EFFECT OF NATURALLY OCCURRING TERPENOIDS ON THE CELL MEDIATED IMMUNE RESPONSES OF METASTATIC TUMOUR BEARING ANIMALS

CHAPTER 8

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1 INTRODUCTION

Immunosuppression and dose-limiting toxicities are the major problems for the success of available cancer therapies (Ratain and Relling, 2001). In addition, for advanced tumours developed from epithelial tissues such as lung, colon, breast, prostate and pancreas, conventional cytoreductive therapies are less successful. Therefore, standard chemotherapy and radiotherapy might negate or reduce the therapeutic benefits. Thus combination of chemotherapy or radiotherapy with immunomodulating agents may provide a strategy for overcoming the immunosuppressive effects of chemotherapy or radiotherapy.

Progressive tumour growth in human and animal models is frequently accompanied by concomitant immunosuppression regardless of tumour location and etiology. (Chattopadhyay et al., 1986; Nelson and Nelson, 1987). One explanation for immunosuppression by tumours is due to decrease in cytotoxic T lymphocytes and natural killer cell activities. (Santin et al., 2000; Tsavaris et al., 2002). There are several evidences for the production of soluble factors by developing neoplasia affecting the function of host cells involved in the immunity (Nelson and Nelson 1987; Parhar and Lala, 1998). In addition, the production of factors in abnormal amounts by tumour-bearing hosts may alter normal cytokine network and cause a deleterious imbalance of immune system (Handel et al., 1997).

Plant and plant products have been the basis of treatment of human diseases since time immemorial. Many of them such as *Viscum album* (Kuttan and Kuttan, 1992), *Tinospora cordifolia* (Mathew and Kuttan, 1999), *Withania somnifera* (Davis and Kuttan 2000), *Panax ginseng*, (Sing et al, 1984). *Piper longum Linn.* and Piperine (Sunila and
Kuttan, 2004) have found to possess immunomodulatory activity. They are nontoxic compared to other class of immunomodulators and have high interest in the research field. Plant derived immunostimulating drugs can enhance cell-mediated immune responses and natural killer cell (NK cell) activity, facilitating the killing of the tumour cells by the body (Antony et al., 2000; Lala et al., 1985) Cytokines are a unique family of growth factors. Secreted primarily from leukocytes, cytokines stimulate both the humoral and cellular immune responses. Cytokines are important mediators of immune responses and are found to stimulate immune cells. Most biological agents that enhance NK cytotoxicity do so via their common ability to induce IFN γ (Ehrhardt et al., 1997), increase the activity of Tc cells, macrophages and NK cells (Ehrhardt et al., 1997). IL-2 and GM-CSF are other important cytokines involved in the immune cell activation. (Misawa et al., 2000)

The effect of terpenoid compounds on cell mediated immune system has not been studied in a systematic way. In the present chapter we report the stimulatory activity of triterpenoids ursolic acid and glycyrrhizic acid on cell-mediated immune system of metastatic tumour bearing animals.

2 MATERIALS AND METHODS

2.1 Cell lines

B16F-10 melanoma cells, K562 human leukemic cells and Sheep red blood cells were used for this study.

2.2 Animals: C57BL/6 mice (5 weeks old, 20-25g males) were used for immunological studies.
2.3 Reagents:

RPMI-1640 medium containing 10% FCS

Radioactive materials: Tritiated thymidine and Na$_2$Cr$^{51}$O$_4$

All other reagents used were of analytical reagent quality.

2.4 Terpenoid compounds:

Different concentrations of limonene, perilllic acid, ursolic acid and glycyrrhizic acid suspended in light paraffin oil and intraperitoneally administered. Five doses of limonene administered at a concentration of 100 μmoles/Kg body wt/dose/animal. Perilllic acid, ursolic acid and glycyrrhizic acid were intraperitoneally administered at a concentration of 50 μmoles/Kg body wt for 5 consecutive days.

2.5 Determination of the effect of terpenoids on natural killer cell (NK cell) activity, antibody dependent cell mediated cytotoxicity (ADCC) and antibody dependent complement-mediated cytotoxicity (ACC) in metastatic tumour bearing animals.

C57BL/6 mice were grouped into five (12 nos./group). All the animals were induced metastasis by injecting B16F-10 cells ($10^6$ cells/animals) intravenously. Group I, II, III and IV animals were treated with 5 doses of limonene, perilllic acid, ursolic acid and glycyrrhizic acid respectively. Group V animals were kept as untreated tumour bearing controls. Animals were sacrificed at different time periods after the tumour inoculation and spleen and blood was collected. Spleen cells were processed to single cell suspension and used as effectors for NK and ADCC. Serum was separated from the blood, heat inactivated and used for the ACC assay.
2.5a Determination of natural killer cell activity

Natural killer cell activity was determined by 4h chromium assay as explained in chapter 2 (Kim et al., 1980). Cr\textsuperscript{51} labeled K 562 cells were used as targets and spleen cells from the metastatic tumour bearing animals were used as effector cells.

