Flax seed oil inhibits metastatic melanoma and reduces lung tumor formation in mice

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Summary

Purpose: Cancer is one of the leading causes of mortality worldwide. Tumor cells circulating in the blood evidence the migration of tumor from the site of origin to another site leading to the formation of new metastatic lesion and establishment of metastatic tumors. In the present study, cultured metastatic tumor cells were injected into the C57BL/6 mice through tail-vein injection (TVI) and the anti-metastatic properties of flax seed oil (FSO) were evaluated.

Methods: Pre-administration of FSO in a dose of 0.3 ml/mice/day was performed for 15 days. On 16th and 21st day, mice were challenged with 2x10⁵/100 µl murine B10 melanoma (YAC-1 suspended in sterile PBS) cells and continued with FSO administration until the end of the experimental period (40 days) to assess the effect on lung metastasis. At the end of experimental period, mice were sacrificed for plasma and lung tissue samples for biochemical and marker studies. Activities of marker enzymes (AST and ALT), enzymic antioxidants (superoxide dismutase/SOD and catalase/CAT), levels of non-enzymic antioxidants (glutathione), oxidation-stress marker (malondialdehyde/MDA) and cytokines (TNF-alpha, IL-2, IFN-gamma and MMP-9) were assessed.

Results: Elevated marker enzyme activities in serum and altered enzymic and non-enzymic antioxidants were recovered during FSO treatment. Altered metastatic markers levels favoring the formation of metastatic lesions were observed in the disease group. FSO administration re-altered the levels of these markers in the treatment group contributing to better control of metastasis development.

Conclusion: These results support the protective role of FSO against lung cancer metastasis.

Key words: cytokines, enzymic antioxidants, flax seed oil, marker enzymes, melanoma, metastasis

Introduction

A most leading cause for deaths worldwide is due to different types of cancer. In end-stage disease, cancer cells migrate from the primary site to other organs through circulation creating metastatic lesions leading to the formation of secondary tumors. This process includes primary tumor dissociation, migration, invasion, adhesion and proliferation at a distant site [1]. Invasion of tumor cells to the extracellular matrix (ECM) is an important event in the process of metastasis [2]. Inhibitors and modulators of cell proliferation and/or differentiation have been efficiently used as antitumor agents [3], which may also lead to toxicities. Surgical removal of tumors from the primary site could also benefit the patients providing long-term disease-free survival, but the presence of circulating tumor cells contributes to the development of metastasis in secondary sites [4]. Liver macrophages and Kupffer cells, residing in the sinusoids of the liver were responsible to clear-off invading bacteria, senescent blood cells, foreign cells etc, found in the blood circulation
and were the first-line of defense against circulating tumor cells [5]. However, they were not very effective in clearing circulating tumor cells without the presence of monoclonal antibodies and thus, co-localization of Kupffer cells with tumor cells were observed in a previous study [6].

Extremely dense vascular surface area is an unique feature that makes the lungs as a most common microenvironment suitable for the development of metastatic outgrowth [7]. It has been proved that the macrophages and hematopoietic progenitor cells prepare the niche in pre-metastatic organs to enhance the metastasis and secondary tumor growth [8,9]. Initiation of metastasis involves mobilization of hematopoietic progenitor cells from bone marrow to the target niche and colonization in response to factors secreted by primary tumors [10,11]. Colonized hematopoietic progenitor cells at the site of metastatic niche express VEGFR1, CD133, CD34 and c-kit to precondition the microenvironment prior to the dissemination of tumor cells from the primary site [12,13].

Approximately, more than a million of cancer cells are shed into the blood stream every day during the metastatic process of a primary tumor [14]. Although most of these cells are detected in the blood of end-stage cancer patients [15], they fail to form metastasis due to rapid destruction [16]. Furthermore, antibodies could also play a role in lung metastasis. In a recent study, enhancement of lung colonization of B16-F1 cells was reported during administration of an antimelanoma IgM antibody [17]. With this scientific knowledge, inhibition of metastatic processes could be a leading approach to control cancer metastasis [18].

