+ B lymphocytes (Figure 3c). Treatments with different natural substances in cord blood mononuclear cells show significantly altering the phenotypic expression of CD56+NK cell, with wheat enhancing the expression the most (Figure 3d). Treatments with different NHSs in cord blood MNCs show significantly altering the phenotypic expression of CD34+ hematopoietic stem cells, with GSP Ebeing the most (Figure 3e). Treatments with 11 different NHSs in cord blood MNCs show significantly altering the phenotypic expression of all the 5 different CDs together, at a glance on the Figure 2f.

Among 55 measures, there were 3 with relative high values: 2.3, 2.2, and 1.8. The Z score was obtained for the highest measure of 2.3, which was 3.80 times of the standard deviation. The implication is that either this observation is actually outlier or the population distribution is far away from a normal distribution. Of importance in this context is the fact that 2.3 is the measure of the ratio of wheat grass extract. This ratio of sample % vs. control % appears to outperform as compared with that of the rest of ten NHSs.

There were 55 measures having values less than 2.0 while there were two measures having each ratio greater than 2.0. Due to discrepancy of the number of these two different groups, Fisher's exact probability test was done. It reveals that the difference between these two groups is statistically highly significant (two-tailed tailed $p < 0.000003$, degree of freedom = 53).

The results of the effect of wheat grass extract on CD56+NK-cell cytotoxicity were expressed as ratios of survival of K562 cells of the treated groups versus the control. For CD56 NK cells, the ratio of samples % vs. controls % of wheat grass extract 2.3 was the highest exposure, which revealed a ratio of sample % vs. control % of cell gate4 1-51, and 1.613, respectively ($p < 0.01$, Figure 4 TPA). The difference of distributions among various CDs lies in the fact that CD56NK cells are all in normal distribution (Figure b).

The results of wheat grass extract on CD56+NK cell cytotoxicity were expressed as ratios of survival of K562 cells of the treated groups to that of controls. For CD56+NK cells the ratio of sample % vs. controls % of wheat grass extract 2.30 is the highest as aforementioned.

Figure 3: The 55 biomeasurements of 5 Cd monocytes treated with 11 natural health substances in clustered columns with a 3D visual effect without data transformation.

The lysis of K562 cells

Studies of the lysis of K562 cells after enrichment of CD56+NK cells revealed that NK-cytotoxicity increased by 2.68 ($p < 0.05$) and 29.09 ($p < 0.01$) after pre-treatment with 100 and 10 (ig/mL of wheat grass extract, respectively as compared to those of the untreated controls. The cytotoxicity at an E/T ratio of 5:1 was not significant, compared to the controls. When E/T ratios were as high as 80:1, no high cytotoxic effect was observed. Such a situation is likely due to over-saturation of cell numbers (data not shown).

The NK-cytotoxicity increased significantly by the ratio of 1.51, and 1.613 ($p < 0.01$) respectively when pre-incubated with 1 and 10 uML TPA (Figure 4). Treatment with wheat grass extracts produced comparable cytotoxic effects to the treatment with TPA. The latter was used as the positive control and NK cytotoxicity enhanced by wheat grass extract was comparable to that of this positive control.

Individual variance of cell subtypes

Regarding individual variance of cell subtypes, it was found the ratio of sample % vs. control was 2.30 while for the concentration of 10 ug/ml were 29.09 for wheat grass extract (100(ug/ml). These data implies that the phenotypical changes of NK cells might be the cause of enhancement of the NK- cells. The ratios of CD14CD56 NK cells sample % vs. control % for wheat grass extract in the concentrations of 100 and 500 jig/ml were 1.79, and 1.89, respectively. Both are quite close to each other, which implies that the peak of the effect is around the concentration of 100 |ug/ml

Figure 4: The differences in the ratios of sample % vs. control % of CD26CD14 monocytres/macrophages when using different wheat grass extract concentrations of 100, and 500 ug/ml, respectively.

(Figure 5). The largest response ratio was noted at the lowest level of CD14CD56 NK cells. The populations of CD14CD56 NK cells were about 11 times higher in the concentration of 10|ag/ml than that of l00ug/ml (29.09: 2.68 =10.85: 1). In the presence of higher concentrations, the CD14CD56 marker expression decreased by 10.85%.

There are differences on CD26+ CD56+ NK cells in ratios [of sample% vs. control %] segmented by different fractions of cell size, i. e. >30 kDa vs. > 10 kDa, respectively.

Figure b: The p values of various CDs after Kolmogorov-Smirnov test, before transformation with logarithm.