2.5b Determination of antibody dependent cellular cytotoxicity (ADCC)

ADCC was determined by 4h chromium release assay as described in chapter 2 (Kim et al., 1980). Chromium labelled SRBC was used as target cells and spleen cells from animals were used as effector cells. Anti SRBC antibody was raised in rabbit and was used as the source of antibody in ADCC assay.

2.5c Determination of antibody dependent complement mediated cytotoxicity (ACC)

Serum from the above animals was used for the determination of ACC activity explained in chapter 2. Serum samples were incubated along with fresh rabbit serum as a source of complement and EAC cells as target cells (10\textsuperscript{6}) at 37\textdegree C for 3h and percentage cell death was determined by trypan blue exclusion method (Chapter 2).

2.6 Determination of the effect of terpenoids on cytokine production by metastatic tumour bearing animals

C57BL/6 mice were grouped into five (9 nos./group) and all the animals were injected with B16F-10 cells (10\textsuperscript{6} cells/animal) intravenously. Group I, II, III and IV animals were treated with 5 doses of limonene, perillic acid, ursolic acid and glycyrrhizic acid respectively. Group V animals were kept as untreated tumour bearing controls. Blood was collected by tail bleeding on 7\textsuperscript{th} and 21\textsuperscript{st} day after tumour inoculation. Serum separated and used for the estimation of cytokines such as IL-1\beta, IL-2, IL-6, GM-CSF and TNF-\alpha using respective Elisa kits.
3 RESULTS

3.1 Effect of naturally occurring terpenoids on natural killer cell activity of metastatic tumor bearing mice

The effect of terpenoids on the NK cell activity of metastatic tumour bearing animals is shown in figure 8.1. There was a significant enhancement of the NK cell activity in terpenoids treated metastatic tumour bearing animals. In terpenoids treated group maximum cell lysis was obtained on 4th day (60.2%, 43%, 50.3% and 44.7% cell lysis respectively for glycyrrhizic acid, ursolic acid, limonene and perillic acid) after the tumour inoculation. In control animals the maximum cell lysis (25% cell lysis) was obtained only on 16th day.

3.2 Effect of terpenoids on ADCC of metastatic tumour bearing animals

The effect of naturally occurring terpenoids on ADCC activity is given in figure 8.2. Intraperitoneal administration of terpenoids clearly enhanced the ADCC activity in metastatic tumour bearing animals. In tumour alone treated control animals maximum cell lysis was obtained only on 16th day (20% cell lysis). But in the case of terpenoids treated metastatic tumour bearing animals, the maximum lysis was obtained on 12th day and it was 47%, 44.7%, 33.9% and 32.5% cell lysis respectively for glycyrrhizic acid limonene, perillic acid and ursolic acid treated groups.

3.4 Effect of terpenoids on ACC activity in metastatic tumour bearing animals

ACC was also enhanced by the terpenoids treatment. Maximum cell lysis was observed on 17th day for all the compounds. The maximum ACC activity was obtained in glycyrrhizic acid treated metastatic tumour bearing animals (26.52%). Where as in ursolic
Fig 8.1 Effect of terpenoids on NK cell of metastatic tumour bearing animals

% Lysis

Days after tumour inoculation

CONTROL  LIMONENE+T  GLYCYRRHIZIC ACID+T  URSOLIC ACID+T  PERILLIC ACID+T
Fig 8.2 Effect of terpenoids on ADCC

Days after tumour inoculation

% Lysis

1st 2nd 3rd 4th 6th 8th 10th 12th 14th 16th 18th 20st 21st

CONTROL LI LIMONENE+T GLYCYRRHIZIC ACID+T URSOLIC ACID+T PERILLIC ACID+T
Fig 8.3 Effect of terpenoids on ACC

Days after tumour inoculation

% Lysis

1st 2nd 3rd 5th 7th 9th 11th 13th 15th 17th 19th 21st

- Glycyrrhizic acid
- Limonin
- Ursolic acid
- Perillic acid
- control
acid, perillic acid and limonene treated groups maximum cell lysis were 22.9%, 21.3% and 22.2% respectively. But in metastatic tumour bearing control animals, the maximum cell lysis was observed on 19th day and it was only 13.4% (Fig.8.3).