Phenolic compounds containing antioxidants were used in the prevention or control of deleterious health effects and are therefore very useful for the reduction of risk of chronic diseases [19] including various cancers and inflammatory disorders [20]. Antioxidants containing dietary factors have been proposed to prevent and treat many cancers, in particular melanoma [21]. Flax seed is well known for its antioxidant, glycemic and lipid lowering effects due to its components linolenic acid, lignans and its degradation products [22]. Flax seed products were reported to benefit in lipid profile changes in animal studies and human clinical studies [23]. Flax seed contains high amounts of soluble fibres or mucilage [24], high amounts of α-linolenic acid [25], plant lignin secoisolariciresinol diglucoside [26] and small amounts of other lignans, namely pi noenosinol, lariciresinol and matairesinol [27]. With this information, the present work has been designed to study the antimetastatic effects of FSO via TVI in a mouse metastatic melanoma model.

**Methods**

**Cell culture**

Murine B16 melanoma YAC-1 cells were obtained from American Type Culture Collection (ATCC), VA 20110, USA, and were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) in an atmosphere of 5% CO₂ and 37°C. After confluence, cells were harvested using trypsin digestion and re-suspended in phosphate-buffered saline (PBS) for further use. TVI to the experimental mice was done immediately, to avoid loss of viability of re-suspended cells.

**Experimental animals**

Male mice of C57BL/6 strain, weighing 20±2 g and aged 8 weeks were purchased from Vital River Laboratory Animal Technology Company Ltd, Beijing, China, and were acclimatized before initiating the experimentation. Animals were provided with 12:12 hrs of light:dark cycles with normal pelleted feed and purified drinking water ad libitum. Animal Care and Usage Committee’s approval was obtained earlier and the prescribed study protocol was followed for handling laboratory animal experimentations of the present study.

**Dosage schedule and in vivo grouping**

Animals were grouped into 4 subgroups of 6 animals each. Group I served as the normal control group, administered only PBS. Group II animals served as drug controls with administration of FSO at a dose of 0.3 ml/mice/day from day 0 to day 40 (until the end of the experimental period). This dose of FSO was confirmed that could provide a flax seed content of 1.5% to the body weight. Group III animals were administered cultured murine B10 melanoma cells at a dose of 2x10⁵ cells in 100 μl of sterile PBS on day 16 and day 21 of the experimental period via TVI. Group IV animals were administered FSO as in group II and TVI as in group III until the end of the experimental period. At the end of the experimental period, all the animals were sacrificed after overnight 12-hr fasting. Plasma samples were collected for different assays and immediately stored at -20°C until further use. Collected lung tissues were immediately rinsed with ice-cold normal saline and were processed for analyses.

**Marker enzyme estimation**

Measurement of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the plasma and tissue was done using UV-VIS1 spectrophotometer (Thermo Scientific, Hudson, USA).
Enzymic antioxidants assays

SOD and CAT were assayed using standard protocols according to the manufacturer’s instructions from Calbiochem Inc., CA, USA.

Glutathione assay

Reduced glutathione was determined by the method of Ellman (1959) in plasma and lung tissue [28]. The amount of reduced glutathione was expressed as mg/dl of plasma or mg/g of lung tissue.

Oxidative stress marker assay

Oxidative stress marker and lipid peroxidation (LPO) was determined in plasma and lungs by the thiobarbituric acid reaction as described by Ohkawa et al. in 1979 [29]. The level of lipid peroxides was expressed as millimoles TBARS/ml of plasma or nmoles TBARS/g of tissue.

Cytokine assay

Cytokine levels such as tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2), interferon-gamma (IFN-γ) and matrix metalloproteinase-9 (MMP-9) were determined according to the manufacturer’s instructions using ELISA kit procured from Diaclone SAS, France and R&D Systems Inc.

Statistics

All results were expressed as means±SD in each group. The level of significant difference between groups (p<0.05) was determined using one-way ANOVA test. Comparisons were done between control vs FSO, control vs TVI, and TVI vs FSO+TVI groups.

Results

Body weight changes

Body weight of the experimental animal groups was measured during the initial (day 0) and final day (day 40) of the experimental period (Figure 1). No significant difference in body weight was noticed in the experimental groups of animals on day 0. After the treatment regimen, at the end of the experimental period (day 40), body weight of the animals was measured and no significant changes were observed among groups. However, a decreasing trend (7.24%) in body weight was observed in the TVI group of animals during the experiment.

