Study on the Apoptosis of Renal Cancer Cells and the Expression of FAS/APO-1 and BCL-2 Genes Induced by Kanglaite Injection

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Abstract

Purposes: To investigate the anti-tumor mechanism of Kanglaite Injection (KLT). Materials and Methods: The inhibiting action of KLT on renal cancer cells was examined by MTT method. Cell apoptosis was assayed by TUNEL method. The expression of gene Fas/Apo-1 and Bcl-2 was analyzed by immunohistochemistry method. Results: The IC\textsubscript{50} of KLT for inhibition of renal cancer cells was 19.31\mu/ml. KLT at the concentrations of 5\mu/ml and 10\mu/ml could effectively induce apoptosis of cancer cells with apoptotic percentage of 31.30% and 89.76% respectively. The percentage of apoptotic cells would decrease with higher concentrations of KLT, for example less than 10% of cancer cells were apoptotic in the group of 15\mu/ml or 20\mu/ml. Immunohistochemistry analysis showed that the expression label index for Fas/Apo-1 gene was 74.6% in KLT treatment group while it was 21.2% in control group. The expression label index for Bcl-2 gene was 25% in control group while it was 6.6% in 10\mu/ml KLT treatment group. Conclusion: The mechanism of the anti-tumor action of KLT can be found in its ability to induce apoptosis and necrosis of tumor cells. KLT of the concentration \leq 10\mu/ml mainly induces apoptosis of tumor cells. The optimal concentration for cell apoptosis induction is 10\mu/ml. More than 10\mu/ml of KLT will cause necrosis of tumor cells. The anti-tumor action of KLT might be explained by its ability to increase Fas/Apo-1 gene expression and inhibit Bcl-2 gene expression.

Key words: Kanglaite; apoptosis, Fas/Apo-1, Bcl-2

Kanglaite Injection is an anti-tumor agent prepared from Semen Coicis, a traditional Chinese medicine. Pharmacodynamic and clinical investigations have shown that KLT has a definite therapeutic effect of inhibiting the growth of many tumors. But its action and mechanism on renal cancer cells have not been reported. The purposes of this study are to investigate the anti-tumor mechanism of KLT in renal cancer cells and serve as guidance for clinical combined therapy of tumor.

1.Materials and Methods

1.1 Drugs and Cell Culture

Kanglaite injection (10%) and emulsion for control were supplied by Zhejiang Kanglaite Pharmaceutical Co., Ltd. Blue tetrazolium [3(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H tetrazolium-bromide, MTT] was purchased from Sigma, U.S.A. Dimethyl sulphoxide (DMSO) was purchased from England. APO-DIRECT Kit was purchased from Boehringer Mannheim, U.S.A.
The granulocyte renal carcinoma cell line (GRC-1) was supplied by the Institute of Urinary Surgery, the First Hospital of Beijing Medical University. The cells were cultured and passaged in RPMI-1640 culture medium containing 15% calf serum at 37°C, 5% CO₂ in an incubator.

Flow cytometer: FAC Scan (Beton Dickson, U.S.A) was supplied by the analysis department, the First Hospital of Beijing Medical University.

1.2 Blue Tetrazolium Reduction Method (MTT Method)

Inoculate 0.2ml logarithmic growth phase tumor cells of 1×10⁵/ml into each pore of a 96-pore culture plate. Establish saline water control group and experiment groups of different KLT concentrations with 6 parallel pores each group. The cells were cultured in an incubator at 37°C, 5% CO₂ for 72 hours. Add 10μl MTT (5mg/ml) to each pore 4 hours before termination of the experiment. The culture medium should be removed before test. 200μl dimethyl sulfoxide (DMSO) was added into each pore. The absorbance (O.D.value) of each pore would be measured by enzyme marker photometer at 570nm wavelength. The inhibition rate for cell proliferation was calculated with the following formula:

\[ \text{Inhibition Rate(%) = (1 - mean O.D.value of experiment group / mean O.D.value of control group)} \times 100\% \]

The IC⁰₅ was calculated with logarithmic probability graphic method.

1.3 Assay of Cell Apoptosis with Terminal Deoxynucleotidyl Transferase Labeled (TUNEL) Immunofluorescence staining Method

APO-DIRECT Kit was applied. Renal carcinoma cells were routinely cultured. KLT injection of 0μl/ml, 5μl/ml, 10μl/ml, 15μl/ml and 20μl/ml were added respectively to each experiment group when a approximate single-layer of cells formed. The cells would be cultured for another 48 hours, then digested. Washed them twice with 1×10⁶ cells suspension and phosphate buffer solution (PBS), then fixed with 5% paraformaldehyde for 15 minutes and washed again with PBS. Added 50μl TUNEL labeled fluorescence mixed staining solution to the cell sediment and kept by lukewarm bath at 37°C for 1 hour, washed with PBS and added Propidium Iodide (PI) 20μl to react at room temperature for 30 minutes for assay.

