Research Article

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INFLUENCE OF SOME TRADITIONAL CHINESE MEDICINES (TCMS) ON CYTOKINE-INDUCED KILLER CELLS PROLIFERATION AND ANTI-TUMOR FEATURES IN VITRO

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ABSTRACT

Some traditional Chinese medical (TCM) formulations shown to have anti-tumor actions in patients with various solid tumours. However, the mechanisms of the potential anticancer action are rarely reported. In the present study, we found that extracts from pharmaceutical raw materials consisting of ROR (Red ginseng, *Ophiopogon japonicus*, *Radix Astragali*) could significantly improve the proliferation rate and cytotoxic activity of cytokine-induced killer (CIK) cells, which is a heterogeneous population of polyclonal T lymphocytes sharing NK (Natural Killer) phenotype and functional properties. The extracts were prepared using modified ethanol-water reflux method. Pharmacodynamic test results showed that 5% ROR-adding CIK Cells group performs 2.8 folds proliferation multiples than that of no ROR-adding CIK cells group, meanwhile the cytotoxic activity to K562 tumor cell has also a significant difference between them. In conclusion, ROR-adding CIK cells group exhibits stronger proliferation and cytotoxicity than no ROR-adding CIK cells group, and the results provided a new experimental evidence for TCM's application in CIK-immunotherapy.

Keywords: cytokine-induced killer cell, TCM, cell proliferation, cytotoxic activity

INTRODUCTION

Immunotherapy stimulating the immune system and enhancing the patient's own anti-tumor ability is an alternative and promising way to treat cancer. Cytokine-induced killer (CIK) cells are a heterogeneous population of polyclonal T lymphocytes sharing NK (Natural Killer) phenotype and functional properties. Initially described by Schmidt-Wolf et al. in 1991¹, CIK cells are endowed with a potent MHC-unrestricted anti-tumor activity and can be efficiently expanded in vitro from bone marrow or peripheral blood mononuclear (PBMC) by the timed addition of IFN- γ , Ab anti-CD3 and IL-2²⁻⁴. The first property is the possibility

of CIK cells to be *ex vivo* expanded to adequate numbers for effective and multiple *in vivo* infusions. CIK cells are therefore, suitable for the immunotherapy against tumor cells, especially residual tumor cells.

Traditional Asian Medicine has been documented for centuries in China, Korea, Japan, and other countries in Asia⁵. The traditional practice has been used extensively in therapy and prevention of various disorders and diseases, including cancer. Recent studies of traditional Chinese Medicine (TCM) in cancer have been focused on molecules extracted and purified from herbs. Furthermore, Some traditional Chinese medical formulation, main ingredients including phytohemagglutinin, isoflavonoid,

saponins and polysaccharide etc., used as an adjuvant in cancer treatment or as a phytochemical immune modulator to strengthen the host defense system, have shown extensively to have anticancer actions in patients with various tumours⁶⁻¹¹. In the last decades, the effects of redginseng extracts, the active components of *Panax ginseng*, were evaluated on transplantable tumors, proliferation of lymphocyte, two-stage model and rat liver lipid peroxidation¹⁰. Various bioactive components extracted from *Ophiopogon japonicus* (Thunb.) KerGawl tuber, such as homoisoflavonoid, saponins and polysaccharide, was also evaluated¹¹. *Radix astragali* (Huang qi), dried root of *Astragalus membranaceus* Bge. Var. mongholicus, was considered as a tonic in the traditional Chinese medicine. Many researchers found that *R. astragali* could activate the cytotoxicity of lymphocytes to increase the secretion of IL-2 and IFN- γ ¹². The sound reason seems to be caused by containing several bioactive constituents such as isoflavonoids, saponins, and polysaccharides¹³⁻¹⁴.

Thus, in present study three immune function-enhancing agents' formulation consisting of Redginseng *O. japonicus*, *R. Astragali* (abbreviated as ROR), were investigated for the effect on proliferation and cytotoxicity of CIK cells using a modified extraction method. The aim was to determine the feasibility of generating a sufficient number of CIK cells to assess the toxicity of this regimen, and to evaluate its ability to exert anti-tumor effect.

MATERIALS AND METHODS

ROR extraction and solution preparation

The ROR extracts preparation was a slight modification of the method described by previous study¹⁵. 300 grams of dry and minced ROR (100 grams each agent) were treated by hot reflux extraction with 1.8 L ethanol-water (80:20, V/V) solution for 1.5h. Extract solution of ROR was concentrated to dryness using by the ethanol solvent in a rotary evaporator (Shanghai SENCO Science & Technology Co., Ltd., China) at 60°C. Subsequently, sterilized water was added, and then the liquid was centrifuged with 2500 rpm for 15 min to obtain the sample solutions. Then the collected solution was concentrated and filtered by 0.45 μ m filter until the crude ROR concentration reached to 5g/ml. Finally the pyrogen were removed by activated charcoal and pH monitored to

7.0, the final ROR extracts were sterilized by 0.22 μ m filter. All other chemicals were of analytical grade and purchased from commercial sources.

