The Effect of KLT on the Sensitivity of Biological Therapy of Colon Cells

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[Abstract]

Purposes
Study the possibility of KLT as a new biological response modifier (BRM).

Methods
Using MTT method to observe the sensitization action of fused protein composed by monoclonal antigen and superantigen mediated tumor biological therapeutic effect by KLT. Using flow cytometry and RT-PCR method to detect the expression of tumor cell surface molecules before and after pretreated with KLT.

Results
KLT could not only increase sensitization of tumor cell’s biological therapeutic action caused by the fused protein, but also unregulated the expression of functional Fas at the tumor cell surface.

Conclusions
KLT possesses the sensitive action of biological therapy and is a potential new type BRM.

National II type new drug Kanglaite (KLT) is an injections emulsion produced by an efficient antitumor component which was isolated from traditional Chinese medicine-semen coicis. It has been demonstrated that KLT was effective against a variety of tumors in experimental studies and has definite efficacy in clinical treatment. The mechanism of its action contained cell arrest at G2/M stage in cell cycle, induction of apoptosis and improvement of immunity. Thus, KLT is an ideal diphasic, broad-spectrum new antitumor agent in recent clinical treatment. On the basis of our previous finding of the antitumor activity of fused protein composed by monoclonal antigen and superantigen, sensitization of KLT to this fused protein mediated biological therapeutic effect in colon cells was further observed. And the preliminary mechanism was also explored.

1. Materials and Methods
1.1 Reagents and Other Materials

KLT provided by Kanglaite pharmaceutical CO., Ltd (Zhejiang, PRC); fused protein C 242Fab-SEA composed by antitumor monoclonal antigen and superantigen, obtained from Sweden pharmacia & Upjohn Immunology center; anti Fas. Fasl antibody (Santa Cruz company products), anti-lCAM-1 and FITC labeling one antibody (products of ZYMED company) purchased from Beijing Zhongshan Co.
1.2 Tumor Cell Culture
Human colon cancer cell line colo205 were obtained from American Tissue collection CO., and maintained in RPM1-1640 complemented with 10% bovine serum, cultured in 5% CO₂ incubator at 37°C.

1.3 Isolation of Peripheral Blood Lymphocytes (PBL)
Blood obtained from healthy donor, diluted with equal volume of Hanks solution. According to Ficoll density gradient centrifuging method, collect PBL, regarding as effectors.

1.4 Observation of the Synergistic Effect of Biological Therapeutic Action (BTA) by KLT
MTT method was used. Using untreated colo 205 cells as target cells of control group and cells pretreated with KLT (dose less than IC₅₀) as target cells. The ratio of effector-to-target cells was 10:1. The final concentrations of fused protein were 0, 0.01, 0.1, 1.0 and 10.0μg/ml respectively. Five pores for each group, experiment repeated 3 times. The calculating equation was as follows:

Inhibitory rate (%) = (OD_{E+T} - OD_{E+T+drug}) / OD_{T}

OD_{T}: OD value of control target cell pore
OD_{E+T}: OD value of target cells and effector cells pore
OD_{E+T+drug}: OD value of target cell, effector cells and fused protein pore.

1.5 Detection of Cell Surface Molecules
To detect the expression level of colo 205 cell surface molecules before and after pretreated with KLT by using flow cytometry. Added monoclonal antigen 100μl into each tube, reacted for 30 min in the dark, washed with PBS for 3times, then added FITC labeling double antigen and reacted for 30 min in the dark, washed with PBS 3 times. At last, cells were fixed with 0.5ml of 1% polyaldehyde for assay.

1.6 Cytotoxicity of Colo 205 Cell by Anti Fas Antibody
MTT method was used. Untreated colo 205 cells were regarded as control group, pretreated colo 205 cells with KLT as tested groups. Five pores for each group and experiment repeated 3 times. The calculating equation was as follows:

Inhibitory rate (%)=(OD_{T} - OD_{T+drug}) / OD_{T}

OD_{T}: OD value of control group pore
OD_{T+drug}: OD value of pore of tumor cells treated with drugs

1.7 The Effect of KLT on the Expression of Fas Gene
RF-PCR method was used. Extract cellular total RNA by using one step of Trizal reagent. Add 5×RT buffer solution 4μl, each of dNTP 0.5 mmol, dTT 50 pmol, AMV 9u, Rnasin 32u, RNA 3.915 μg into 20 μl of reverse transcription system. And incubate for 90 min at 42°C.
PCR reactive system was 50µl which contained cDNA 5µl, 10×PCR buffer solution 5µl, each of dNTP 0.5mM, MgCl₂ 3.7mM, one pair of Fas primer 10 pmol of each, one pair of β-actin 10 pmol of each, Taq enzyme 1.5u. Reactive condition: predenatured for 120 sec at 94℃, Then 30 sec at 94℃, 30 sec at 55℃, 60 sec at 72℃, total was 30 cycles, extended 600 sec at 72℃. Taking 15µl of PCR product ran on an agarose (1.5%) gel electrophoresis.

