The Role of Tumor-Associated Ingredients in Serum on Antitumor Immunity

Abstract

Antigen mutation and metastasis are responsible for the low curative rate and high postoperative recurrence of cancer. More efficacious and enduring anti-tumor immune response needs to be further researched. In this study, we aimed to analyze the levels and functions of tumor-related cytokines (IL-2, IFN-γ, TNF-α) and antibodies (IgG, IgM, IgA) in serum after the improvement of antitumor immune response on H22-bearing mice by GLSPs. Our data indicated that the levels of IL-2, IFN-γ, and TNF-α were significantly reduced in model group due to the damage of immune system, while those in polysaccharide group showed remarkable promotion. The highest levels of H22-specific IgG and IgM antibodies in sera were respectively found in polysaccharide group and model group, and no IgA antibody against H22 antigen was discovered. The in vivo antitumor test showed that sera and H22-specific IgG antibody of polysaccharide group could not effectively inhibit tumors growth. Thus, we concluded that the functions of IL-2, IFN-γ, TNF-α on cancer cells depended on their enhancement on immunity, IgM antibody could only mitigate the tumor cells proliferation by connecting them, IgG antibody might play an important role in the later period of tumor removal, when tumor cells were induced apoptosis by activated cellular immunity. The level of IgG antibody against tumor antigen in serum might be a positive indicator to reflect the ability of body in eliminating tumor cells, which was consistent with the proportion of CD19+ B cells in spleen. These results demonstrated the functions of tumor-related cytokines and antibodies separately. Further studies are necessary to determine the mechanism of cellular immunity on tumor cells in vivo.

Keywords: Cytokines; Tumor-specific antibodies; Antitumor activity; CD19+ B cells

Abbreviations: GLSPs: Gracilaria Lemaneiformis; Polysaccharides: IL-2: Interleukin-2; IFN-γ: Interferon-γ; TNF-α: Tumor Necrosis Factor-α; Ig: Immunoglobulin; CD: Cluster of Differentiation; DMSO: Dimethyl Sulfoxide; FBS: Fetal Bovine Serum; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Introduction

Cancer has become a major concern for human health owing to the high mortality. Some studies have confirmed that many active substances of plant, such as phenolic constituents [1] and seaweeds sulfated polysaccharides [2], could inhibit the proliferation of cancer cells in vitro through various mechanisms without obvious side effects. However, drugs with fewer side effects always kill off chemotherapy-sensitive cancer cell populations and leave chemo resistant cells behind to re-colonize the tumor bed, ultimately leading to disease relapse [3]. Vaccines for cancer treatment have received an increased interest in recent years, tumor antigen-based cancer vaccines can stimulate tumor-specific immune responses, which have the potential to not only eradicate the cancer, but also generate long-lasting memory responses to guard against tumor recurrence [4]. Immunity system, including cellular immune and humoral immune, plays a critical role in protecting the host from cancer [5]. Cell-mediated antitumor immune response mainly involves the capturing and processing of tumor-derived antigens by dendritic cells, as well as the recognition and elimination of tumor cells by CD4+ and CD8+ T cells [6], which plays a central role against tumors.

In contrast with T lymphocytes involving in cellular immune responses, B lymphocytes take part in humoral immune responses by the production of immunoglobulins, also named antibodies, which can directly recognize to extrinsic invaders and trigger to defeat those 7. The B cells are derived from lymphoid stem cells, undergoing various developmental stages: progenitor B cells, precursor B cells, immature B cells and mature B cells periods, and antigen driven activation of mature B cells leads to generation of plasma cells producing soluble antibodies and memory B cells [7,8].

IgG, the most abundant type of antibody in serum, is found in all body fluids and plays crucial role in anti-tumor and anti-infection immunity [9,10], and the involvement of IgG antibodies also suggest a balanced immune response [11]. The antigen-specific IgM yield good results to the early detection of acute infections while non-specific IgM is not known but could involve a reaction to glycolipids [12]. IgG and IgM are also found in secretions but not equal to the amount of IgA. IgA, the greater part in the secretions of mucosal surfaces, has been found protecting our body from infections [9]. The human mucosal surfaces is protected by IgA that shield is very important for human body and deficiency of IgA may lead to cancer [13]. Tumor-associated antigens can trigger and stimulate mature B cells of spleen to proliferate, differentiate and then release various immunoglobulins IgG, IgA and IgM to serum [14-16].