Marker enzyme assays

Marker enzymes AST and ALT were assayed in the plasma of the experimental animals (Table 1). Significantly increased activities were noted, accounting for 41.76% and 24.05% in the plasma for AST and ALT, respectively. All these alterations were normalized during FSO treatment.

Enzymic antioxidants assays

Enzymic antioxidants include SOD and CAT that defend against oxidative stress. In Table 2, activities of enzymic antioxidants were significantly decreased by 31.5% and 30.33% for SOD and CAT, respectively, in the lungs of TVI administered mice. During FSO treatment, these abnormalities were significantly restored to normal. No significant difference was noticed in the FSO-alone administered group.

Glutathione assay

GSH is an indicator for the redox status and healthiness. In the present study, a decrease of 10.83% and 26.79% in GSH levels in plasma and lungs, respectively, were observed during TVI (Table 3). Significantly normalized level was noticed

<table>
<thead>
<tr>
<th>Specification</th>
<th>Control</th>
<th>FSO</th>
<th>TVI</th>
<th>FSO+TVI</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (U/L)</td>
<td>18.2±2.5</td>
<td>16.7±0.9</td>
<td>25.8±2.1*</td>
<td>22.5±1.8*</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>15.8±1.3</td>
<td>14.1±1.8</td>
<td>19.6±1.4*</td>
<td>17.4±1.4*</td>
</tr>
</tbody>
</table>

Results are means±SD for 6 mice. Comparisons: control with FSO, control with TVI and TVI with FSO+TVI. *p ≤0.05 For abbreviations see text.
in the lungs during FSO treatment. Conversely, no significant normalization was noticed in the plasma GSH level in the FSO-treated group.

**Oxidative stress marker assay**

LPO, assessed by the levels thiobarbituric acid reactive substance (malondialdehyde) in the plasma and lung tissue, was performed to quantify the oxidative stress produced by TVI in the experimental group of animals (Table 3). Significant increase in the levels of LPO was noticed in the lung tissue (50.8%). This alteration was normalized during FSO treatment. Conversely, no significant alterations in the level of plasma LPO were noticed in all of these groups of animals.

**Cytokine assay**

TNF-α, IL-2, IFN-γ and MMP-9 are the inflammatory markers that depict the progression of disease and the status of the immune response of the host. Abnormal alterations in the levels of these cytokines were noticed during TVI (Figure 2-4). Significant reversal of these alterations during FSO treatment indicated the protective effect of FSO against the metastatic tumor development.

**Discussion**

Animal models for metastatic tumor formation were established by intravenous injection of highly metastatic, in vitro cultured tumor cells. Extracellular matrix invasion and epithelial-mesenchymal transition (EMT) are the most important processes during metastasis [30,31].

Cellular mechanism of toxicity produced by TVI could be the reason for the marker enzymes’ elevation in plasma and decrease in liver [32,33]. Normalization of these marker enzymes during FSO treatment could be beneficial due to the protective role of the drug. Oxidative injury due to the generation of reactive oxygen species (ROS) plays an important role in many life-style related diseases such as cancer, arthritis, neurodegenerative and coronary heart diseases etc [34]. ROS has been suggested to play an important role in inflammation [35]. Inflammation can essentially accelerate and help neoplastic progression [36]. MDA is one of the important indicators for oxidative damage, which is produced from the peroxidized polyunsaturated fatty acids [37]. According to the present report, it could be concluded that FSO inhibited the excess production of ROS and reduced the incidence of metastatic events in the lungs.

SOD and CAT are the most important enzymic antioxidants present in a cell [38]. Respiratory system is endowed with enzymic antioxidant defense enzymes to maintain proper redox balance [39]. Protection of macromolecules against the oxidative damage is exclusively done by antioxidants enzymes in the cell [40]. Disturbances in the balance between ROS and antioxidant defense were depicted and FSO played an efficient role in the restoration of this imbalance.