Figure 5: The different effects of CD14+CD26+ macrophages on the ratio of sample % vs. control % with positive controls using TPA in different concentrations.

Discussion and Conclusion

The statistical distribution of various CDs with the 55 bio-measurements before transformation with the logarithm was de-

termined. Without transformation, the difference of distribution among the various CDs lies in the fact that these five CDs were in normal distribution.

Upon Kolmongrov - Smirnov test, the entire 5 CDs were still all in normal distributions.

The entire 5 5 measures after transformation with the logarithm, for variables of all CDs, reveals that normal distribution parameters estimated: mean =- 0.15971796, and Standard Deviation = 0.53148599. The Kalmongrove-Smirnoff test was carried out in order to test normality: p value = 0.288, which is greater than the significant level of 0.05; therefore, null hypothesis of Kalmongrove-Smirnov test is not rejected. The distribution is normal.

On the other hand, scattered plot with the quantiles of the scores after logarithmic transformation of the horizontal axis and the expected normal scores on the vertical axis. The steps in constructing a QQ plot are as follows: First, the data is sorted from smallest to largest. A plot of these scores against the expected normal scores should reveal a straight line. The expected normal scores are calculated by taking the z-scores of (I -Vi)/n where I is the rank in increasing order. For variables, all CDs of entire 55 measures (after taking log-transformation), the parameters are as following: Normal distribution parameters estimated: mean =- 0.15971796, and Standard Deviation (SD) = 0.53 148599. After performing the Kalmongrove-Smirnov test to test normality: p value = 0.288, which was greater than 0.05, the significant level set. Curvature of the points indicates departures of normality. This plot is also useful for detecting outliers. The outliers appear as points that are far away from the overall pattern of points. (The plot is not shown here.).

The out-performance of CD56 NK cells treated with wheat grass extract

Quite interesting, as well, important to note that the explanation of more than two SD (standard deviations) away from the mean is that the measurement of 2.3, as the effect of treatment with wheat grass extracts on CD56 NK cells, after logarithmic transformation is not a statistical outlier of this distribution, but actually is the best performance among these 5 5 biomeasurements to the extent that the ratio of sample % vs. control % is concerned. Such an inference is supported with Box- plots and Histogram after transformation with the logarithm. Whereas two other measurements of the ratio of sample % vs. control %, for CD3 T lymphocytes, 0.11 treated with Quercetin, and that for the identical CD3 T lymphocytes, 0.16 treated with GSPE respectively are the actual outliers of this population distribution. (Plots and the Histogram are not shown). Of importance in this context is the fact that 2.3 is the measure of the ratio of wheat grass extract. The ratio of 2.3 of sample % vs. control % does the best out-perform as compared with the rest of 10 other natural substances, and not to be regarded as an outlier.

Such a ratio of 2.3 of sample % vs. control % does the best outperform as compared with the rest of 10 other natural substances in this pilot study, and not to be regarded as any outlier. Among totally 55 such measures, 2.3 is the value of CD56 NK cells treated with wheat grass extract, as being indicated. Whereas two other measurements of the ratio of sample % vs. control %, for CD3 T lymphocytes, 0.11 treated with Quercetin, and that for the identical CD3 T lymphocytes, 0.16 treated with GSPE respectively are the actual outliers of this population distribution, they are represented by the two isolated columns situated at the most left-handed side in the Histogram. Hence, the UCB samples from six different individuals (participants) after treatments with wheat grass extract respectively exhibited a significant immune response, predominantly involving CD56 NK cells in the current pilot study.

Lyses of K562 cells after enrichment of CD56+ NK cells vs. that after the treatment with the positive control TPA

NK cell cytotoxicity increased significantly by the ratio of 1.51, and 1.613 ($p < 0.01$) respectively in preincubation with 1 uML and 10 uML TPA, which is the positive control (Figure 4).

Individual variance of cell subtypes

For wheat grass extracts, in the concentration of lOOug/ml, the ratio of sample % vs. control was 2.68, while that in the concentration of 1 Oug/ml was 29.09. These data may indicate that the phenotypical changes of NK cells might be the cause of enhancement of the NK-cell cytotoxicity effect, and treatment with high concentrations of wheat grass extract may decrease the NK cell growth (Figure 5). Our impression is as following: for samples treated with wheat grass extract, on CD56+ NK cells, the ratio of sample % vs. control % was 2.3, the highest among all the 55 measures. We believe that wheat grass extract was advancing cellular immunophenotypic expression, and promoting the cytotoxicity of CD56+ NK cells. (The proposed mechanism see details in the section of Result.).

