Effect of combined treatment with recombinant interleukin-2 and allicin on pancreatic cancer

Cong-Jun Wang · Chao Wang · Jiang Han · Yong-Kun Wang · Lin Tang · Dong-Wei Shen · Yi Zhao · Rong-Hua Xu · Hui Zhang

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Abstract This study aimed to evaluate the efficacy of combined treatment with recombinant interleukin-2 (rIL-2) and allicin on pancreatic cancer and explore the potential immunological mechanism. A total of 60 C57/BL6 nude mice pancreatic cancer xenograft models were randomized into four groups of 15 mice per group: control group, allicin treatment group, rIL-2 treatment group, combined treatment with allicin and rIL-2 group. Mice in each group were treated with saline, rIL-2, allicin, or combination of rIL-2 and allicin by weekly i.v injection for four weeks. After four weeks of treatment, eyeballs of the mice were extracted and blood was drawn, percentages of CD4⁺ T, CD8⁺ T and NK cell were analyzed by FACS, IFN-γ level was detected by ELISA. One mouse in each group was sacrificed to measure the weight and volume of the tumor and prepared to the paraffin section of tumor tissue. Apoptosis of the tumor cells was analyzed by TUNEL and FACS. Other mice continued to receive treatment, survival period were compared between each group. We observed a significant suppression of xenograft growth and a significant prolonged survival time in the combined treatment with allicin and rIL-2 group ($P < 0.05$). The most amount of apoptotic cells were observed in the combined therapy group ($P < 0.05$). The percentages of CD4⁺T, CD8⁺T and NK cell and serum IFN-γ level increased significantly in the combined treatment group compared with other groups ($P < 0.05$). Combined treatment with allicin and rIL-2 resulted in suppression of tumor growth and prolonged survival time possibly through activation of CD4⁺T, CD8⁺T and NK cell.

Keywords Recombinant interleukin-2 · Allicin · Pancreatic cancer · Combined treatment

Introduction

Pancreatic cancer is a common type of digestive system malignancies. Due to lack of early diagnostic method and effective treatment, the cure rate is extremely low. Diallyl trisulfide, the major constituent of allicin, has multiple medicinal values, including anti-viral, anti-bacterial and anti-fungal effect. Allicin also has immunomodulative effect including enhancing the activity of NK cell and IL-2 production, and boosting proliferation and activity of T cell [1, 2]. Previous study showed that allicin contained an immunomodulator that modulated cytokine patterns towards a Th1-type response and the development of an effective cell-mediated response [3]. IL-2, a small glycoprotein mainly produced by CD4⁺T cell, has manifold biological activities including enhancing the killing activity of CD8⁺T cell and NK cell and activating immune cell to produce other cytokines [4, 5]. RIL-2, which is produced by genetic technology, with the same or similar structure as...
IL-2 can boost T cell proliferation and differentiation, induce the generation of cytotoxic T lymphocyte (CTL), enhance the activity of NK cell, induce the generation of lymphokine-activated killer cell (LAK) and tumor infiltrated lymphocyte (TIL), activate B cell to proliferate, differentiate and secrete antibody, and induce the production of IFN-γ and other cytokines [6]. Currently, rIL-2 is used as an adjunctive therapy for ascites and hydrothorax caused by carcinomas. Low dose and short course of treatment with rIL-2 alone can not result in appreciable clinical benefit, however, high dose of IL-2 can lead to toxicity. Therefore, a combination of rIL-2 and anti-viral drug treatment would be a better strategy for pancreatic cancer. Both allicin and rIL-2 can boost cell mediated immunity, which plays an important role in tumor immune. This study aim to evaluate the efficacy of combined treatment with recombinant interleukin-2 (rIL-2) and allicin in pancreatic cancer xenograft model and explore the potential immunological mechanism.

