Effects of Some Natural Immunomodulatory Compounds in Combination with Thalidomide on Survival Rate and Tumor Size in Fibrosarcoma-Bearing Mice

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Abstract
Purpose: Despite significant advances have been achieved in cancer therapy, response to conventional treatments like surgery, radiotherapy and chemotherapy varies among individuals. Immunotherapy is known to be an effective strategy for patients who are resistant to the currently available interventions.

Methods: Ninety-six male Balb/c mice (aged 6-8 weeks) were selected and divided into twelve groups of eight. Approximately, 1x106 of WEHI-164 cells were injected to each mouse for tumor genesis. Five immunotherapy treatments were considered in this study, including Heat Shock Proteins (HSP), Bacillus Calmette-Guérin (BCG), Bifidobacterium, Immuno-Modulator Drug (IMOD) and Thalidomide. After tumor formation, the groups were treated with one or more of these therapies. Tumor size and survival rate was regularly monitored.

Results: Depending on the treatment group, tumor sizes were different. In some groups, combined treatments demonstrated more inhibitory effects on tumor growth rate. The mice in group (IMOD+ Thalidomide) had the lowest survival rate but group (BCG+ HSP+ Thalidomide) survived until the end of the experiment.

Conclusion: The (HSP+ BCG+ Thalidomide) group exhibited satisfactory outcomes and two third of the mice in this group went into complete remission. Some combination therapies in test groups had significant impacts on survival and tumor growth rate.

Introduction
Nowadays, multidisciplinary strategies that utilize surgery, chemotherapy and radiation have drastically improved the survival rate of the patients suffering from cancer.1 However, since many patients are resistant to conventional therapies the mentioned malignancy remains a leading cause of mortality worldwide.2 On the contrary, immunotherapy represents a variety of effective applications to improve overall survival and decrease treatment- associated morbidity.3,4 In this regard, we have assessed the efficacy of some immunotherapy approaches, alone and combination with each other, in mouse tumor models.

HSPs are intracellular molecules that act as chaperones for antigens.3 From the immunological point of view, HSPs are involved in stimulation of the innate and adaptive immune systems. The ability of HSPs in binding to the antigenic peptides and transporting them to the antigen presenting cells (APC) on MHC-I is the basis for their potential role in the generation of peptide-specific T lymphocyte responses.5,7 HSPs, as anti-tumor vaccines are involved in phase II and III clinical trials for cancer immunotherapy.8,9 The chemical composition of Bacillus Calmette-cell Guérin (BCG) walls (BCG-CW) demonstrates immunoadjuvant and anti-tumor activities.10 The activation of immune cells and dendritic cells (DC) and the identification of the receptors on the surface of DC for BCG-CW are responsible for its immunological activities.11 BCG-CWS up-regulates a maturation marker, CD83, and costimulators, CD80 and CD86, as well as MHC levels, and secretion of IL-12 and TNF-α by DC.12

In general, tumor progression is accompanied by angiogenic factors like VEGF, bFGF and TGF-β.13 These factors could be arrested by Thalidomide, Thiols, and Polyphenolic components.14,15 In other words, Thalidomide plays its role by prohibiting the formation of new vascular network from existing blood vessels.16,17 By this mechanism, Thalidomide can delay oxygen and...
nutrients delivery to tumors and restrain the cell division.\textsuperscript{18} Bifidobacteria are gram-positive, obligate an aerobes naturally found in large intestine and the lower small intestine of human and some mammals.\textsuperscript{19} These non-pathogenic bacteria show inhibitory effects on colon, mammary, liver and HPV-associated cervicalcancers.\textsuperscript{20,21} Bifidobacteria can only proliferate in anoxic environments and thus preferentially tend to accumulate in such regions of solid tumors.\textsuperscript{17} For this reason, they could also be employed in cancer gene therapy.\textsuperscript{22} Immuno-Modulator Drug (IMOD) is an anti-AIDS drug, which is composed of two chemical components and three herbal extracts.\textsuperscript{25} It is shown that, this drug has immuno-modulatory and anti-AIDS effects.\textsuperscript{23,24} In this study, we have tried to find an appropriate combination of the aforementioned strategies to prevent the invasive cancer, to decrease the tumor size and increase the survival rates.

