Effects of *Rosa roxburghii* Extract on Proliferation and Differentiation in Human Hepatoma SMMC-7721 Cells and CD34$^+$ Haematopoietic Cells

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Rosa roxburghii Tratt is an herbal medicine with anticancer potential. This study investigated the effects of an ethanol extracts and a triterpene (CiLi triterpene) of *Rosa roxburghii* on proliferation and differentiation of human hepatoma SMMC-7721 cells and in umbilical cord blood CD34$^+$ hematopoietic progenitor cells. The CiLi triterpene and ethanol extracts inhibited the proliferation of hepatoma cells in a concentration- and time-dependent manner and decreased the release of alpha-fetoprotein from hepatoma cells. Apoptosis was increased only at the highest dose of the ethanol extract in hepatoma cells. The Cili triterpene and ethanol extracts of *Rosa roxburghii* did not affect the differentiation of cord blood CD34$^+$ cells to granulocytes and monocytes, as evidenced by flow cytometry analysis of CD11b and CD15. Thus the Cili triterpene and ethanol extracts of *Rosa roxburghii* are effective in the inhibition of human hepatoma SMMC-7721 cell growth, without affecting the differentiation of CD34$^+$ cells.

Key words —— *Rosa roxburghii* extract, proliferation, differentiation, human hepatoma SMMC-7721 cells, umbilical cord blood, CD34, hematopoietic cells

INTRODUCTION

Rosa (*R.*) *roxburghii* Tratt, also called CiLi in Chinese medicine, is a plant of which the fruit juice has been used as a medicinal remedy for a variety of diseases. CiLi is rich in vitamin C and vitamin E and contains biologically active components such as superoxide dismutase, polysacchairide, roxburic acid, roxburic glycoside, and catechin.[1)] *R. roxburghii* Tratt has been shown to have beneficial properties for cardiovascular, gastrointestinal, urinary, and reproductive functions, including anti-aging, free radical scavenging, immunologic regulation, stress tolerance, *etc.*[2–5)]

R. roxburghii Tratt juice has been shown to inhibit the growth of Ehrlich ascites tumor xenograft and human leukemia K562 cells *in vitro*.[6,7)] Clinical investigation showed that a CiLi mixture can reduce bladder carcinoma incidence in benzidine- exposed population and prevent postoperative tumor relapse.[8)] CiLi juice is effective against pro-nitroso dimethyl amine-induced hepatocellular cancinoma in mice.[9)] Resent studies have also shown that CiLi ethanol extract inhibited the growth of gastric carcinoma cells *in vitro*.[10,11)] However, little is known about the effect of CiLi on hepatoma cells.

Chemotherapeutic agents are unable to distinguish cancer cells from normal cells and often produce myelosuppression. Bone marrow toxicity is commonly produced by chemotherapeutic agents, including neutropenia and severe pancytopenia.[12)] CiLi is relatively safe, no apparent toxicity report is available, and little is known about its effects on bone marrow cells, especially at anticancer doses. This study was aimed at examing the growth-inhibitory effect of the extracts from *R. roxburghi* on

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hepatoma SMMC-7721 cells *in vitro*. Furthermore, we evaluated the effects of a CiLi triterpene and ethanol extracts on the proliferation and differentiation of human umbilical cord blood (UCB) $CD34^+$ hematopoietic stem/progenitor cells (HSPCs) with granulocyte-macrophage colony stimulating factor (GM-CSF) for screening for potential bone marrow depression.

MATERIALS AND METHODS

Materials—— The CiLi triterpene (CT, purity 98%) and CiLi ethnol extracts (CiLi) of *R. roxburghii* Tratt were provided by Key Laboratory of Chemistry for Natural Products of the Chinese Academy of Science of Guizhou, China. CT and CiLi were dissolved in 5% ethanol (final concentration) to 100 µM and 100 µg/ml, respectively, and further diluted in culture medium. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and trypan blue were from Sigma (St. Louis, MO, U.S.A.). The alpha-fetoprotein (AFP) radioimmunoassay kit was from Jiuding Biotech. Co. Ltd. (Tianjin, China).

SMMC-7721 Cell Culture—— SMMC-7721 cells were purchased from the Institute of Cell Biology of the Chinese Academy of Science of Shanghai. Cells were maintained in RPMI 1640 medium (Gibco BRL, Grand Island, NY, U.S.A.), supplemented with 10% newborn calf serum, penicillin 100 U, and streptomycin 100 µg/ml, in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cells were treated with various concentrations of CT (10 nM–10 µM) and CiLi (0.01–10 µg/ml) for 24 hr to 96 hr. 5-Flurouracil (5FU, 100 µM) was used as a positive control and 0.05% ethanol as vehicle control. Cells at 80% confluence were used for experiments.

