Does Panax notoginseng (Sanqi) inhibit H22 tumor growth via improving the host's cellular immunity?

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Abstract

Ginsenoside Rg3 has many functions, including anti-tumor activity, antioxidant activity, and immune system modulatory activity. Interestingly, There are two stereoisomers exist in Rg3, 20(S)-ginsenoside Rg3 [20(S)-Rg3] and 20(R)-ginsenoside Rg3 [20(R)-Rg3], respectively. These two isomers have different pharmacological effects due to their stereospecificity. In this study, we compared their effect of stimulating the ConA to induce lymphocyte proliferation and increase the cytokines IL-2 and IFN-γ levels in mice and improve the cellular immunity of the host. The study predicted that ginsenoside (Rg3) could increase Th1-type cytokines interleukin-2 and interferon-γ and stimulate ConA-induced lymphocyte proliferation in mice, thereby improving cellular immunity in H22 mice. The effects of the Rg3 R-form were significantly greater than Rg3 S-form.

Keywords: Panax notoginseng, ginsenoside, cellular immunity, cytokine, Ginsenoside Rg3, Immunosuppression, stereoisomer, stereospecific, liver cancer

1. Introduction

Panax notoginseng, a traditional Chinese medicine with a long history and high clinical value, is becoming increasingly popular in China due to its possible therapeutic effects and restorative properties. There have been many modern research studies on the effectiveness of Panax ginseng saponins (PNS) in treating many cardiovascular diseases such as atherosclerosis, hypertension, myocardial ischaemia and aortic endothelial hyperplasia. (Peng et al., 2018).

The main bioactive components of Panax notoginseng include saponins, salviae, flavonoids, polysaccharides and fatty acids (Wang et al., 2006). Among them, the saponins are the most significant for anti-cancer, anti-hyperlipidaemia, anti-hyperglycaemia, anti-inflammatory response, anti-depression, neuroprotection, antioxidant and promotion of bone formation (Wang et al., 2006).

Liver cancer is ranked second only to cardiovascular disease as a cause of cancer-related death worldwide. The main features of liver cancer are high recurrence rates, low early detection rates and poor treatment outcomes. (Wu et al., 2014). The main treatment options for malignancies are surgery, systemic chemotherapy, radiotherapy, molecular targeted therapy and immunotherapy. In addition, Chinese herbal medicine is also available. For early stage malignant tumours, especially solid tumours such as gastric cancer, lung cancer and colorectal cancer, surgery is the preferred treatment. The early stages of HCC usually have no or mild symptoms and are usually not easily detected. (Wu et al., 2014), and it is usually only in the late stages that it is detected and treatment is initiated. Due to the short median survival time, the opportunity for curative treatment of the tumour such as resection, transplantation or local ablation has disappeared and in the advanced stages of the tumour there is usually a shift to the use of systemic anti-cancer treatments such as chemotherapy. Chemotherapy such as cyclophosphamide (CTX) can bring about side effects in treatment. Apart from the usual toxic side effects of chemotherapy such as nausea, vomiting, hair loss, constipation and diarrhoea, it can also seriously deplete bone marrow progenitor cells ,the consequence of myelosuppression is a decrease in whole blood cells, including leukocytes, hemoglobin and platelets, which are prone to infection, anemia, dizziness and fatigue. As a result, the therapeutic effect can also be greatly compromised by limiting the dose of the drug in order to reduce side effects. (Liu et al.). In general, the immune system can eliminate malignant non-self cells through cellular immunomodulation. However, some malignant non-self cells can evade immune surveillance and form tumours when the immune system is compromised. Therefore, promoting cellular immunity through immunostimulatory drugs is a feasible approach, as it can inhibit tumour growth without damaging the host.

In Panax notoginseng, two components can improve the host's cellular immunity. Both NPPN and 2 stereoisomeric pairs involve in Rg3 have function of immuno-stimulation.