Materials and Methods

Study design

Ninety-six male inbred Balb/c mice, aged from six to eight weeks, were purchased from Pasture Institute (Tehran, Iran) and housed under conditions of constant temperature and humidity according to institutional ethical guidelines. The mice were randomly divided into 12 groups of eight as following: 1: Control, 2: BCG, 3: HSP, 4: Thalidomide, 5: Bifidobacterium, 6: IMOD, 7: BCG+ Thalidomide, 8: IMOD+ Bifidobacterium, 9: IMOD+ HSP, 10: IMOD+ Thalidomide, 11: HSP+ BCG+ Thalidomide and 12: IMOD+ Bifidobacterium+ HSP+ BCG+ Thalidomide. Approximately 1×10\(^6\) cells/100µl of WEHI-164 cells were injected to create fibrosarcoma tumors in mice. Consequently, tumors were generated after 11 days.

### Treatment method

Table 1 represents detailed information about the medication groups. Drugs were Injected twice a week from the 11\(^{th}\) day intratumorally and subcutaneously. In control group, 200 µl of PBS was injected to mice. The BCG used for injection was diluted with PBS in a ratio of 1:1. In HSP group, WEHI-164 cells were first stimulated to produce HSP. To release HSP, WEHI-164 cells were cultivated in flasks and incubated in a 42°C water bath for 60 min. The cells were trypsinated after 12-hour incubation at 37°C and washed thrice with PBS. Finally, cell suspension containing 5×10\(^5\) cells/100µl PBS was prepared. Cell lysates were produced by five times freeze and thaw and HSP-70 expression was detected using western blotting assay. For this purpose, equal amounts of lysate proteins (extracted from 5x10\(^5\) WEHI-164 cells) were fractionated into 10% SDS-PAGE gels and transferred into a polyvinylidene fluoride (PVDF) membrane. After being washed, the membrane was blocked with 5% skim milk at room temperature for 1h and incubated with the mouse monoclonal antibody against HSP-70 (1:1000 R&D systems) at 4°C overnight. After washing, the membrane was incubated with horseradish peroxidase-conjugated anti-mouse antibody (1:1000, Sigma) at room temperature for 1h. Finally, the immune-reactive bands were detected by ECL (Amersham Pharmacia Biotech Inc, USA) (Figure 1). After the confirmation of HSP production, lysates were injected twice a week, intratumorally and subcutaneously. Thalidomide was solved in DMSO/PBS solvent according to the manufacturer’s recommendations. The lyophilized Bifidobacteria were solved in sterile PBS for injection. The bacterial suspension was prepared in a ratio of 1:10. IMOD was purchased from Pars Roos Company, Tehran-Iran and diluted in a ratio of 1:10 with 5% dextrose.\textsuperscript{23}

<table>
<thead>
<tr>
<th>Treatments</th>
<th>BCG</th>
<th>HSP</th>
<th>Thalid.</th>
<th>Bifidobacter.</th>
<th>IMOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1- Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2- BCG</td>
<td>200 µl</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3- HSP</td>
<td>-</td>
<td>200 µl</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4- Thalidomide</td>
<td>-</td>
<td>-</td>
<td>200 µl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5- Bifidobacterium</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>200 µl</td>
<td>-</td>
</tr>
<tr>
<td>6- IMOD</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>200 µl</td>
</tr>
<tr>
<td>7- BCG+ Thalidomide</td>
<td>200 µl</td>
<td>-</td>
<td>100 µl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8- IMOD+ Bifidobacterium</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100 µl</td>
<td>200 µl</td>
</tr>
<tr>
<td>9- IMOD+ HSP</td>
<td>-</td>
<td>100 µl</td>
<td>-</td>
<td>-</td>
<td>150 µl</td>
</tr>
<tr>
<td>10- IMOD+ Thalidomide</td>
<td>-</td>
<td>-</td>
<td>100 µl</td>
<td>-</td>
<td>200 µl</td>
</tr>
<tr>
<td>11- HSP+ BCG+ Thalidomide</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12- IMOD+ Bifidobacterium+ HSP+ BCG+ Thalidomide</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
</tbody>
</table>

### Tumors size

The size of tumors was measured weekly on special day and the volume was calculated by the following formula:

\[
\text{Tumor volume (mm}^3\text{)} = (\text{width})^2 \times (\text{length}) \times \pi/6.
\]

The survey of tumors was continued until the end of the 14\(^{th}\) week.
Effects of Some Immunomodulators on Survival and Tumor Size

**Figure 1.** HSP-70 expression of heat shocked tumor cells. Western blot analysis of HSP-70 expression in WEHI-164 cells, maintained at 37°C (2), heat treated 42°C, 60 min, (12 h recovery in 37 °C) (1).