There are many other kinds of active antitumor-related ingredients such as IL-2, IFN-γ, and TNF-α. IL-2 participates in...
antitumor immune response of differentiation, clonal expansion and activation of cytotoxic and helper T lymphocytes, and it can also stimulate B cells to divide and produce antibodies [17]. IFN-γ, the uppermost cytokine implicated in anti-tumor immunity, plays a central role in the recognition and elimination of transformed cells [18]. TNF-α is a multifunctional cytokine acts as a key mediator for local inflammation and in the development of cancer, studies on TNF-α inhibition therapies in experimental autoimmune encephalomyelitis suggested a potential effect of reducing disease severity and progression by administration of an TNF-α blocking agents [19,20]. These results suggest that various antibodies and cytokines involve in the enhancement of immunity and the elimination of tumor cells either directly or indirectly.

In a previous study, we successfully proved that a Gracilaria lemaneiformis sulfated polysaccharide (GLSPs) exhibited a strong antitumor activity on H22-bearing mice by improving both specific and nonspecific cellular immune response [21]. However, the tumor-related antibodies and cytokines levels in peripheral blood of mice have not been detected, and the mechanisms of antitumor humoral immunity are not investigated. In this study, we preliminary researched the direct and indirect effects of tumor-associated cytokines and antibodies on H22 hepatoma cells, and deduced the important role of IgG antibody in antitumor immune response.

**Experimental**

**Materials and chemicals**

Gracilaria lemaneiformis sulfated polysaccharide (GLSPs) was prepared by our own laboratory [21], dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were provided by Sigma Chemical Co. (St. Louis, MO, USA). Medium RPMI-1640 was obtained from Hyclone (Thermo Scientific Inc, USA). Fetal bovine serum (FBS) was purchased from Hangzhou Sijiqing Corp (Hangzhou, China). Cytokine detecting ELISA kits were provided by Lufeng Bio-Technique Co. Ltd (Shanghai, China). Rabbit Anti-mouse IgG/HRP antibody, Goat Anti-Mouse IgM/HRP antibody and Goat Anti-Mouse IgA/HRP antibody were from Beijing bioss biological technology Co. All the chemicals and agents were of analytical grade.

**Animals and cell lines**

Seventy eight-week-old female ICR mice with an average weight of 20.0±2.0 g were provided by the center of Experimental Animals of Academy of Military Science (Beijing, China), animals were raised in a pathogen-free environment with a controlled temperature (23±2°C) and a relative humidity (50±5%) on a 12-h light/dark cyde with standard pellet diet and tap water supplied ad libitum throughout the experimental period. All experimental procedures were approved by the Local Ethics Committee for Animal Care and used at Tianjin University of Science and Technology.

Thirty ICR mice were randomly divided into three groups. Blank and model groups were both applied saline solution orally throughout the experimental period. Polysaccharide group was given GLSPs with 600 mg per kilogram of body weight per day [21]. After 14 days of gavage, all the mice were transplanted with H22 hepatoma cells provided by the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China) in the right forelimb armpit with 0.2 mL of 5×10^6 cells except blank group. All groups were administrated for another 2 weeks and sacrificed to provide blood samples for further research.

**MTT assay**

The direct cytotoxic effects of mice serums on H22 hepatoma cells were evaluated by MTT. H22 hepatoma cells were seeded in 96-well plates and added with physiological concentrations (10%) of serums in each group except control. Subsequently, MTT reagent (1 mg/mL) in phenol red-free 1640 medium was added to each well and plates were incubated for additional 4 h at 37°C in humidified atmosphere of 5% CO2. The intracellular formazan product was dissolved in 100 μL of DMSO after medium removal, and the absorbance was measured at 570 nm and mitochondrial dehydrogenase activity was realized as described [22].