Methods

Materials and apparatus

The pancreatic cancer cell line BXPC-3 was purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. C57/BL6 mice, 4–6-week-old, (20 ± 2) g, half male and half female were purchased from Experimental Animal Centre of SIBS (Shanghai, PR China). RPMI-1640 medium was purchased from Gibco. Fetal bovine serum was purchased from Hangzhou Sijiqing biotec. rIL-2 was supplied by Shanghai Fuzhong biotec. Allicin was obtained from Shanghai Hefeng biotec. Immunohistochemistry kit was from Santa Cruz. Mouse IFN-γ ELASE kit was provided by Shanghai Yaji Biotec. TUNEL kit was provided by Shanghai Yibaiju Biotec. All monoclonal antibodies (anti-CA19-9, anti-CD4+, anti-CD8+, anti-CD16+ and anti-CD56+) were provided by Becton Dickinson. Second antibody was provided by Wuhan Boshide Biotec.

Fluorescent microscopy was provided by Olympus (Japan). CO2 incubator was obtained from SANYO (Japan). FACS Calibur was from Becton Dickinson (USA).

Murine xenograft model and grouping treatment

BXPC-3 cell line was cultured in RPMI-1640 medium supplemented with 10 % fetal bovine and incubated in CO2 incubator containing 5 % CO2 at 37 °C. Xenograft model in C57/BL6 nude mice was established by subcutaneous inoculation of 2 × 106 exponentially growing BXPC-3 cells into the lower back of each mouse. Mice were randomized into four groups of 15 mice per group: control group, allicin treatment group, rIL-2 treatment group, combined treatment with allicin and rIL-2 group. Mice were weekly intravenously injected with 10 mg/kg allicin in allicin group, 20 μg/kg rIL-2 in rIL-2 group, 10 mg/kg allicin combined with 20 μg/kg rIL-2 in combination therapy group or saline in control group for 4 weeks. Four weeks after treatment, eyeballs of the mice were extracted and blood was drawn and then anti-coagulated with heparin. One mouse in each group was sacrificed to measure the weight and volume of the tumor and prepared to the paraffin section of tumor tissue. Other mice continued to receive treatment and survival time was observed.

Calculation of inhibitory rate

The longest perpendicular tumor diameter a (mm) and shortest perpendicular tumor diameter b (mm) were measured by caliper, the tumor volume was estimated using the following formula:

\[
V = \frac{4}{3} \pi r^3 (r = (a+b)/4)
\]

Inhibitory rate (%) = \frac{[1 - (mean starting tumor volume of treated group – mean ending tumor volume of treated group)/(mean starting tumor volume of control group – mean ending tumor volume of control group)]}{100}.

TUNEL assay

TUNEL assay was performed to detect in situ apoptosis on tumor tissue section using TUNEL kit according to the manufacturer’s instructions. Typical cell morphological changes of apoptotic cells include stained by brown and nuclear shrinkage accompanied by chromatin condens. The percentage of apoptotic cells was determined by counting 1,000 cells from 10 fields (100 cells/field) under fluorescent microscopy.

Analysis of apoptosis of tumor cell by FACS

We prepared single-cell suspensions of tumor tissue by mechanic dispersion. Cells were fixed with 3 % paraformaldehyde for 20 min at 4 °C. The cells were washed with PBS three times, and then permeabilized with Triton X-100. After that the cells were washed with PBS three times, and resuspended in PBS at the concentration of cells is 10⁶ cells/ml. The cells were split into two halves, 10 μl mouse anti-human CA19-9 monoclonal antibody was added into one half, 10 μl PBS was added into the other half, and incubated at 4 °C for 1 h. Then the cells were washed with PBS three times and blocked with 2 % BSA at...
room temperature for 15 min, then, washed with PBS once. After centrifugation, the supernatant was aspirated. 50 μl pre-titrated FITC labeled rabbit anti-mouse antibody (1:100) was added into the cells, and incubated at 4°C for 15 min. Then the cells were washed with PBS three times, and supernatant was aspirated followed by resuspension of the cells with 500 μl PBS and flow cytometric analysis.