Panax notoginseng crude polysaccharide (CPPN) can enhance the host immune system, thereby effectively

extending the life span of tumour-bearing. The NPPN that extracted from CPPN combine with CTX, the inhibition of its tumor suppressive effect becomes more pronounced due to its ability to reduce the side effects associated with CTX treatment, such as myelosuppression and immunosuppression(Liu et al. 2021)

Among the many active constituents of Panax ginseng, ginsenosides have the most significant function and have very good activity for immunomodulation, anticancer and antioxidant compared to other components of Panax ginseng. In recent years, hundreds of different ginsenoside samples have been isolated and identified, with Rg3 showing the most effective results There is much evidence that Rg3 has antitumour and immunomodulatory pharmacological effects with low toxicity in vivo and in vitro. (Wu et al., 2011). The stereospecific role of ginsenosides (Rg3) in the immune system is well worth investigating. For the two stereoisomers of Rg3, Rg-3 S form and Rg-3 R form, they exhibit disparate pharmacological effects due to the structures difference of the two isomers. However, both had significant inhibitory effects on the growth of H22 transplanted tumours. Not only that, the cellular immunity of H22 mice was also significantly improved after Rg3 treatment. This may be due to the increase levels of Th1-type cytokines interleukin-2 (IL-2) and interferon-γ (IFN-γ) and stimulate ConA-induced lymphocyte proliferation (Wu et al., 2014). This study will demonstrate this idea by comparing the antitumor and immunomodulatory activities of 20(s)-Rg3 and 20(R)-Rg3 using a mouse model of H22 liver cancer. Splenocyte proliferation and cytokine production can measure the immunomodulatory activity of Rg3 (S) -form and Rg3 (R)- form.

2. Methods

2.1. Experimental materials

20(S)-Ginsenoside Rg3 and 20(R)-Ginsenoside Rg3 are active pharmaceutical ingredients extracted from the roots of Ginseng.

For Panax notoginseng, according to previous literature and traditional Chinese processing methods, its medicinal properties will change when baking, cooking, boiling and frying In these processing processes, steaming method is the most commonly used, and it is generally carried out at 100 ℃. (Peng et al., 2018)

As Panax ginseng is processed, some of the original saponins are degraded and a considerable number of stereoisomers of saponins exist as secondary saponins are produced. 20(S)-Ginsenoside Rg3 and 20(R)-Ginsenoside Rg3 are the isomers produced after degradation.

(Peng et al., 2018). Hank's balanced salt solution (HBSS) were used to prepare culture medium.

2.2. Cell line

This experiment will use Hepatocellular carcinoma cell line (H22)

Establishment and grouping mouse models

H22 cells were resuspended in normal saline and injected into the peritoneal cavity of mice. 7 days later, ascites tumor cells were extracted and mixed with normal saline until they were dispersed to a concentration of 5×106 cells/ml. On day 1, the diluted ascites tumor cell suspension was injected subcutaneously into the right axilla of KM mice to establish a solid tumor animal model.

After transplantation of H22 cells, mice were divided equally into three groups of five each: 20(S)-Rg3 group, 20(R)-Rg3 group and model group. 5 normal mice (without transplantation of H22 cells) would serve as the normal group. The next day, the S-form group and R form groups will be injected intraperitoneally with 20(S)-Rg3 and 20(R)-Rg3 (3 mg/kg body weight) (3 mg was tested when doing titration time course experiments to avoid false negative results due to suboptimal concentrations or long experimental times), and the normal and model groups will be injected with equal volumes of normal saline (0. 2 mL/ 10 g BW), and each of the four groups will be done once a day for 10 days. Injection of 3 mg/kg Rg3, has been clinically proven to be effective in the treatment of ovarian cancer (Wu et al., 2014).

2.3. In vivo antitumor assay

24 hours after the last test administration. The animals were fasted and the mice in each group were killed after weighing.Then, the thymus and spleen were removed and splenocyte proliferation and cytokines were collected for assay. Dissected solid tumor and weighted. (Wu et al., 2014) The inhibition rate can be calculate the following formula:

$$\text{Inhibition rate of tumor growth (\%)} = \left(1 - \frac{\text{Average tumor weight of administrartion group}}{\text{Average tumor weight of the model group}}\right) \times 100\%$$

2.4. Lymphocyte proliferation stimulation measurement

Preparation of mouse spleen lymphocytes: The collected mouse spleens were separated into small pieces in HBSS under aseptic conditions, fat and connective tissue were removed, and filtered through a 200-mesh sieve to obtain a homogeneous cell suspension. After centrifugation (1000×g, 4°C, 10 min), the cells were washed 3 times with phosphate-buffered saline (PBS) and

resuspended in complete medium. The cell suspensions were then inoculated onto 96-well plates. The medium was separated on a centrifuge plate and 150 μ L acidic isopropanol working solution (144) μ L isopropanol and 6 μ L 1 M HCl were added to each well. After 10 minutes of incubation, absorbance was measured at 570 nm with a microplate reader. Splenocyte proliferation capacity was calculated according to the following formula: splenocyte proliferation capacity = absorbance value of non-ConA cultures minus absorbance value of ConA cultures. (Wu et al., 2014).