**Evaluation of IL-2, IFN-γ and TNF-α in peripheral blood**

The serums of sacrificed mice in each group were prepared for assessments of IL-2, IFN-γ and TNF-α using an ELISA kit (Hufeng Biotech. Co., Shanghai, China). Under the manufacturer’s instruction, the plate was read at 450 nm on a Bio Rad Model 680 Microplate Reader.

**Evaluation of H22-specific IgG, IgM and IgA in serums**

The H22 hepatoma cells were resuspended with coating buffer and inoculated in a 96-well elisa plate with 0.1 mL of 2×10^5 cells at 4°C overnight, the mixtures were transferred and incubated with blocking buffer for 1 h at 37°C. After washing with PBST, the H22-specific IgG, IgM and IgA antibodies in serums of mice were detected by adding HRP conjugated rabbit anti-mouse IgG, goat anti-mouse IgM and IgA respectively. The absorbance at 450 nm with a reference 630 nm was read with BioRad Model 680 Microplate Reader.

**Flow cytometry assay of CD19+ B cells**

Proportions of CD19+ B cells in peripheral blood and spleen were examined using a flow cytometer. A small amount of blood and splenocytes suspension were collected and incubated with anti-mouse PE-CD19 monoclonal antibodies (Sanjian Biotech. Co., Tianjin, China) for 20 min in the dark. Thereafter, the uncombined antibodies and erythrocytes were removed via adding hemolysin solution and PBS washing. After centrifugation (1000 rpm for 5 min), the samples were resuspended in 0.5 mL of PBS buffer: Antigen expression was measured by flow cytometry and analyzed by CellQuest Pro software (Becton Dickenson, FACS Caliber, USA).

**Morphology of the H22 hepatoma cells cocultured with serums**

H22 hepatoma cells (2×10^5 cells/well) were inoculated in 6-well plates and added with 10% serums of each group, after incubated at 37°C with 5% CO2 for 24 h and 48 h respectively, the cells were photographed and analyzed by light microscopy (Nikon, Japan).

**Role of H22-specific IgG antibody on body antitumor immunity**

Forty mice were also randomly divided into four groups with ten mice each group. Blank control group were subjected to
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no disposal, model control group were transplanted with H22 hepatoma cells with 0.2 mL of 5 ×10^6 cells, sera interventions group A was intravenously injected with 0.2 mL sera of mice in polysaccharide group after transplanted with the same amounts of H22 hepatoma cells, sera interventions group B was treated substantially identical to model group except the transplanted H22 hepatoma cells were incubated with 10% sera of mice in polysaccharide group at 37°C for 1 h and washed twice for uncombined IgG antibodies removal by saline.

The tumor size (volume, V) was calculated from the tumor length (L) and width (W) which were measured with a caliper, using the following formula: V=\frac{LW^2}{2} [23]. Following 3 weeks feeding, mice were weighed and sacrificed by cervical dislocation. Liver, spleen and thymus were collected and weighed, and apparatus indexes were expressed as viscera weight relative (mg) to body weight (g).

Statistical analysis

Data were presented as the mean±standard deviation (S.D.) for all treatments. Significance was calculated by one-way ANOVA or Student’s t-test, p < 0.05 were considered statistically significant.

Results

Toxic effects of sera on H22 hepatoma cells

We first evaluated the in vitro antitumor activity of sera in each group on H22 hepatoma cells, but the results did not reveal any significant inter-group differences. Whereas compared to control, a significant decrease in cell viability was observed in sera-treated groups by both 24 h and 48 h of incubation (Figure 1).

Cytokine detection

As is known to us, levels of IL-2, IFN-γ and TNF-α in serum are closely associated with the antitumor immune response in vivo. In this study, there were statistical differences in these groups according to the results. As shown in Table 1, the IL-2, IFN-γ and TNF-α levels in sera of model group were significantly reduced compared to blank group (p<0.05), while the polysaccharide exhibited a strong immunoregulatory activity in balancing the levels of these cytokines in vivo, insured that the amounts of IL-2, IFN-γ and TNF-α were significantly higher than model group (p < 0.05).