Analysis of percentages of CD4+T, CD8+T and NK cell in peripheral blood by FACS

CD16 and CD56 double positive cells were regarded as NK lymphocytes. 4 weeks after treatment, eyeballs of the mice were extracted and blood was drawn and then anti-coagulated with heparin. We prepared 50 μl whole blood, and 10 μl FITC labeled anti-CD4, anti-CD8, and FITC labeled anti-CD16 and Cy3 labeled anti-CD56 antibody were added and incubated at room temperature in dark for 20 min. After that, 1 ml red blood cell lysis buffer was added and incubated at room temperature in dark for 20 min to eliminate red blood cells. After centrifugation, supernatant was aspirated, and the cells were resuspended in 1 ml PBS. 300 μl were taken for FACS analysis.

Detection of IFN-γ level in peripheral blood by ELISA

Four weeks after treatment, eyeballs of the mice were extracted and blood was drawn and then anti-coagulated with heparin. We prepared 50 μl whole blood, and 10 μl FITC labeled anti-CD4, anti-CD8, and FITC labeled anti-CD16 and Cy3 labeled anti-CD56 antibody were added and incubated at room temperature in dark for 20 min. After that, 1 ml red blood cell lysis buffer was added and incubated at room temperature in dark for 20 min to eliminate red blood cells. After centrifugation, supernatant was aspirated, and the cells were resuspended in 1 ml PBS. The concentration of the optical density was determined, using a microplate reader set to 450 nm.

Statistical analysis

The results were analyzed with independent sample t test using SPSS 17.0 software, and presented as x ± s. P < 0.01 or P < 0.05 represented a statistically significant difference.

Results

Effect of combined treatment with rIL-2 and allicin on pancreatic tumor growth

Tumor was palpable at 3 weeks after inoculation. Mice were sacrificed 4 weeks after treatment. Tumor size and weight reduced significantly in all experimental groups compared with control group. The best tumor suppression effect was observed in the combination therapy group (Table 1). The mean tumor weight before treatment in each group was 1.2 ± 0.09 g, no significant difference among each group. After 4 weeks of treatment, tumor weight in rIL-2 group was 70.2 % of control group, and tumor weight in allicin group was 55.6 % of control group, tumor weight in the combination therapy group was 57.1 % of allicin group and 50.2 % of rIL-2 group. The mean tumor weight before treatment in each group was 0.46 ± 0.12 cm³, no significant difference among each group. After 4 weeks of treatment, the mean tumor volume in control group was (2.67 ± 0.15) cm³, the mean tumor volume in rIL-2 group was (0.98 ± 0.09) cm³, with the tumor inhibition rate of 81.3 %; the mean tumor volume in allicin group was (1.22 ± 0.13) cm³, with the tumor inhibition rate of 70.5 %; the mean tumor volume in combined therapy

<table>
<thead>
<tr>
<th>Group</th>
<th>Tumor volume (0 days)</th>
<th>Tumor volume (4 weeks)</th>
<th>Tumor inhibition rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.46 ± 0.8</td>
<td>2.67 ± 0.15</td>
<td>–</td>
</tr>
<tr>
<td>rIL-2</td>
<td>0.45 ± 0.6</td>
<td>0.98 ± 0.09**</td>
<td>81.3 %</td>
</tr>
<tr>
<td>Allicin</td>
<td>0.45 ± 0.0</td>
<td>1.22 ± 0.13*</td>
<td>70.5 %</td>
</tr>
<tr>
<td>rIL-2 + Allicin</td>
<td>0.47 ± 0.9</td>
<td>0.59 ± 0.06**</td>
<td>90.5 %</td>
</tr>
</tbody>
</table>

* Represent significantly different (P < 0.05) compared with control
** Represent extremely significantly different (P < 0.001) compared with control

Fig. 1 Survival time and number after 4 weeks of treatment. Pancreatic cancer xenograft model mice were randomized into four groups: control group, allicin treatment group, rIL-2 treatment group, combined treatment group. Survival time and number was observed in each group.
group was (0.59 ± 0.06) cm³, with the tumor inhibition rate of 90.5%. Both allicin treatment and rIL-2 treatment significantly inhibited tumor growth \((P < 0.05)\). A combination of allicin and rIL-2 treatment showed incremental tumor inhibition effect from using allicin or rIL-2 alone, resulted in the smallest mean tumor volume, compared with other groups \((P < 0.05)\).