**Statistical analysis**
One-way, two-way, or repeated measures ANOVA were used to identify significant group differences. To assess survival rates, the Kaplan-Meier model was used. Values of p < 0.05 were considered significant in all studies, and all p values were two-tailed.

**Results**
Despite the fact that, all mice were injected with the same dose of WEHI-164 cells, one mouse in each of following groups including HSP, Thalidomide, IMOD + *Bifidobacterium*, IMOD+HSP and Thalidomide+IMOD groups did not develop any fibrosarcoma tumors and excluded from the study. According to the results, tumor size differed among the treatment groups, dependent on the drugs administered. Mean sizes of tumors are also represented in Table 2. Table 3, indicates survival situation among the test groups. The tumors are also dependent on the drugs administered. Mean size of tum.

**Table 2.** Means of tumor sizes in various groups during the experiment.

<table>
<thead>
<tr>
<th>Weeks</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>191.6±112</td>
<td>475.7±23</td>
<td>593.7±49</td>
<td>698.3±47</td>
<td>931.3±98</td>
<td>1712±123</td>
<td>2680±187</td>
<td>3990±405</td>
<td>4688±308</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>BCG</td>
<td>34.3±9</td>
<td>184.6±17</td>
<td>637.6±14</td>
<td>1495.3±108</td>
<td>2594.6±180</td>
<td>3943.3±185</td>
<td>5006.6±186</td>
<td>5926±205</td>
<td>6304±189</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>HSP</td>
<td>455.6±15</td>
<td>1759.6±50</td>
<td>1126.3±94</td>
<td>1997.3±124</td>
<td>2573.3±145</td>
<td>2973±110</td>
<td>4582.6±354</td>
<td>6568.6±240</td>
<td>7812±217</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Thalidomid</td>
<td>280±34</td>
<td>469.3±42</td>
<td>610.6±84</td>
<td>769.6±94</td>
<td>1586.3±104</td>
<td>2829±131</td>
<td>3971±158</td>
<td>4191.5±284</td>
<td>5570±291</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td><em>Bifidobacterium</em></td>
<td>243.3±39</td>
<td>557.4±55</td>
<td>1069±91</td>
<td>1573±106</td>
<td>2267±187</td>
<td>1823±128</td>
<td>3324.3±204</td>
<td>4397.3±315</td>
<td>4480±301</td>
<td>4605±294</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>IMOD</td>
<td>175.6±14</td>
<td>243±12</td>
<td>537.6±45</td>
<td>909.3±54</td>
<td>1521±94</td>
<td>2398.6±170</td>
<td>3421.6±182</td>
<td>3848±204</td>
<td>4720±208</td>
<td>4156±267</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>BCG &amp; Thalidomid</td>
<td>221.0±24</td>
<td>671.6±80</td>
<td>1093.6±102</td>
<td>1423.6±134</td>
<td>1208.3±158</td>
<td>1916.3±142</td>
<td>2033.6±245</td>
<td>2073.3±214</td>
<td>2171.3±194</td>
<td>2100±218</td>
<td>2149±187</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>IMOD &amp; <em>Bifidobacterium</em></td>
<td>194.6±34</td>
<td>589.3±56</td>
<td>1218.7±80</td>
<td>1847±84</td>
<td>2974.6±107</td>
<td>5619±204</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>IMOD &amp; HSP</td>
<td>237.6±45</td>
<td>422.6±34</td>
<td>794.6±64</td>
<td>1130±108</td>
<td>2784±278</td>
<td>4512.6±207</td>
<td>5053±324</td>
<td>6332±219</td>
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<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>IMOD &amp; Thalidomid</td>
<td>353.6±67</td>
<td>979.3±85</td>
<td>1539.6±120</td>
<td>2749±6240</td>
<td>4445±290</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>BCG, HSP &amp; Thalidomid</td>
<td>148.3±12</td>
<td>203±19</td>
<td>331.6±45</td>
<td>416.3±52</td>
<td>420.1±43</td>
<td>411.3±32</td>
<td>234±24</td>
<td>286.3±27</td>
<td>294.3±20</td>
<td>218.3±22</td>
<td>206.3±17</td>
<td>172.6±15</td>
<td>156±15</td>
<td>57±14</td>
</tr>
<tr>
<td>BCG, HSP, Thalidomide, IMOD &amp; <em>Bifidobacterium</em></td>
<td>215.6±27</td>
<td>515.6±35</td>
<td>666.3±74</td>
<td>987.3±86</td>
<td>996.3±102</td>
<td>1397±94</td>
<td>2252.6±145</td>
<td>2703.3±120</td>
<td>3166.6±124</td>
<td>3088±118</td>
<td>2700±126</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