3.4 Effect of terpenoids on the cytokine production by metastatic tumour bearing animals

As shown in table 8.1, 7th day after tumour inoculation, TNF-α level of metastatic tumour bearing control animals was drastically elevated to 241.5 pg/ml which was significantly reduced by the administration of glycyrrhizic acid (102.6 pg/ml), perillic acid (117.6 pg/ml), limonene (124 pg/ml) and ursolic acid (142.3 pg/ml). The level of GM-CSF was also reduced by terpenoids treatment. The maximum inhibition of serum GM-CSF levels was obtained in limonene treated (10.02 pg/ml) metastatic tumour bearing animals and followed by in the treatment with perillic acid (18.7 pg/ml), glycyrrhizic acid (20.3 pg/ml) and ursolic acid (22.5 pg/ml) treated metastatic tumour bearing animals compared to control animals (37.9 ± 1.1 pg/ml). The highly elevated level of IL-6 (370.1 pg/ml) in control animals was reduced by the treatment of glycyrrhizic acid (313 pg/ml) ursolic acid (299 pg/ml) limonene (314.4 pg/ml) and perillic acid (318 pg/ml). The lowered level of IL-2 in the untreated control animals (24.9 pg/ml) was enhanced by the treatment with limonene (39.1 pg/ml) glycyrrhizic acid (37.9 pg/ml) ursolic acid (35.9 pg/ml) and perillic acid (32.6 pg/ml).

On 21st day after tumour inoculation, the enhanced level of TNF-α in metastatic tumour bearing animals was further enhanced to 334.9 pg/ml, which was significantly reduced by the treatment of glycyrrhizic acid (96.4 pg/ml) ursolic acid (84.7 pg/ml) perillic acid (98.6 pg/ml) and limonene (108.6 pg/ml). The enhanced level of IL-1β was also
Table 8.1 Effect of terpenoids on serum cytokine levels in metastatic tumour bearing animals

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Serum cytokine level (pg/ml)</th>
<th>7th day</th>
<th>21st day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IL-2</td>
<td>IL-1β</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td>27.4±1.8</td>
<td>16.0±2.1</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>24.9±0.6</td>
<td>44.8±0.6</td>
</tr>
<tr>
<td>Limonene</td>
<td></td>
<td>38.1±0.8*</td>
<td>34.2±2.1*</td>
</tr>
<tr>
<td>Perillic acid</td>
<td></td>
<td>32.6±2.1*</td>
<td>33.6±1.6*</td>
</tr>
<tr>
<td>Ursolic acid</td>
<td></td>
<td>35.98±3.2*</td>
<td>36.4±3.1*</td>
</tr>
<tr>
<td>Glycyrrhizic acid</td>
<td></td>
<td>37.9±2.3*</td>
<td>33.4±1.1*</td>
</tr>
</tbody>
</table>

Animals were injected with B16f-10 cells (10⁶) intravenously. Animals were treated with 5 consecutive doses of limonene, perillic acid ursolic acid and glycyrrhizic acid. Blood was collected by tail bleeding on 7th and 21st day after tumour inoculation. Serum separated and used for assays.

(*P<0.01 Compared with tumour alone treated control)
effectively reduced by the treatment of glycyrrhizic acid (20.1 pg/ml), ursolic acid (21.2 pg/ml), limonene (20.3 pg/ml) and perillic acid (19.8 pg/ml) compared to control animals (60.3 pg/ml). The elevated level of GM-CSF in control animals (21.8 pg/ml) after 21 days was reduced by the treatment of limonene (7.9 pg/ml), perillic acid (10.9 pg/ml), glycyrrhizic acid (12.6 pg/ml) and ursolic acid (22.5 pg/ml). The level of IL-6 was highly elevated in metastatic tumour bearing animals (559.8 pg/ml). Treatment with glycyrrhizic acid (411 pg/ml), limonene (412.3 pg/ml), perillic acid (398.5 pg/ml) and ursolic acid (386.9 pg/ml) could effectively reduce the same. Drastically lowered level of IL-2 in control animals (7.4 pg/ml) was significantly enhanced by the treatment of glycyrrhizic acid (24.5 pg/ml), ursolic acid (30.5 pg/ml), perillic acid (31.4 pg/ml) and limonene (32.9 pg/ml).