Tumor cell lines and culture conditions

The K562 cell line, a highly undifferentiated human erythroleukemic cell line which does not express MHC class I molecules, was provided by Chinese Academy of Medical Sciences, Beijing, were maintained in RPMI 1640 medium containing 10% of FCS, 100 U/ml of penicillin and 100 U /ml of streptomycin at 2×10^6 cells/ml.

ROR solution Co-culture with CIK cells

Briefly, 50mL heparinized peripheral blood was obtained from an eligible patient. The peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (ρ : 1.077 $\pm$ 0.002) density centrifugation. The cells were divided individually into six groups with 2×10^6 cells/ml. Group 1 were incubated with routine CIK cells culture method. The PBMCs were then incubated in fresh serum- free medium (Takara, Japan) with IFN- γ (1000 U/mL; Shanghai Clonbiotech, China). After 24 hours of incubation in a humidified atmosphere with 5% CO₂ at 37°C, biochemically purified murine monoclonal T3 antibody (100 ng/mL, muromonab-CD3; Janssen Pharmaceutica, Titusville, NJ), IL-2 (1000U/ mL; Sihuan Pharmaceutical Co., Beijing, China), and IL-1 α (1000 U/mL; eBioscience, San Diego, CA) were added. IL-2 was added every 3–4 days regardless of cell concentration.

Group 2-6 were treated with different ROR solutions concentration (1%, 3%, 5%, 7%, 9%) on day 0. The same regimes, as above, were followed after 24h incubation. Different concentration ROR solutions were co-cultured in a 640-cm² bag for investigating the effect of ROR. Each of the individual culture bags was sampled on day 11 for sterility testing, and then, on day 14, the cells were harvested and washed twice in saline water with 1% human serum albumin.

Proliferation assessment

The cells proliferation of eight groups were assessed by using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma] assay, which is based on the conversion of MTT to MTT-formazan by mitochondrial

enzymes. Viable cell numbers were determined by the trypan blue exclusion method using a hemacytometer.

Chromium release cytotoxicity assay

The ability of CIK cells to lyse K562 (MHC devoid target cells) was examined using a standard chromium release cytotoxicity assay¹⁶. Two million K562 cells were labeled with 50 μCi $^{51}\text{CrO}_4$ (Amersham International, UK) for 1 h at 37 °C, 5% CO_2 . For effector/target (E/T) titration assays, ROR co-cultured CIK cell and ^{51}Cr K562 cells were plated at 10^4 cells/well in round-bottom 96-well plates with the following E/T ratios (1:1, 5:1, 10:1 and 20:1) for 4h incubation. Supernatant was harvested after 4 hours incubation at 37°C and 5% CO_2 . Spontaneous release was determined by incubating target cells with an equal volume of media only (no effector cells) and

maximum release was determined by incubating target cells in medium containing 1% aqueous solution of NP-40. Cells were co-incubated for four hours at 37°C, and then 100 μl of supernatant was transferred to another microplate. Microplate counting of supernatants was measured by gamma counting with Packard Cobra 5010 (Perkinelmer, USA). The percent specific lysis was calculated as follows: $[(E - M)/(T - M)] \times 100$; T= maximal release, M = spontaneous release, E = experimental release.

Statistical analysis

Data are expressed as means $\pm$ SD. Duncan's multiple range tests was used to determine the difference among groups. *P*-values of less than 0.05 were considered significant.

Table 1: Cytotoxic activity of routine culture CIK and ROR-adding CIK groups against K562 cells

Group	Effector-Target (E/T) ratio			
	20:1	10:1	5:1	1:1
routine culture CIK	83.43 $\pm$ 0.98 a	65.25 $\pm$ 2.37 a	42.73 $\pm$ 2.45 a	10.19 $\pm$ 0.93 a
1% ROR-adding CIK	85.51 $\pm$ 1.35 a	69.38 $\pm$ 2.75 a	49.24 $\pm$ 3.12 b	11.21 $\pm$ 0.78 a
3% ROR-adding CIK	84.22 $\pm$ 0.72 a	66.33 $\pm$ 1.31 a	48.37 $\pm$ 1.78 b	10.45 $\pm$ 1.21 a
5% ROR-adding CIK	85.57 $\pm$ 0.89 b	70.19 $\pm$ 1.67 b	47.96 $\pm$ 3.25 a	11.32 $\pm$ 1.14 a
7% ROR-adding CIK	84.74 $\pm$ 1.03 a	69.26 $\pm$ 1.97 a	45.71 $\pm$ 1.75 a	10.78 $\pm$ 0.91 a
9% ROR-adding CIK	83.14 $\pm$ 1.27 a	64.62 $\pm$ 2.48 a	42.26 $\pm$ 2.93 a	10.93 $\pm$ 1.82 a