Survival time and number in each group

As shown in Fig. 1, all mice in control group died on day 40. While no improvement in survival time was observed in rIL-2 group compared with control group, survival time and survival number significantly increased in allicin group and combined treatment group. The best effect was observed in the combined treatment group, with 60% mice survived on day 55.

Apoptosis of tumor cells

In Figs. 2 and 3, after 4 weeks of treatment, apoptosis index (AI) of tumor cells was 9 ± 3.7 in control group. Compared with control group, apoptosis of tumor cells was significantly increased in other groups, with the AI of 15 ± 5.0 in rIL-2 group, 14 ± 3.3 in allicin group, and 23 ± 4.3 in the combined treatment group. The highest AI was observed in the combined treatment group.

Analysis of T cell subsets and NK cell percentages in peripheral blood

FACS result showed that percentages of CD4+T, CD8+T and NK cell in peripheral blood significantly increased in 3 treated groups compared with control group \((P < 0.05)\), with the most significant increase in the combined treatment group. While there was comparable increase in T cell and NK cell in allicin group, increase in T cell percentage was significantly higher than NK cell in rIL-2 group (Table 2).

Effect of combined treatment with rIL-2 and allicin on NK cell activity

ELISA result showed that there was a substantial increase in IFN-\(\gamma\) level in the combined treatment group, compared with other groups \((P < 0.05)\), suggesting that combined treatment with rIL-2 and allicin increased the activity of NK cell in addition to the number of NK cell (Fig. 4).

Discussions

Due to the extremely low cure rate, the incidence rates of pancreatic cancer are almost equal to its mortality rates. The mean survival time is no longer than 6 months, and the 5-year survival rate was less than 5% [7–11]. Extensive research work has been carried out to identify new agents and treatment to combat pancreatic cancer. In pancreatic cancer patients, both adaptive and innate immune systems are depressed [12, 13]. Pancreatic cancer cells contribute to immune suppression through production of immune suppressive cytokines (e.g., TGF-\(\beta\), IL-10) and by expressing surface molecules that mediate immune suppression (e.g., Fas ligand (Fas-L), programmed death-1 ligand (PD-L1) and indolamine-2, and 3-dioxygenase (IDO) [14]. In addition, immune suppressive cells such as cancer-associated fibroblasts (CAFs), tolerogenic DCs, myeloid-derived suppressor cells (MDSCs), immunosuppressive tumor-associated macrophages (TAMs), and Treg cells in the tumor environment also contribute to the inhibition of antitumor immunity by various mechanisms, including depletion of arginine and elaboration of reactive oxygen species (ROS) and nitrogen oxide (NO) [15]. Finally, an immunosuppressive tumor microenvironment induced by pancreatic cancers suppresses CD8+CTL function through secretion of IL-10 and TGF-\(\beta\) from Treg cells [16, 17]. To improve the prognosis of pancreatic cancer patients, immunotherapies that struggle against pancreatic cancer cells with boosting antitumor immunity (both innate and adaptive immunity) as well as depletion of Treg cells may tip the balance in favor of immunostimulation. In this study, we evaluated the efficacy of combined treatment with recombinant interleukin-2 (rIL-2) and allicin on pancreatic cancer and explored the effect on the boosting of antitumor immunity.

Several studies have shown that allicin may reduce the risk of various types of malignancies, including cancer of...
the lungs, stomach, colon and liver, and the protection effect correlate with the content of diallyl trisulfide [18, 19].
Previous study has shown that allicin suppresses growth of PC-3 human prostate cancer xenograft in vivo [20].
Administering aged allicin to patients with advanced colon cancer, liver cancer and pancreatic cancer, both the number and activity of NK cell improved significantly, but no improvement in survival time was observed [21]. Administration of exogenous cytokines, rIL-2 can result in significant tumor inhibition effect, however, high dose of rIL-2 may lead to severe toxicity. In this study, we observed a 63.3% reduction in tumor size, and a 39.1% increase in