2.5. Cytokine assay

To quantify IFN-γ and IL-2 secretion from the spleen and thymus, the tissues were homogenized in normal saline in a 1:9 ratio on ice. The homogenate was centrifuged for 15 minutes to extract the supernatant, which was then used to measure cytokine levels. The supernatant and the above-mentioned serum were diluted in a 1:10 ratio with Laemmli sample buffer and used to evaluate IFN-γ and IL-2 activity.

3. Result

Possible result of the effects of 20(S)-Rg3 and 20(R)-Rg3 on lymphocyte proliferation and tumor growth in H22 mice (the overview of seven possible result is shown in the table 1)

3.1. Possible result 1: Applying both (S)-form and (R)-form ginsenoside inhibit the tumor growth by promoted lymphocyte proliferation induced by Concanavalin A (Con A) in H22-bearing mice, 20(R)-Rg3 has higher lymphocyte proliferation

Both 20(S)-Rg3 and 20(R)-Rg3 were activated in all samples, which showed a significant decrease in tumor weight. The rate of inhibition in tumor increase, (R)-form ginsenoside have a higher inhibition rate. Proliferation of lymphocyte are all increase significantly ($P < 0.05$). Lymphocyte proliferation of model group are all decrease significantly when compare to the normal group. Proliferation rate of Splenocyte in the (R)-form ginsenoside–treated group was higher than that in the (S)-form ginsenoside–treated group ($P < 0.05$). This result is fully support my hypothesis due to the animal experiment display that (S)-form ginsenoside and (R)-form ginsenoside can promote cellular immunity and (R)-form ginsenoside has a better effect on lymphocyte proliferation.

3.2. Possible result 2: Applying both 20(S)-Rg3 and 20(R)-Rg3 inhibit the tumor growth by promoted lymphocyte proliferation induced by Concanavalin A in H22-bearing mice, 20(S)-Rg3 has higher lymphocyte proliferation

20(S)-Rg3 and 20(R)-Rg3 activate in all of the samples, which showed a significant decrease of tumor weights, the inhibition rate of tumor increase, (S)-form ginsenoside have a higher inhibition rate. proliferation of lymphocyte are all increase significantly($P < 0.05$). Lymphocyte proliferation of model group are all decrease significantly when compare to the normal group($P < 0.05$). Proliferation rate of Splenocyte in the (S)-form ginsenoside–treated group was higher than that in the (R)-form ginsenoside–treated group ($P < 0.05$). This result is partially support my hypothesis duo to the animal experiment display that (S)-form ginsenoside and (R)-form ginsenoside can promote cellular immunity but (R)-form ginsenoside does not has a better effect on lymphocyte proliferation.

3.3. Possible result 3: Applying 20(S)-Rg3 inhibit the tumor growth by promoted lymphocyte proliferation induced by Concanavalin A in H22-bearing mice, but 20(R)-Rg3 does not. 20(S)-Rg3 has higher lymphocyte proliferation

20(R)-Rg3 activate in all of the samples, there was a significant decrease of tumor weights, the inhibition rate of tumor increase. 20(S)-Rg3 does not shows positive result. Proliferation of lymphocyte are all increase significantly ($P < 0.05$). Lymphocyte proliferation of model group are all decrease significantly when compare to the normal group($P < 0.05$). (R)-form ginsenoside silent in all samples proliferation of lymphocyte are all decrease significantly ($P < 0.05$). Lymphocyte proliferation of model group are all decrease significantly when compare to the normal group($P < 0.05$). (R)-form ginsenoside shows same results with model group. Proliferation rate of Splenocyte in the (S)-form ginsenoside–treated group was higher than that in the (R)-form ginsenoside–treated group ($P < 0.05$). This result is partially support my hypothesis due to the animal experiment display that (S)-form ginsenoside can promote cellular immunity but (R)-form ginsenoside does not display that.