All data were expressed with mean $\pm$ SD. Different letters differed significantly in height ($P < 0.05$) compared with CIK

Figure 1: The proliferation multiples of CIK cells with different ROR-adding treatments. The bars with different lowercase letters differed significantly in height ($P < 0.05$) based on ANOVA. Error bars: SD.

RESULTS

Proliferation assessment

The CIK proliferation multiples of each group in day 14, based on the initial PBMCs populations 2×10^6 cells/ml, are illustrated in Fig 1. The proliferation multiples (610 ± 20) presented 5% ROR-adding group was the highest among all groups. The proliferation multiples of other five groups were assessed as no ROR-adding group (200 ± 14), 1% ROR-adding group (234 ± 29), 3% ROR-adding group (421 ± 27), 7% ROR-adding group (180 ± 27), 9% ROR-adding group (120 ± 24) respectively. 5% ROR-adding group is 2.8 folds than the routine culture method of CIK cells. CIK proliferation rate of 5% ROR-adding group and 3% ROR-adding group were significantly higher than those of other four groups ($P < 0.05$) and there was significant difference between those two groups ($P < 0.05$). The proliferation rate of 9% ROR-adding group was the lowest, even less than the control, no ROR-adding CIK cell group.

Cytotoxicity of CIK cells against tumor cells

An effector-target (E/T) cell ratio of CIK and ROR-CIK groups were investigated against the K562 cell line. As it showed in table 1, the cytotoxic activity of CIK cells from different groups was growing with the E/T cell ratio increasing. The 5% ROR-adding CIK cell group obtained the highest level of cytotoxic activity (85.57 ± 0.89 %) at 20:1 E/T ratio, (70.19 ± 1.67 %) at 10:1 E/T ratio, simultaneously. Furthermore, cytotoxic activity of CIK cells from 3% and 5% ROR- adding CIK groups (both) had significant difference in comparison control (no ROR- adding) CIK cell ($P < 0.05$). Additionally, the cytotoxic activity of CIK cells from 1% and 3% ROR-adding CIK groups were higher than control CIK group, however 5% was not at 5:1 E/T ratio. At 1:1 E/T ratio, there were no significant differences among these groups on the cytotoxic activity of CIK cells.

DISCUSSION

CIK cells are promising for the immunotherapy against residual tumor cells because of the in vitro proliferation. Therefore, the quality and quantity of CIK cell in vitro should play a crucial role for its potential immunotherapy. Although ingredients of ROR have not been identified instantly however the good proliferation and high

anti-tumor effect were demonstrated in the present study. Comparison with different concentration ROR-adding and routine culture method, CIK cells groups stimulated by IFN- γ , muromonab-CD3, IL-2 etc. showed that CIK populations of the former treatments changed earlier and much more continuous than that of the later. Previous studies on phytohemagglutinin (PHA) found that appropriate concentration of PHA could activate the Ca^{2+} -dependent pathways to secrete IL-2 inducing the CIK cells proliferation⁶. This could also be an appulsive reason for our results. Various inducers in ROR co-culturing with cytokines, the proliferation function was activated strongly to promote the CIK populations. Consequently, not only the ingredient of ROR but also the optimum dose and the pre-stimulation time should be worthy for further investigation.

Obviously, stronger cytotoxic activity could provide much more confidence for clinical immunotherapy. In this study, 5% ROR-adding CIK cells group was illustrated to have higher cytotoxic activity against K562 cell line based on the effector-target factor. However, the differences were not always significant. At 5:1 E/T ratio, the cytotoxic activity of CIK cell from 5% ROR-adding group has no difference with the control (no ROR-adding group), unlike that from 1% and 3% group. The possible reason could be inhibitors in ROR solutions. So the low concentration of ROR-adding should be considered mild before all ingredients were identified.

Conclusively, the appropriate concentration ROR-adding could improve the CIK proliferation multiples and its anti-tumor effect. It seems that the anti-tumor effects of TCMs could be triggered through inactivating the T lymphocytes, such as CIK cells. TCMs, especially fuzheng drugs can activate immune cell activity and phagocytosis is enhanced while improving immune function, thereby it indirectly improve the quality of life of cancer patients and prolong the survival.

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