FcR receptor blocking in Fc regions (FcR)

By definition, F means a fragment of the immunoglobulin's fragment; while Fc means the crystallisable fragment. During the experimental process of using magnetic labeling, microbeads were conjugated to monoclonal antibodies that have exposed Fc regions (FcR). At such regions, there was binding taking place non-specifically to FcR receptors. Hence, certain FcR blocking reagent is used to block unwanted binding of antibodies to human cells expressing Fc receptors such as B cells, monocytes, and macrophages. With these receptors blocked, the microbeads will only bind to their target antigen, thus increasing the specificity of the magnetic labeling.

The Fc receptor block reagent does not alter the binding behaviour - like affinity, avidity etc. of the antibodies (e.g. microbeads or fluorochrome conjugate). The expectation of increasing of specificity means in this case that unwanted binding to Fc receptors is subdued. Under our observation, in many cases e.g. rare cell isolation adding or leaving out certain Fc receptor reagent, showed different results in the flow cytometry examination. Without Fc receptor blocking reagent, negative cells can even shift when staining with a fluorochrome conjugate has been performed. Adding the Fc receptor blocking reagent does reduce or abolish such a shifting.

Since one Fc includes H chain; this portion of molecular structure has some natures, at least, as following: a] the fragment can combine with the complement Clq. b] containing carbohydrate, and c] deciding whether or not that the immunoglobulin may go through the placenta. As well, d] related with opsonization. Hence, Fc receptor blocking is, at least in immunology, a widely accepted standard method, especially when we are working with rare cells, such as CD56 NK cells. Blocking reagents against Fc receptors is a standard routine for all stainings performed. It is done to be absolutely certain that the positive staining signal is really due to the specific binding of the antibody to its recognized antigen and not just the Fc part binding to the Fc receptor. In this study, the cell source was a pregnant woman immediately after delivering baby, which may contain less Fc receptor positive cells or cells with less up- regulated Fc receptors than from a patient in general.

The limitations of study

The lack of using purified compounds but employing instead the crude extracts in the natural substance assay is inherently a limitation of this study (Figures are not shown.).

Conversely, NK-T cells comprise less than 0.1% of lymphocytes in adult peripheral blood and umbilical cord blood [6]. Both NK-cells and NK-T cells may play important roles in protecting the newborn against infection, which certainly deserves further study. Additionally, a category of NK cells which are activated by post-interleukin 2 activities have distinctively been called Lymphokine-activated killer (LAK); cells. They have been reported to produce a soluble, yet unidentified protein, in mixed tumor-adherent MNC cultures of PB, which substantially reduced the induction of LAK cells in the culture [8]. Other proposes that the existence of a postoperative immunosuppression cascade consist of increases in cytokines and immunosuppressive proteins decreases in both the helper and the cytotoxic T-cell populations, and the development of suppressor T-cell activities postoperatively [9]. We have not yet addressed to these issues except evidently in the current study, the first author (Tang, B) has discovered the complexity Cd3 T lymphocytes' normality in its distribution. See figure b [10-12], Klersy, andria.

Often, biological mechanisms induce log-normal distribution [11,12]. This study has 11 treatments with 1 sample of CD56 cells per treatment with one of each of the 11 natural health substances (one single number as an outcome per treatment), and same for each of the rests of 4 CDs. Unfortunately, because of a shortage of umbilical cord blood we do not have multiple measures within treatment variance, but merely one outcome per treatment. Hence, we could not do statistics on the differences in effects between treatments, but to merely express the finding in a purely descriptive manner.

Interleukin 12 can stimulate the activated Tc cells, and advances the differentiation of the NK cells [13,14]. Modulating immunological functions in lymphocytes of umbilical cord blood with natural health substances is very interesting and if successfully accomplished, could advance our knowledge in controlling human defense against wide varieties of diseases. Nevertheless, the results presented in this report thus far still fall short of this aim.

With regards to the important finding of the current study discovered by the first author Dr. Tang, that is, in the presence of higher concentration of wheat grass extract, the CD 14CD56 markers' expression activity has been decreased about by 11%, which has never been previously reported or published anywhere else.

In conclusion, this pilot study shows that wheat grass extracts likely orchestrates and promotes the in vitro maturation, and the naive fate of CD56+ NK cells in the hUCB. Hopefully, the future identification and purification of molecules of the crude wheat grass extract, will hopefully to be active for augmenting CD56 NK cell function, with opsonization of Fc, and thus may be expected to open a new avenue of cancer immunotherapy that stimulates innate immunity. Such an important factor should be highly considered both immunologically and clinically.

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