3.4. Possible result 4: Applying 20(R)-Rg3 inhibit the tumor growth by promoted lymphocyte proliferation induced by Concanavalin A in H22-bearing mice, but 20(S)-Rg3 does not. 20(R)-Rg3 has higher lymphocyte proliferation

20(S)-Rg3 activate in all of the samples, the tumor weight's was decrease significantly, the inhibition rate of

tumor increase. 20(R)-Rg3 does not shows positive result. Proliferation of lymphocyte are all increase significantly ($P < 0.05$). Lymphocyte proliferation of model group are all decrease significantly when compare to the normal group ($P < 0.05$). (S)-form ginsenoside silent in all samples proliferation of lymphocyte are all decrease significantly ($P < 0.05$). Lymphocyte proliferation of model group are all decrease significantly when compare to the normal group ($P < 0.05$). (S)-form ginsenoside shows same results with model group. Proliferation rate of Splenocyte in the (R)-form ginsenoside–treated group was higher than that in the (R)-form ginsenoside–treated group ($P < 0.05$).This result is partially support my hypothesis due to the animal experiment display that (R)-form ginsenoside can promote cellular immunity but (S)-form ginsenoside does not display that.

3.5. Possible result 5: Applying both 20(S)-Rg3 and 20(R)-Rg3 does not inhibit the tumor growth by promoted lymphocyte proliferation induced by Concanavalin A in H22-bearing mice

20(S)-Rg3 and 20(R)-Rg3 inactivate in all of the samples, there was no reduction of tumor weights, the inhibition rate of tumor does not change. Proliferation of lymphocyte are all decrease significantly($P < 0.05$). Lymphocyte proliferation of model group are all decrease significantly when compare to the normal group. (S)-form and (R)-form ginsenoside are all shows same results with model group. This result does not support my hypothesis due to the animal experiment does not display that (S)-form and (R)-form ginsenoside can promote cellular immunity and does not display (R)-form ginsenoside has a better effect on lymphocyte proliferation.

3.6. Possible result 6: Applying both 20(S)-Rg3 and 20(R)-Rg3 inhibit the tumor growth by promoted lymphocyte proliferation induced by Concanavalin A in H22-bearing mice, 20(R)-Rg3 and 20(S)-Rg3 has similar lymphocyte proliferation

20(S)-Rg3 and 20(R)-Rg3 activate in all of the samples, both of them had a significant reduction of tumor weights, the increasing of inhibition rate are similar. Proliferation of lymphocyte are all increase significantly($P < 0.05$). Lymphocyte proliferation of model group are all decrease significantly when compare to the normal group. Proliferation rate of Splenocyte in the (R)-form ginsenoside–treated group was similar with the (S)-form ginsenoside–treated group ($P < 0.05$). This result is partially support my hypothesis due to the animal experiment display that (S)-form and (R)-form ginsenoside can promote cellular immunity has a similar effect on lymphocyte proliferation.

3.7. Possible result 7: Applying both 20(S)-Rg3 and 20(R)-Rg3 promote the tumor growth by inhibits lymphocyte proliferation induced by Concanavalin A in H22-bearing mice, 20(R)-Rg3 and 20(S)-Rg3 has similar lymphocyte proliferation

20(S)-Rg3 and 20(R)-Rg3 activate in all of the samples, both of them had a significant promotion of tumor weights, the increasing of promotion rate are similar. Proliferation of lymphocyte are all decrease significantly($P < 0.05$). Lymphocyte proliferation of model group are all decrease significantly when compare to the normal group. Splenocyte proliferation in the (R)-form ginsenoside–treated group and in the(S)-form ginsenoside–treated group ($P < 0.05$) are all shows negative result. This result is contradicting to my hypothesis due to The animal experiment display that (S)-form and (R)-form ginsenoside will inhibit cellular immunity and has a negative effect on lymphocyte proliferation.