2. Results

2.1 Using MTT method to detect the synergistic effect of biological therapeutic action (BTA) on colo 205 cells by KLT: Fused protein markedly inhibits the growth of colo 205 cells in vitro which could be augmented by KLT.

<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration of Fused Protein (µg/ml)</th>
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<tbody>
<tr>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td>Control Group</td>
<td>14.2±1.7%</td>
</tr>
<tr>
<td>Pretreated with KLT Group</td>
<td>32.3±5.6%</td>
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*p<0.05, *p<0.01

2.2 To detect the expression of cell surface Fas in colo 205 cells, pretreated with KLT by using FCM. Before pretreated with KLT the expression percentage of colo 205 cell surface Fas was 21.1±3.4%, after pretreated with KLT, it elevated to 60.4±7.8%, but the expression of ICAM-1, FasL was no difference before and after pretreated with KLT.

2.3 The cytotoxicity of anti Fas antibody on colo 205 cells. Detected by MTT method: The cytotoxicity of anti Fas antibody in untreated colo 205 cells was weak, but it was much potent when cells pretreated with KLT.

<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration of Antibody (µg/ml)</th>
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<tr>
<td></td>
<td>0.001</td>
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<tr>
<td>Central Group</td>
<td>5.7±0.8%</td>
</tr>
<tr>
<td>Pretreated with KLT Group</td>
<td>17.5±5.7%</td>
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*p<0.05, **p<0.01

2.4 Expression of Fas Gene Detected by RT-PCR

After pretreated with KLT 4hr, then continued to incubate for 24 hr, the expression of Fas showed obviously. When cell group pretreated with KLT only for 2 or 4 hr, or untreated with KLT group, no PCR products appeared (0.46kb).

3. Discussion

Fused protein composed by antitumor monoclonal antigen and superantigen depending to specific orientation characteristics could make superantigen SEA target on tumor site to exert
its potent activity in stimulating T-cell. Thus, the inhibition of tumor growth could be obtained through superantigen dependent cell-mediated cytotoxicity (SPC). Fused protein, as a new biological therapeutic agent of cancer, has been entered in clinical trials. However, the genetic unstability of tumor cells is responsible for the producing a cell group with highly heterogeneity. Design a combined treatment regimen from multiple aspects and different levels is needed to effectively control the genesis and development of cancers. In the present study, we intended to explore the possibility of the combination of this fused protein and KLT which was a common used diphasic antitumor agent. The results indicated that KLT pretreated colo 205 cells was more sensitive to this fused protein mediated SDCC. Tumors could not be effectively expelled owing to the presence of a deep immune inhibitory “dark hole” at the tumor sites which make the tumor specific CTL activated or the activated CTL could not exert its cytotoxin action. The mechanism was very complex, might be related with the following factors: underexpression or unexpression of tumor specific/relative antigen of tumor cell; aberrant expression of tumor cell surface molecule MHC and associated stimulating molecule, such as B7 etc. The function of APC insufficient; the presence of free immune inhibitor etc. Recent studies further demonstrated that the aberrant expression of tumor cell surface Fas influence the activated CTL to kill tumor cells potently. The main mechanism of CTL's cytotoxin action was that CTL combined and activated the surface Fas, then initiated the basic apoptotic program in target cells and induced apoptosis. In addition, activated target cells were not only under expressed or unexpressed Fas, and also highly express Fas L which could either escape from immune effector or kill immune cells actively. At last, the tumor cells could survive abnormally.

KLT itself could block the progression of cell cycle to inhibit the proliferative activity of tumor cells by the dose of KLT used in sub lethal and less than IC₅₀ in our experiment. So the results showed that KLT not only exerted its direct inhibition of proliferative action by arresting cells in G₂/M stage, but also could modify the tumor cell in low concentration, then activate CTL easily.

During the experiments, we studied the change of tumor cell surface molecules before and after pretreated with KLT by using FCM and RTF-PCR. It demonstrated that KLT could definitely elevate Fas mRNA level of colo 205 cells and upregulate the expression of Fas protein on the cell surface which was responsible for the sensitizing of BTA by KLT. Since the expression of ICAM-1 located on colo 205 cell surface was not changed significantly, KLT pretreatment might not be helpful for reorganization of adhesive process of immune cells. Besides, no high expression of Fas L on colo 205 cell surface in our study was found which means the high expression of Fas L was not universally occurred in tumors. The possible reason is that the differentiated degree of same kind of tumor cell is variable and during the process of carcinogens, the pressure of immune selection is existed.

According to the characteristics of Fas which was easily activated by related antibody, we observed the cytotoxin action of anti Fas antibody on colo 205 cells by using MTT method.
Since KLT pretreated colo 205 cells were killed easily, it indicated that unregulated expression Fas was functional Fas, could induce cell death. Our experiments also showed KLT sensitized the effect of biological therapy which was related to the elevating Fas mRNA level of target cells and unregulate the expression of functional Fas protein on the cell surface.