Table 1: Results of IL-2, IFN-γ and TNF-α level in sera of mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>IL-2(pg/mL)</th>
<th>IFN-γ(ng/L)</th>
<th>TNF-α(ng/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank group</td>
<td>4.93±0.16</td>
<td>143.16±1.33</td>
<td>49.77±0.95</td>
</tr>
<tr>
<td>Model group</td>
<td>2.94±0.14</td>
<td>128.82±3.03</td>
<td>40.93±1.20b</td>
</tr>
<tr>
<td>Polysaccharide</td>
<td>5.66±0.33c</td>
<td>159.66±3.12c</td>
<td>50.72±1.68c</td>
</tr>
</tbody>
</table>

Note: bp<0.05 compared with blank group. cp<0.05 compared with model group.

Evaluation of H22-specific IgG, IgM and IgA in peripheral blood

Table 2 shows the levels of H22-specific IgG, IgM and IgA antibodies against H22 hepatoma cells antigens (n=10). The levels of specific IgG and IgM antibodies were significantly higher than control (p<0.05), which indicated that there were different amounts of H22-specific IgG and IgM antibodies in sera of each group, while no IgA antibody associated with H22 tumor antigens in sera was found in this study. The H22-specific IgG and IgM antibodies levels in model group increased significantly compared to blank group. When compared with model group, the level of H22-specific IgG antibody in polysaccharide group was remarkably higher while the amount of H22-specific IgM antibody showed no significant difference.

Table 2: Results of tumor-specific antibody levels in peripheral blood.

<table>
<thead>
<tr>
<th>Groups</th>
<th>IgG</th>
<th>IgM</th>
<th>IgA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.24±0.01</td>
<td>0.32±0.01</td>
<td>0.14±0.01</td>
</tr>
<tr>
<td>Blank group</td>
<td>0.30±0.02a</td>
<td>0.43±0.02a</td>
<td>0.15±0.02</td>
</tr>
<tr>
<td>Model group</td>
<td>0.37±0.04ab</td>
<td>0.53±0.05ab</td>
<td>0.15±0.01</td>
</tr>
<tr>
<td>Polysaccharide</td>
<td>0.51±0.03ac</td>
<td>0.49±0.002a</td>
<td>0.16±0.01</td>
</tr>
</tbody>
</table>

Note: ap<0.05 compared to control. bp<0.05 compared with blank group. cp<0.05 compared with model group.

Distribution of CD19+ B cells in peripheral blood and spleen

CD19 is a B cell specific member of the immunoglobulin superfamily expressed from the pro-B-cell stage until plasma cell differentiation in both human subjects and mice, and functions as a B-cell receptor (BCR) co receptor. CD19 also plays an essential role in regulating B-cell activation thresholds and antibodies levels, thereby influences B-cell selection and differentiation [24-26]. Figures 2 & 3 showed the distribution and proportion of CD19+ B cells in peripheral blood and spleen. Results showed that the percentage of CD19+ B cells in peripheral blood of blank group was notably ascended relative to the model group (p<0.05), while the polysaccharide could effectively maintain the amounts of CD19+ B cells in blood of H22-bearing mice (p<0.05). The proportion of...
CD19+ B cells in spleen of model group was significantly increased compared to blank group (p<0.05), while reduced significantly relative to polysaccharide group (p<0.05).

**Coculture of H22 hepatoma cells and serums**

As shown in Figure 4, the appearance of H22 hepatoma cells was not influenced by the addition of serums, while the cells bunched together and some darkness appeared on the surface compared to control, indicating that there were tumor-specific antibodies binding to the antigen of H22 hepatoma cells in serums. Results showed that serums in both model group and polysaccharide group exhibit a greater degree on cell aggregation compared to blank group.

**Effects of H22-specific IgG antibody on H22 hepatoma cells in vivo**

The changes in body weight of mice treated with the serums of polysaccharide group are presented in Figure 5A. As shown, compared to blank group, the body weight of model control group was higher in the later period due to H22 hepatoma cells proliferation in vivo. However, no significant differences were found in serums intervention groups compared with model control group, which indicated that ingredients in serums cannot decrease the weight of solid tumors in H22-bearing mice.