1.4 Immunohistochemical Analysis

Logarithmic growth phase tumor cells were treated with 10μl/ml KLT and 10μl/ml blank emulsion respectively for 48 hours then digested. Smears were made and kept in a moist box at 37°C for 5 hours, washed twice with PBS then fixed with cold acetone for 15 minutes and stained with SP method. Meanwhile, PBS and normal rabbit serum were used for blank control [The SP kit, Fas/Apo-1 and Bcl-2(100) univalent antibody were supplied by Zhongshan Biotechnology Co.] The evaluation method of gene expression intensity: the positive expression for gene Fas/Apo-1 was that the membrane appeared brown yellow in color and
gene Bcl-2 expressed positively when the cytoplasm appeared yellow in color.

5 HP visual fields (×400) were selected for observation and their average cell numbers were calculated. The label-index (LI) of Fas/Apo-1 gene in membrane and the LI of Bcl-2 gene in cytoplasm were calculated respectively according to the following formula:

\[ LI = \frac{\text{number of positive cells}}{\text{total number of cells counted}} \times 100\% \]

1.5 Statistics Analysis

SAS software was applied for T-test.

2. Results

2.1 Inhibition Effect of KLT on Renal Carcinoma Cells

After the GRC-1 cells were treated with KLT injection for 48 hours, it was observed that KLT had the effect of inhibiting the proliferation of GRC-1 cells \( (IC_{50} = 19.3\mu l/ml) \).

2.2 Assay of Apoptosis of Renal Carcinoma Cells Induced by KLT

After the GRC-1 cells were treated with KLT of different concentrations for 48 hours, apoptosis of cells induced by KLT was assayed by TUNEL-flow cytometric scan method. When the GRC-1 cells were treated with KLT at the concentration of 5\( \mu l/ml \) and 10\( \mu l/ml \), the rate of positive cells in M1 area was 31.30% and 89.76% respectively while that of the control group was 1.02%. When the GRC-1 cells were treated with KLT at the concentration of 15\( \mu l/ml \) and 20\( \mu l/ml \), the percentage of positive cells in M1 area was 1.76% and 8% respectively with no significant difference compared with control group (See Figure 3).

2.3 Effect of KLT on Gene Fas/Apo-1 and Bcl-2

The expression of Fas/Apo-1 gene in GRC-1 cells was weak in emulsion group and the LI was 21.20%. The Fas/Apo-1 gene expression in GRC-1 cells was remarkably intensified in KLT group and the LI was 74.60%.

Bcl-2 gene expression could be measured in the cytoplasm of renal carcinoma cells of the control group and the LI was 25%. The Bcl-2 gene expression was decreased in KLT groups when GRC-1 cells were treated with increased KLT concentration. The Bcl-2 gene expression arrived at the lowest level when cells were treated with KLT at the concentration of 10 \( \mu l/ml \) whereas the LI was 6.6% (See Figures 1 and 2).

3. Discussion

Renal carcinoma is very difficult to manage. Due to the high expression rate of multiple drug resistant gene (in about 87% cases), the effect of chemotherapy for renal cancer is far from satisfactory. There is still no ideal therapy for renal carcinoma at present.
Apoptosis of tumor cell is an independent process of cell suicide due to the cracking of DNA by activated endonuclease, forming 50-300 kbp base segments and then splitting into smaller segments (200 bp). Electrophoretic analysis will reveal a characteristic ladder appearance. Apoptosis could also be assayed by labeling fluorescein isothiocyanate (FITC-dUTP) to the single-chain or double-chain of 3-hydroxyl group-terminal of the 200 bp segments through terminal deoxynucleotidyl transferase (TdT). Quantitative analysis of apoptotic cells can be made according to the fluorescent intensity. Differentiation of apoptosis from necrosis can also be made by this method with a higher sensitivity and specificity than gel electrophoresis method.

The tumor-cell killing action of KLT for a variety of tumors both in vivo and in vitro has been reported by many researchers. Investigations on the cell proliferation cycle show that KLT has the retardation action for tumor by blocking the cells at the G2+M phase and decreasing the percentage of the DNA synthesis phase (S phase) cells. Yang Hua et al. demonstrated that human erythroleukemia cells were induced into apoptosis after they were treated by KLT 10 μl/ml for 6 hours. When the cells were treated by higher KLT concentrations, injury of tumor cell membrane, PI high staining and increase of cell necrosis were observed. Apoptosis of human colon carcinoma cells SW1116 induced by KLT 10 μl/ml was also detected by TUNEL technique. The study reveals that the effective concentration of KLT to induce apoptosis of tumor cells is 10 μl/ml.