MTT Assay—— Cells were seeded in 96-well culture plates and treated with various concentrations of CT and CiLi for 24–96 hr, and then incubated with MTT 5 mg/ml, 10 µl per well, at 37°C for 4 hr. The culture medium was carefully aspirated and dimethylsulfoxide 150 µl was added to dissolve the blue formazan product. Cell viability was determined from the value of the optical density (OD) at 490 nm on an enzyme-lined immunosorbent spectrophotometer (Tecan, Untersbergstrass, Australia). Living cells were also evaluated using the trypan blue dye-exclusion method.

AFP Radioimmunoassay—— SMMC-7721 cells were seeded in 24-well plates for 24 hr, and then 100 µl supernatant of culture solution was collected and assayed with ^{131}I-labeled antibody against AFP, using q γ-radio-immunity counter (Gcr-2010, Kedachuangxin CO. Ltd., Tianjin, China).

Apoptosis Determination—— SMMC-7721 cells were cultured for 24 hr and treated with the indicated concentration of CT and CiLi in triplicate. The cells were stained by dual-color fluorescence of Annexin V/fluorescein isothiocyanate (FITC, Jingmei Biological Engineering Co. Ltd.) and propidium iodide. Apoptotic cells were counted with flow cytometry (BD FACS Calibur, San Jose, CA, U.S.A) in 10^4 cells after incubation for 15 min in the dark.

Collection of UCB and Magnetic-activated Cell Sorting (MACS) $CD34^+$ HSPCs in Cord Blood—— UCB samples were obtained after full-term cesarean delivery from healthy donors following hospital ethical regulations and approval by the Institutional Human Research Committee. Blood samples were diluted 1:1 in phosphate-buffered saline (PBS). Mononuclear cells (MNCs) were enriched from cord blood using density-gradient centrifugation with Histopaque (Sigma, St. Louis, MO, U.S.A). After incubation of MNCs with CD34 antibody of MACS MicroBeads (Miltenyi Biotec, Germany), $CD34^+$ cells were selected on the MACS columns and MACS separators for culture.[13, 14] Cells before and after MACS were subjected to dual-color fluorescence in FACS analysis with conjugated monoclonal antibodies including mouse $IgG_1\kappa$/FITC and phycoerythrin (PE) isotype controls. Anti-CD11b-PE, anti-CD15-FITC, and anti-CD34-PE were from Becton Dickinson Immunocytometry Systems (San Jose, CA, U.S.A.).

Cell Proliferation and Differatiation Assays in Primary $CD34^+$ HSPCs—— Magnetic bead-separated human cord blood $CD34^+$ and $CD34^-$ cells (2000 cells/ml) were maintained in a 37°C humidified atmosphere of 5% CO_2-95% air in Stemline Hematopoietic Stem Cell Expansion Medium (Sigma) for 14 days,[15, 16] supplemented with 10% fetal bovin serum (Invitrogen, Carlsbad, CA, U.S.A.) and GM-CSF (Sigma, final concentration 200 U), penicillin 100 U/ml, and streptomycin 100 µg/ml. Cells were seeded into 24-well plates (Falcon Biosciences, Franklin Lokes, NJ, U.S.A) at a density of 2000 cells/well. Cells were treated with CT (1 µM, 10 µM,), CiLi (1 µg/ml, 10 µg/ml), or 0.5% ethanol (vehicle control) or culture medium (control group), $CD34^-$ cells were used as a negative control. The cells were counted using the try-

pan blue dye-exclusion method. The cells were collected and evaluated using flow cytometry for the expression of CD11b, CD15, and CD34 antigens.

Statistical Analysis —— Results are expressed as mean ± SEM from three to five repeated wells of each experiment, with a minimum of three independent experamants. Statistical significance was determined using Ducann's multiple test. $p < 0.05$ was to represent a statistically significant difference.