The changes of solid tumor volume in each group are presented in Figure 5B. The results showed that tumor volume of serums intervention groups had no significant differences compared to model control group, which was consistent with the changes of body weight. As Figure 5C shown, the model control group had higher liver, spleen indexes and lower thymus indexes than blank control group, suggesting that H22 solid tumors caused severe damage to the liver, spleen and thymus. Our results indicated that both serums intervention group A and B could not significantly mitigate the injury of viscera indexes from solid tumors, suggesting that serums of polysaccharide group had no direct antitumor activity on H22-bearing mice, which indicated that no further relative detection was required.

**Discussion**

We present a comprehensive analysis of the inhibition effects of serums on H22 hepatoma cells in vitro and the serums levels of tumor-related indicators of each group. Previous studies have shown that the levels of IL-2, IFN-γ and TNF-α in serum of tumor-bearing mice were significantly reduced or increased [27], and the same trend was found in our study. However, physiological concentration of serums in all groups showed no significant difference on inhibition effect, which indicated that cytokines like IL-2, IFN-γ and TNF-α exhibited a vital effect on tumor-related
immunomodulatory [28-30] except the direct cytotoxicity on tumor cells.

Subsequently, we observed the serums levels of specific IgG, IgM and IgA antibodies against H22 hepatoma cells antigens in each group (n=10). IgM forms pentamers that aggregate invaders and facilitate phagocytosis [31], and aggregation of the cells would mitigate H22 hepatoma cells proliferation. We found that levels of IgM antibody in these groups were consistent with the inhibition effects and the degree of aggregation on H22 hepatoma cells, concluding that tumor-specific IgM could inhibit H22 hepatoma cells directly by causing cells agglutinate in a dose-dependent manner, indicated that IgM antibody was not the vital factor in antitumor immune response due to lack of specific characteristics. We also detected the levels of IgA antibody in each group against H22 antigen, and discovered no significant difference compared with control.

IgG molecules can participate in antitumor response by activating the cellular immunity [10] and complement system [32]. We found that the level of H22-specific IgG antibody was independent of serums cytotoxicity in vitro while consistent with the body antitumor ability, which revealed that the amounts of H22-specific IgG antibody in serum could be a good indicator of evaluating the ability to eliminate H22 hepatoma cells. In order to confirm the relationship of B cells and tumor-related antibodies, we detected the proportions of CD19+ B cells in peripheral blood and spleen, and only discovered a significant positive correlation between CD19+ B cells proportion in spleen and IgG antibody levels in serums, which indicated that spleen was responsible for B cell differentiation and antibodies secretion, while peripheral blood simply played a role of transporting B cells.

There are two serum intervention groups that may explain the role of H22-specific IgG in antitumor immunity described here. It has been proposed that IgG antibodies are involved with identification of antigens [33], while in our study various indexes of mice in serum intervention group A and B exhibited no obvious differences compare to model control group. It is independent, and then apoptotic cells were combined with H22 antigen, and discovered no significant difference compared with control.

Normal tumor cells exhibit a weak antigenicity, while apoptosis make it more immunogenic, which explains that tumor-specific IgG level in serums of polysaccharide group is significantly increased compared to model group. In both mice and humans, induction of antigen-specific T-cell immunity correlates with protection against infection and control of tumor growth [34], we can conclude that activated cellular immunity is responsible for apoptosis of H22 hepatoma cells, and then tumor-specific IgG antibodies begin to take effects on the process of leukocyte phagocytosis.

Conclusion

In the present study, we found that IL-2, IFN-γ, TNF-α, IgG, IgA showed no direct inhibition effects on H22 hepatoma cells in vitro, while IgM could suppress its growth by causing cells agglutinate. Moreover, we demonstrated that the levels of H22-specific IgG antibody were consistent with the ability of eliminating tumor cells and the proportion of CD19+ B cells in spleen, speculating that H22 hepatoma cells were induced apoptosis by cellular immunity independently, and then apoptotic cells were combined with more tumor-antigen specific IgG antibody, which might be benefit for the process of leukocyte phagocytosis and immunological memory.

Conflict of Interest

We have no potential conflicts of interest to declare.

Acknowledgement

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References

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