* Significant reduction in tumor size compared to control group.
# not compared because of unavailability of control group.
NA: Not Assessed.

Figure 2. Comparison of survival in BCG, HSP and Thalidomide (combination therapy) and control groups. Despite control group, most of the mice in BCG, HSP and Thalidomide group went into complete remission.
Discussion
In most treatments used currently, only certain aspects of cancer, which overcome the immune system is considered. Whereas, a cancer evasion from immune system is happened by several mechanisms like secreting angiogenic factors, reducing cytokine secretion, surface antigen secretion. In this study, five immunotherapy approaches, alone and in combination with each other were applied to test groups and the outcomes were compared with those of the control group. Among test groups, BCG + HSP + Thalidomide demonstrated the most satisfactory results. Five out of the eight mice in this group survived and went into complete remission. Furthermore, the inhibition of the tumor size from 7th week was obvious in this group. The positive impacts seem to be due to individual and interactive features of the treatments. For instance, the chemical structure of the CWS of Mycobacterium bovis BCG strain (BCG-CW) has biological activities, especially immune-adjuvant and antitumor activities. Because of the availability and easy usage, it is considered as an immunotherapeutic tool. BCG also increases the expression of CD80, CD83, CD86, MHC, IL-12 as well as TNF-α secretion from the dendritic cells. On the other hand, heat shock proteins (HSPs) are employed to inhibit the aggregation of other damaged proteins and assist with refolding and reactivation of the damaged proteins under stress conditions. Several studies have investigated HSPs as tumor rejection antigens. In some cases, exogenous stress proteins seem to act as vehicles for the delivery of antigens to professional antigen-presenting cells (APC), consequently in cross-priming. It has been proved that HSPs can cooperate with various receptors on APCs resulting in HSP-peptide uptake, antigen cross priming, secretion of pro-inflammatory cytokines and maturation of the dendritic cells. Accordingly, the association of some stress proteins seems to induce both innate and adaptive immunity several folds. Some HSPs from tumor cells have been discovered to be able to start particular immunity against the tumor, through combination of the entire antigens and delivering them to APCs on MHC-I to activate tumor-antigen-specific CTL. The interaction of HSPs with APCs results in the secretion of pro-inflammatory cytokines like Interferon-α by dendritic cells (DCs) and macrophages. Moreover, presence of these molecules leads to decreased tumor size and increased survival rate. Angiogenic factor secretion from the tumor cells is the most important way to develop tumors in body and metastatic invasion to organs far from the primary tumor. Cancer cells by producing angiogenic factors such as VEGF, bFGF and TGF-β cause new vessels and expand the existing vessels. Thalidomide is one of the preventative factors that prohibit the formation of new vascular network from existing blood vessels. One of the goals of the angiogenesis inhibition is reducing blood flow to the tumor in order to limit nutrients and oxygen supplies for malignant cells.

Despite the fact that each of these drugs could individually result in anti-tumor effects, none of them was able to inhibit the tumor growth or improve the survival rate solely. In groups with only one treatment, since a few mechanisms of tumor repression were involved, anti-tumor impacts was not significant. In binary group BCG + Thalidomide, the average survival time of the mice was higher than individual groups and the mean size of tumors was lower than monotherapeutic and control groups. In the contrary, drugs in combination therapy especially with more than two drugs had the ability to inhibit the tumor development via different mechanisms.

Conclusion
In trilogy group BCG + HSP + Thalidomide, the anti-tumor effects were more than the other groups. This
could be due to the synergistic effects of drugs and involvement of multiple inhibitory mechanisms including angiogenesis repression, improved tumor antigens presentation and the immune system fortification. Complementary studies to discover the mechanisms of action are encouraged.

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Conflict of interest
The authors report no conflicts of interest.

References