RESULTS

CT and CiLi Inhibiton of SMMC-7721 Cell Growth

The viable cell numbers of SMMC-7721 cells treated with CT and CiLi were detected using the MTT test (Fig. 1). The OD values decreased with increased CT and CiLi concentrations and with time ($p < 0.05$ compared with vehicle group). The percentage of inhibition was calculated base on the OD value. When cells were treated with CT 1 μM and 10 μM and CiLi 1 μg/ml, and 10 μg/ml for 24 hr, the inhibition percentages of cell proliferation were 13.2% and 21.7% and 18.6% and 19.2%, respectively. The inhibition percentages increased to 47.9%, 52.6%, 28.8%, and 36.8%, respectively, 96 hr after treatment. The 5FU (100 μM) group showed 28.8% and 58.0% inhibition at 24 hr and 96 hr (data not shown), respectively.

Effects of CT and CiLi on Growth Curves of SMMC-7721 Cells

Growth curves of SMMC-7721 cells were gradually shifted to the right following the increased concentration of CT (from 10 nM to 10 μM) and CiLi (from 0.01 μg/ml to 10 μg/ml), especially in the CT treatment groups, in a similar pattern to the MTT test (data not shown).

Effects of CT and CiLi on Level of AFP in Cultured SMMC-7721 Cell Medium

The concentrations of AFP decreased with CT and CiLi treatment in a dose-dependent manner compared with the vehicle control in culture medium of SMMC-7721 cells (Fig. 2). The level of AFP did not differ between vehicle and medium treatment ($p > 0.05$). 5FU had no significant effect on AFP levels (data not shown).

Apoptosis Assay with CT and CiLi

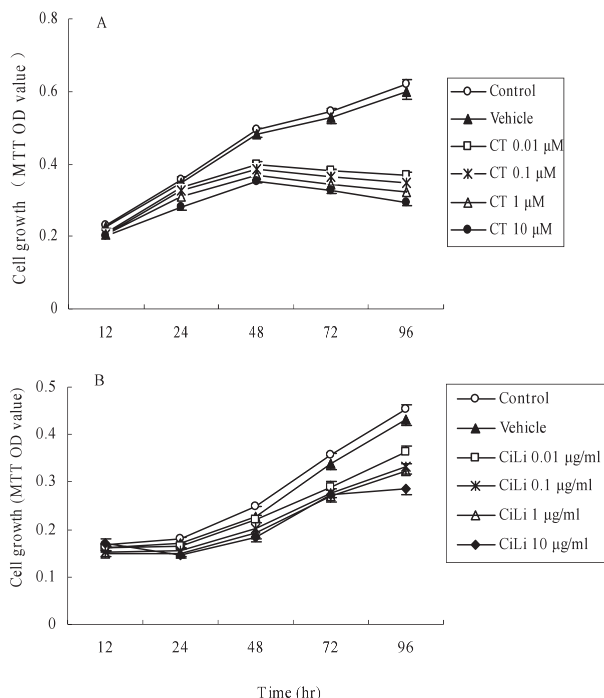

Fig. 1. Growth Inhibition Effects of CT and CiLi Extracts on Human Hepatoma SMMC-7721 Cells in the MTT Test

Mean ± SEM, five wells per group and repeated three times. $^*p < 0.05$ compared with vehicle control.

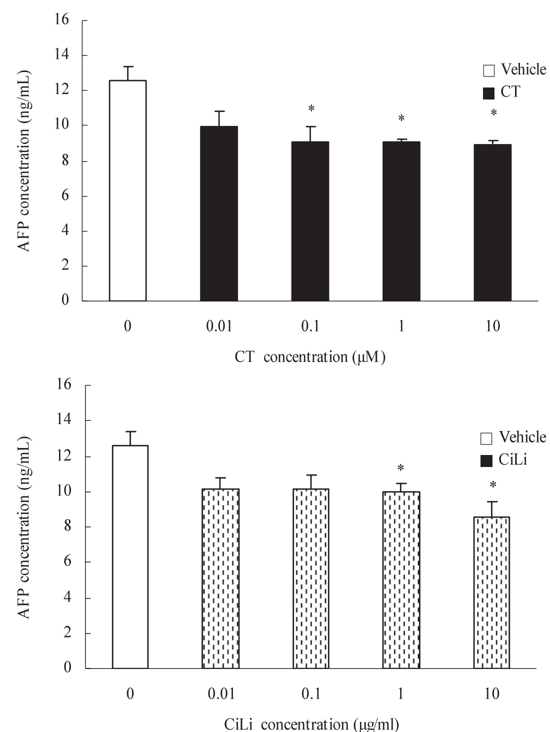

Fig. 2. AFP Changes in Cultured Human Hepatoma SMMC-7721 Cell Medium with CT and CiLi Treatment after 24 hr in Radioimmunoassay

Mean ± SEM, $n = 3$. $^*p < 0.05$ compared with vehicle control.