Fig. 3 Apoptosis of tumor cells analyzed by FACS. a Control group; b rIL-2 treatment group; c allicin treatment group; d combined treatment group

Table 2 The percentages of CD4+T, CD8+T and NK cells in peripheral blood at 4th week after of treatment

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>rIL-2</th>
<th>Allicin</th>
<th>rIL-2 + allicin</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+</td>
<td>33.70 ± 7.09</td>
<td>59.75 ± 9.11**</td>
<td>50.87 ± 6.89**</td>
<td>69.61 ± 11.01**</td>
</tr>
<tr>
<td>CD8+</td>
<td>12.98 ± 2.09</td>
<td>24.53 ± 5.23</td>
<td>21.04 ± 4.13**</td>
<td>30.03 ± 7.49**</td>
</tr>
<tr>
<td>NK</td>
<td>25.78 ± 12.45</td>
<td>34.47 ± 9.07*</td>
<td>46.57 ± 11.34**</td>
<td>59.03 ± 15.22**</td>
</tr>
</tbody>
</table>

* Represent significantly different (P < 0.05) compared to control
** Represent extremely significantly different (P < 0.001) compared to control
apoptosis of tumor cells in rIL-2 group, but no improvement in survival rate and survival time, possibly due to the toxicity of rIL-2. In allicin group, tumor size reduced by 54.3%, while apoptosis of tumor cells increased by 34.8%, both survival rate and survival time increased significantly. In the combined treatment group, tumor size reduced by 77.9%, while apoptosis of tumor cells increased by 60.9%, both survival rate and survival time increased significantly. Recent study has shown that anti-tumor drug may enhance elimination of tumor cells through modulation of both immune system and tumor antigen [22]. Cell-mediated immune response plays important role in tumor tumor immunity. Immune response cytolytic effectors that interact with tumoral cells are innate (such as NK cell) and adaptive (such as CD8+T cell) [23]. Animal study has shown that anti-tumor drug may enhance elimination of tumor cells through modulation of both immune system and tumor antigen [22]. Cell-mediated immune response plays important role in tumor tumor immunity. Immune response cytolytic effectors that interact with tumoral cells are innate (such as NK cell) and adaptive (such as CD8+T cell) [23].

In this study, the numbers of CD4+T, CD8+T and NK cell in peripheral blood increased significantly in all treated group compared with control group, with the most substantial increase in CD4+T cell (106.6%), CD8+T cell (131.4%) and NK cell (129.0%) compared with control group. NK cells play a major role as effector cells of the innate immunity in anti-infection activity and tumor surveillance, studies have shown that the activity of NK cells can be enhanced by IFN-γ, activated NK cells can also secrete IFN-γ [30, 31]. Our results showed that, IFN-γ level in peripheral blood increased significantly in treated group compared with control group, with an increase of 46.1% in allicin group, 63.3% in rIL-2 group, 87.2% in combined treatment group, indicating that a combination of rIL-2 and allicin treatment has incremental benefit of boosting both innate and adaptive immunity.

This study demonstrated that combined treatment with rIL-2 and allicin significantly suppressed pancreatic cancer xenograft growth in C57BL6 nude mice.

Both numbers of tumor infiltrated T cells and peripheral blood T cells and NK cells and IFN-γ level in peripheral blood increased significantly compared with other groups, thus uncovered the immunological mechanism of combined treatment with rIL-2 and allicin for pancreatic cancer. We propose that the combined therapy with rIL-2 and allicin can prolong the survival of pancreatic mice model by triggering an increase in both innate and adaptive immune cells, accompanied by an improvement of their activation state. Our study can shed some light on immunotherapy for pancreatic cancer.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards All human studies have been approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. All persons gave their informed consent prior to their inclusion in the study.

References

Fig. 4 ELISA measurement of protein level of IFN-γ (pg/ml) in peripheral blood of mouse after 4 weeks of treatment. *Represent significantly different (P < 0.05) compared with control; **Represent extremely significantly different (P < 0.001) compared with control.