4 DISCUSSION

Tumour development, outgrowth and metastasis are under the surveillance of the immune system. The fate of the host-tumour interactions depends on the balance between the intrinsic metastatic potential of the tumour and strength of the host immune response (Cooper et al., 2001). One of the major objectives of immunotherapy is to modulate immune response for selected objectives. Cell-mediated immunity is the component of the immune system most responsible for destruction of infected cells and tumour. Macrophages are critical in presenting antigens to helper T-cells (CD-4) cells via MHC II. This activates the helper T-cell to further activate cytotoxic (killer) T-cells and natural killer cells creating a robust immune response. The cells of the cell-mediated immune system depend on signals to communicate with each other in order to mount an aggressive
and orchestrated attack. These signals are transmitted using cytokines, lymphokines, interferons, and other chemical messengers. In this study we evaluated the cell mediated immune response against B16 F10 metastatic tumour in mice by the activation of NK cell, ADCC, ACC and the production of various cytokines by the administration of terpenoid compounds.

The role of natural killer (NK) cells in the induction and regulation of immune responses has been the focus of many investigations. Due to the peculiar immunoregulatory characteristics of NK cells, it may be a potential target for novel immunotherapeutic interventions aimed at the prevention or treatment of infectious disease, cancer and autoimmune disease (Henney, 1981; Mandelboim, 1999). Even though they have no known antigen specific receptors they are able to recognize and kill a limited range of abnormal cells including cancer cells. Activation of NK cells is one of the objectives of tumour immunotherapy. NK cells also produce cytokines, particularly IFN-γ (Kobayashi et al., 1989; Ehrhardt, 1997). The lymphokine IL-2, which was identified as T cell growth factor (Misawa et al., 2000), alone or in combination with IFNs, also promote the lytic activity of NK cells (Caligiuri, 1993) which in turn produces a variety of immunoregulatory molecules that could synergize with IFNs or IL-2 for the induction of antitumour responses (Henney, 1981). Treatment with these compounds has stimulated host defense response, as demonstrated by the enhanced level of IL-2.

ADCC is the cooperative interaction of humoral and cell mediated immune effectors. A number of cells that have cytotoxic potential express membrane receptor for the Fc region of the antibody molecule. When antibody is specifically bound to a target cell these receptor bearing cells can bind to the Fc region of the antibody and thus to the
target cells and subsequently cause lysis of the target cells. Cytotoxic T lymphocytes and NK cells have Fc receptors that are capable to trigger cytotoxic attack to target cells (Henney, 1981; Mandelboim, 1999). Intraperitoneal administration of terpenoids elevated NK cell activity resulted in significant enhancement of ADCC in both tumour bearing as well as normal animals where as untreated tumour bearing animals with low NK cell activity showed decreased ADCC activity.

Complement is a system of plasma proteins that can be activated by antibody, leading to a cascade of reactions that occurs on the surface of pathogens and generate active components with various effector functions. Administration of these terpenoids ursolic acid and glycyrrhizic acid could enhance the ACC activity in tumour bearing animals. Complement proteins are also responsible for cell lysis and mediation of inflammation, serving to attract phagocytic cells and enhance phagocytosis. It plays a major role in cell mediated immune response. Enhancement of ACC shows the activation of cell mediated immune system by the administration of terpenoids.

IL-6 is a key inflammatory mediator produced by many cell types. It is the major inducers of the acute-phase response and fever. Blocking IL-6 may alleviate rheumatoid arthritis and may also be effective in other autoimmune, inflammatory, and bone-erosive diseases. In mice, IL-6 is required for development of oil-induced plasmacytomas (Hilbert et al., 1995) and is involved in tumor cachexia (Strassmann, 1995). In humans, IL-6 is a growth factor for myelomas, (Licastro et al., 1993). In this study administration of terpenoids were shown to reduce the IL-6 production in the metastatic tumour bearing animals. TNF-alpha and IL-1β are also the principle cytokines that mediates acute inflammation. In excessive amounts TNF-alpha also is the principal cause of systemic
complications such as the shock cascade. Interleukin-1s (IL-1) are secreted by stimulated macrophages and induce the synthesis of collagen and collagenase. Blocking IL-1 activity via receptor antagonist, soluble receptors, or newly tailored drugs shows promise in controlling inflammatory diseases, such as rheumatoid arthritis and septic shock. IL-1, probably most effectively if combined with blockade of other inflammatory cytokines, such as TNF and IL-6. GM-CSF binds to receptors on neutrophils, eosinophils, and monocytes, it activates these cells and inhibits apoptosis. Administration of terpenoids decreased the elevated levels of this cytokines. Lymphokine IL-2, stimulates cytotoxic activity of NK and T cells and acting as a cofactor in activating macrophages and B cells. Administration of these terpenoids reduced the enhanced level of TNF, IL-6, GM-CSF and IL-1β and also increases the production of IL-2 in metastatic tumour bearing animals.

The above study revealed immunostimulatory effects of glycyrrhizic acid and ursolic acid on metastatic tumour bearing animals, and these activities may be due to the stimulation of IL-2 production and there by enhanced cell mediated immune responses.