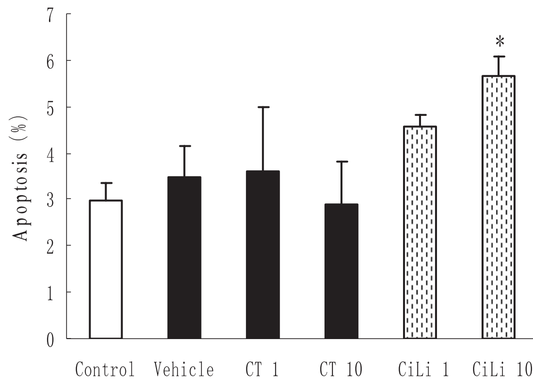

Fig. 3. Apoptotic Cell Percentages of Human Hepatoma SMMC-7721 Cells with CT (1 and 10 μM) and CiLi (1 and 10 μg/ml) Treatment for 24 hr by Flow cytometry

Mean ± SEM, $n = 3$, $^{*}p < 0.05$ compared with vehicle control.

Table 1. Effects of CT and CiLi on Proliferation of Human Umbilical Cord Blood $CD34^{+}$ Hematopoietic Stem Precursor Cells with GM-CSF for 14Days

Group	Concentration	Cell number
control	same volume medium	7780 ± 147
ethanol	0.05%	4720 ± 253
CT 1	1 μM	2644 ± 91#
CT 2	10 μM	2788 ± 91#
CiLi 1	1 μg/ml	1389 ± 60*
CiLi 2	10 μg/ml	2083 ± 72*

Mean ± SEM, three wells per group and repeated three times.$^{*}p < 0.05$, *vs*. ethanol vehicle, $^{\#}p < 0.05$ *vs*. CiLi 2.

CT did not affect apoptotic percentages of SMMC-7721 cells at the concentrations used. Apoptotic cells slightly increased from 3.67 ± 0.79% to 5.65 ± 0.44% with CiLi 10 μg/ml treatment (Fig. 3), but were unchanged at CiLi 1 μg/ml.

Effects of CT and CiLi on Proliferation of Cord Blood $CD34^{+}$ HSPCs

The cord blood $CD34^{+}$ cells used in the study were of high purity (81.6% to 98%). Cell viability was greater than 90% in all cases. Human UCB $CD34^{+}$ HSPCs were cultured with GM-CSF for 14 days. Cell numbers tended to decrease with CT 1 μM and 10 μM, but were not significantly different compared with the vehicle and control groups (Table 1). Cell numbers were higher in CT gourps than in the high-dose CiLi group ($p < 0.05$). Total cell numbers were decreased in the CiLi 1 μg/ml and 10 μg/ml groups as compared with the vehicle and control groups. In the $CD34^{-}$ cell group, many of cells were dead and there were only 520 ± 147 live cells.

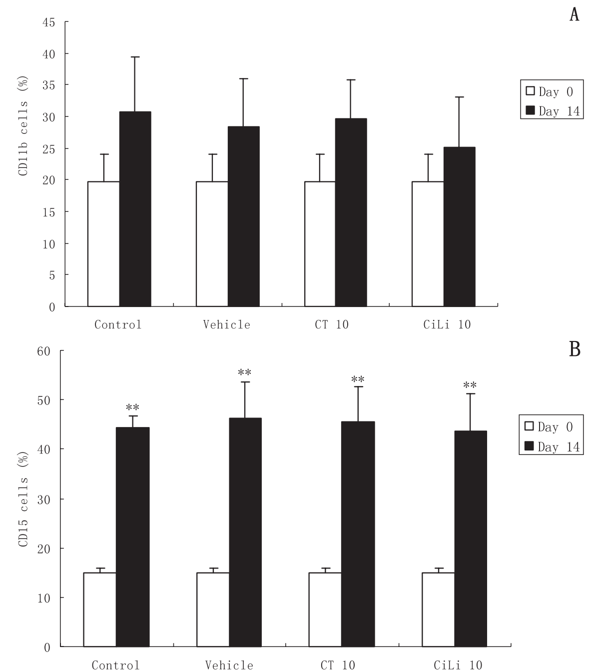

Fig. 4. Effects of CT (10 μM) and CiLi (10 μg/ml) on CD11b (A) and CD15 (B) Immunophenotype of Cells

The analysis was used flocytometry of cord blood $CD34^{+}$ cells in the culture medium with GM-CSF (200 U) before and after 14 days. The experiment was repeated three times per group. $^{**}p < 0.01$ compared with before treatment (day 0).