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**Table 2. Activities of SOD and CAT in the lungs of the experimental groups of mice**

<table>
<thead>
<tr>
<th>Specification</th>
<th>Control</th>
<th>FSO</th>
<th>TVI</th>
<th>FSO+TVI</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD (U/mg protein)</td>
<td>8.35±0.6</td>
<td>8.62±0.8</td>
<td>5.72±0.5*</td>
<td>7.36±0.5*</td>
</tr>
<tr>
<td>CAT (U/mg protein)</td>
<td>1.22±0.1</td>
<td>1.18±0.1</td>
<td>0.85±0.09*</td>
<td>0.96±0.06*</td>
</tr>
</tbody>
</table>

Results are means±SD for 6 mice. Comparisons: control with FSO, control with TVI and TVI with FSO+TVI. *p ≤0.05 For abbreviations see text.

**Table 3. Levels of plasma and lungs LPO and GSH in the experimental groups of mice**

<table>
<thead>
<tr>
<th>Specification</th>
<th>Control</th>
<th>FSO</th>
<th>TVI</th>
<th>FSO+TVI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma LPO (mM TBARS/mL)</td>
<td>9.5±0.8</td>
<td>9.2±0.7</td>
<td>10.3±1.2</td>
<td>9.6±0.9</td>
</tr>
<tr>
<td>Plasma GSH (mg/dL)</td>
<td>1.57±0.08</td>
<td>1.49±0.08</td>
<td>1.4±0.12*</td>
<td>1.39±0.11</td>
</tr>
<tr>
<td>Lungs LPO (nM TBARS/g tissue)</td>
<td>503.7±41.4</td>
<td>489.5±39.5</td>
<td>759.6±71.4*</td>
<td>683.5±52.5*</td>
</tr>
<tr>
<td>Lungs GSH (mg/g tissue)</td>
<td>0.56±0.03</td>
<td>0.55±0.02</td>
<td>0.41±0.03*</td>
<td>0.48±0.05*</td>
</tr>
</tbody>
</table>

Results are means±SD for 6 mice. Comparisons: control with FSO, control with TVI and TVI with FSO+TVI. *p ≤0.05 For abbreviations see text.
TNF-α is a potent regulator of the immune system [41]. Higher expression of VEGF, M-CSF, IL-6 and TNF-α were reported in the serum of B16 tumor-bearing mice [7]. Upregulation of TNF-α along with other cytokines may enhance tumor metastasis [42,43]. Higher levels of circulating TNF-α were reported to be associated with lung cancer [44]. Intra-lesion therapy of IL-2 may be useful for the treatment of metastatic or recurrent melanoma [45]. Due to this increase in IL-2 level, it would be beneficial for the development of immune response against the tumor in vivo. IL-2 could also induce the macrophage production of TNF-α [46], which might have contributed to the elevated level in the plasma of TVI group. In a recent study, it has been proved that FSO was able to decrease the levels of TNF-α [47]. Consistent with these reports, decreased level of TNF-α in the treatment group of our study might be responsible for the reduced formation of metastatic tumor in the lungs. IFN-γ is one among the important inflammatory cytokines that triggers damage during various stresses and its depletion could result in marked reduction in injury and inflammation [48]. In our study, treatment with FSO decreased the elevated level of IFN-γ and thereby suppressed the inflammatory responses that is in-line with the previous reports [49]. Significant reversal of the pathological alterations during FSO treatment indicates the treatment efficiency and could help to trigger the response of the host against the formation of metastatic tumor.

During the metastatic processes, ECM proteins and basement membranes were degraded by MMPs, and specifically MMP-9 overexpression in various cancer cells helps degrade type-IV collagen [50]. In a study, overexpression of MMP-9 was associated with tumor cell progression and metastasis [51]. Tumor cells secrete growth factors and cytokines leading to MMPs production that ultimately increase their invasiveness. Inhibition or decreased level of MMP expression could possibly prevent cancer metastasis [52]. Downregulation of MMP-2 and MMP-9 through the inhibition of ERK activation has been demonstrated to inhibit tumor metastasis in human leukemia cells [53]. Another report has shown that downregulation and decreased activities of MMPs could lower the invasive and metastatic ability of cancer cells [54]. Consistent with these findings, significantly decreased MMP-9 levels could block the invasion/extravasion of cells leading to decreased extravasive phenomena of metastatic cells. However, further studies are warranted to depict the antimetastatic role of FSO in vivo.
References

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Flax seed oil in melanoma and lung metastasis