4. Discussion

In our previous study we compared the antitumor and immunomodulatory effects of 20(R)-Rg3 and 20(S)-Rg3 extracted from ginseng roots. It was reported that Rg3 inhibited the growth of H22 tumours and promoted anti-tumour immune responses with its stereospecificity without causing side effects. In addition, both S-form Rg3 and R-form Rg3 remarkably increased the IL-2 and IFN-γ levels in the immune organs and serum of H22 tumour-bearing mice. As can be seen in table 1, all these results demonstrate that Rg3 can inhibit tumour growth and improve host cellular immunity without causing any severe side effects.

Possible result 1 shows agreement with my hypothesis that ConA promotes lymphocyte proliferation in H22 mice. r-Rg3 has a better effect than S-Rg3 on activated Con-A-triggered T lymphocytes and enhances cytokine secretion. The production of the cytokines IL-2 and IFN-γ is responsible for the immunomodulatory effects on antitumours. IL-2 is one of the most important cytokines regulating cellular immunity as it not only stimulates the proliferation of T cells but also enhances the function of B cells. Antibodies made by B cells can alter the function of antigenic targets on cancer cells, thus promoting NK cell-mediated tumour killing. These immunomodulatory effects are all relative to the fight against cancer. Thus, IL-2 and IFN-γ could play an important role in inhibiting tumour growth and could also be used to treat immune disorders and fight cancer. Results consistent with my hypothesis

showed that the levels of interleukin-2 and interferon-γ were maintained or even significantly increased when R-form ginsenoside and S-form ginsenoside were used in H22 tumour-bearing mice, compared with significantly lower levels of interleukin-2 and interferon-γ in the immune organs and serum of mice after transplantation of H22 tumours in the model group. The reason for the different effects of ginsenoside S- and R-types observed in this study is not clear. In order to further investigate how the stereospecificity of the isomers may have different effects against tumours, we should gain insight into their structure and function.

The inconsistent results for 2, 3, 4, 5 and 6 suggest a potential systematic error in the experimental design. Possible result 2 could be because the quality of R-Rg3 caused it to not show its promotion rate well. possible results of 3, 4 and 5 could be because inactivated Rg3 was used to do the experiment, so it showed similar results to the model group. The 6th possible result could be because the amount of 20(R)-Rg3 injected into the mice was lower than 20(S)-Rg3, so it did not show a better effect on lymphocyte proliferation.

Possible outcome 7 contradicts my hypothesis that it did not promote lymphocyte proliferation and inhibit tumour growth, but rather increased tumour size and inhibited lymphocyte proliferation. It is unlikely that these two results occurred in these ginsenoside studies, as Rg3 can promote .

Production of the cytokines interleukin-2 and interferon-γ, which are relatively well studied, and these two results are more likely to occur in the clinical setting because there may be some genetic mutations or cell lines that we are not familiar with, or there may be specific samples that have not been well studied.

Possible Result on lymphocytes proliferation

Group	Result 1	Result 2	Result 3	Result 4	Result 5	Result 6	Result 7
20(S)-Rg3	+ lower	+ higher	+	-	-	+ similar	-
20(R)-Rg3	+ higher	+ lower	-	+	-	+ similar	-
Normal	-	-	-	-	-	-	-
Model	-	-	-	-	-	-	-

Note: "+" represent a significant increase on promotion of lymphocyte proliferation. "-" represent not significantly difference from negative control, does not or partially support hypothesis."higher" represent group have higher lymphocyte proliferation. "lower"represent group have lower lymphocyte proliferation. "similar" represent group have similar lymphocyte proliferation

5. Conclusion

In summary, this study investigated the effects of ginsenoside Rg3 on the proliferation of splenocytes and the production of cytokines IL-2 and IFN-γ in H22 mice. Also, changes in tumour size were observed after inject the 20(R)-Rg3 and 20(S)-Rg3 to H22 tumour-bearing mice. Our results suggest that Rg3 has a good effect on inducing cellular immunity, which stimulating the secretion of cytokines, effectively inhibits tumour growth and does not cause adverse side effects. Notably, 20(R)-Rg3 had better ability of antitumour and immunomodulatory than 20(S)-Rg3.

The possible controversial result on S-Rg3 and R-Rg3 will also indicate the potential relationship between Rg3 and its stereospecificity, which should be investigate in the future studies on its structure and relative function. There also should be more clinical studies to authenticate the retiree and promotion ability of ginsenoside Rg3 on cellular immunity in treatment of tumor.

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