Effects of CT and CiLi on Cell Differentiation

Human UCB $CD34^{+}$ HSPCs were cultured with GM-CSF for 14 days. The cells expressing CD34 were markedly reduced to 1–2% from 81.6–98% on day 0 in all groups. The percentage of cells expressing CD15 were markedly increased in all groups at day 14 compared with those in culture on day 0. The expression of CD11b was 15.02 ± 0.771 on day 0. On day 14, CD11b tended to increase by 190–213% in all groups (data not shown), but the difference was $p > 0.05$ compared with that on day 0. The percentages of cells expressing CD15 and CD11b were not significantly changed in the CT 10 μM and CiLi 10 μg/ml groups compared with the 0.05% ethanol vehicle group (Fig. 4).

DISCUSSION

These results showed that CT and CiLi inhibited the proliferation of hepatoma SMMC-7721 cells in a concentration- and time-dependent manner. Hepatoma cell inhibition effect was more marked in the CT group than in the CiLi group, clearly indicating that CT and CiLi had antil-liver tumor poten-

tial. The results are consistent with reports in the literature that the juice of *R. roxburghii* Tratt inhibits the growth of K562 cells and Ehrlich ascites xenograft tumors *in vivo* and reduces human bladder cancer incidence.[6,7,9] CT did not affect apoptosis of hepatoma SMMC-7721 cells and apoptosis occurred only at the highest dose of CiLi. This suggests that apoptosis is not a primary mechanism for CiLi-induced growth inhibition of hepatoma cells, and that other mechanisms, such as cell differentiation, could play a role.

Both CT and CiLi significantly decreased the level of AFP in the culture medium. AFP is not only a tumor marker of hepatoma cells, but also a target of chemotherapy and a useful evaluation index of therapeutic effect. The serum AFP concentration is usually correlated with the malignancy of liver cancer. Low AFP levels indicate that differentiation of hepatoma cells was induced.[17,18] Therefore CT and CiLi may also induce hepatoma cell differentiation.

Many of the current anticancer drugs have significant side effects because they cannot distinguish cancer cells from normal cells, producting bone marrow depression. This creates a challenge for cancer chemotherapy. Thus anticancer agents with fewer side effects are desired. Human peripheral blood $CD34^+$ cells have been used as a source of hematopoietic progenitors to evaluate the myelotoxic effects of chemotherapeutic agents in the first stages of drug development.[19] Human UCB $CD34^+$ cells are similar to both peripheral blood and bone marrow $CD34^+$ cells, although their biological characteristics are not identical. For example, GM-CSF was not detected in UCB $CD34^+$ cells.[20,21] Human UCB cells are therefore an attractive alternative to replace peripheral blood and bone marrow cells as a source of hematopoietic stem precursor cells and have been used in clinical transplantation for hematologic recovery following chemotherapy including leukemia.[22] In the present study, we successfully used GM-CSF to stimulate the differentiation of human UCB $CD34^+$ cells to granulocytes and monocytes, as evidenced by decreased $CD34^+$ cells and increased CD15 and CD11b cells 14 days after stimulation. The expressions of the surface molecules CD15 and CD11b were not affected by CT and CiLi, clearly demonstrating CT and CiLi did not affect human UCB $CD34^+$ cell differentiation to granulocytes and monocytes with GM-CSF stimulation. Although CT and CiLi are extrated from *R. roxburghii*, CT shows less inhibition of $CD34^+$ cell proliferation than CiLi, indicating that CT appears to be less toxici hematopoietic stem/progenitor cells, and thus CT may be a better candidate for further drug development. However CiLi inhibits the proliferation of $CD34^+$ HSPCs. This may be an adverse effect of CiLi on hematogenesis in bone marrow although CiLi has a anticancer effect.

In summary, this study clearly demonstrates that CT and CiLi are effective in inhibiting hepatoma SMMC-7721 cell proliferation. This effect was not primarily mediated by induction of apoptosis, but may be due to the induction of hepatoma cell differentiation to reduce AFP production. Both CT and CiLi at the antitumor doses did not affect human UCB $CD34^+$ cell differentiation, and CT is less inhibitory agonist $CD34^+$ cell proliferation, suggest that it is less toxic and deserves further investigation.

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