In our study, the effect of KLT on inhibiting the proliferation of renal carcinoma cells was also observed after the cells were treated with KLT. The IC50 was 19.31 μl/ml. KLT could induce apoptosis of renal carcinoma cells. The apoptotic percentage was 31.30% when the cells were treated by KLT 5 μl/ml and 89.76% when the cells were treated by KLT 10 μl/ml. The apoptotic cell number decreased if the cells were treated with higher concentration of KLT, indicating that high concentration of KLT would cause necrosis of the cells. We believe that there is an optimum dose-effect relationship for the anti-tumor action of KLT and the optimum therapeutic concentration of KLT should be studied for the purpose of obtaining the best therapeutic efficacy.

Fas/Apo-1 gene plays an important role in regulating apoptosis of cells in the immune system. Fas/Apo-1 is a trans-membrane protein, belonging to the nerve growth factors and the TNF receptor superfamily. Fas/Apo-1 molecule is the receptor for the signal transduction of cell apoptosis. After it combines with Fas/Apo-1 antibody or ligand (Fas Ligand, Fasl), gene products for cell apoptosis are activated in cytoplasm and apoptosis of the cell is induced in which Fas/Apo-1 molecule expresses. Although the apoptosis signal transduction from Fas/Apo-1 are not clearly elucidated at present, it has been accepted by scholastic community that the signals activate Fas/Apo-1 and can induce apoptosis of certain tumor cells. If the Fas/Apo-1 signal system is damaged, tumor cells might selectively escape from immune surveillance and gain certain survival advantage. On the surface of activated T-lymphocytes, the expression of Fas/Apo-1 protein is high. There must be enough Fas/Apo-1 expression on cell membrane to induce apoptosis of tumor cells.
The structure of all the links of the signal transduction system for Fas/Apo-1 antigen expression and cell apoptosis must be intact. Fas/Apo-1 molecule expresses extensively in both normal and cancer cells. The expression level of Fas/Apo-1 molecule in cancer cells is an important predictor for cancer diagnosis and treatment. Li Hongjun et al. reports that the expression level of Fas/Apo-1 in bladder carcinoma cell line (T24) and prostatic carcinoma cell line (PC-3M) is high. On the other hand, in bladder carcinoma cell line (BIU-87) and renal carcinoma cell line (RCC-949 and GRC-1), the expression level of Fas/Apo-1 is low or there is no expression.

Our study also reveals that the expression level of Fas/Apo-1 molecule in GRC-1 cells is low so that cancer cells might escape the surveillance of T-lymphocytes with FasL immunization. This might explain the reason why the clinical results of immunotherapy using IL-2 and interferon for renal carcinoma are unsatisfactory. We have found that the expression level of Fas/Apo-1 molecule in GRC-1 cells is markedly increased when the cells have been treated with $10 \, \text{μl} / \text{ml}$ KLT for 48 hours. It is suggested that the increased expression of Fas/Apo-1 gene in GRC-1 cells will make it easier for the activated T-lymphocytes to recognize cancer cells.

Bcl-2 is a proto-oncogene with the specific action of inhibiting apoptosis of cells, therefore playing an important role in the apoptotic process. The anti-tumor activity of many chemotherapeutic agents such as Adriamycin and Cisplatin is due to their ability to decrease the Bcl-2 expression level. Many researchers believe that different sensitivity of leukemia cells to chemotherapeutic agents is correlated with the Bcl-2 expression level. In acute myelemia treatment by IL-6, G-CSF, glucocorticoid and dexamethasone, the Bcl-2 gene expression was suppressed and the sensitivity of Adriamycin and Cytarabine was increased for inducing apoptosis in leukemia cells. In this study, it is revealed that $10 \, \text{μl} / \text{ml}$ KLT has the action of inhibiting the GRC-1 cell Bcl-2 gene expression. Many researchers have reported the sensitivity-enhancing action of KLT for chemotherapy and radiotherapy in treating tumors. It might be suggested that the mechanism underlying the increased tumor cell killing action of chemotherapy or radiotherapy when combined with KLT can be found in the function of KLT to inhibit Bcl-2 gene expression in tumor cells.

References


The Apoptosis of GRC-1 Cells Induced by KLT with Different Concentrations
A. Negative Control  B. Positive Control  C. Emulsion  D. KLT 5μl/ml  E. KLT 10μl/ml  F. KLT 15μl/ml  G. KLT 20μl/ml