Effect of all-trans retinoic acid on growth of xenograft tumor and its metastasis in nude mice

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Keywords: all-trans retinoic acid xenograft tumor metastasis nude mouse gastric cancer cell

Objective To study the effect of all-trans retinoic acid on growth of xenograft tumor and its metastasis in nude mice.

Methods Human gastric cancer BGC-823 and MKN-45 cells were inoculated into spleen subcapsule of nude mice, respectively. The nude mice were subsequently administered with all-trans retinoic acid every other day. Food consuming and body weight of nude mice were measured weekly. Six weeks later, the nude mice were killed. Xenograft tumors in spleen and metastatic tumors in liver were pathologically examined. Microvessel density in the tumors was detected immunohistochemically, and serum carcinoembryonic antigen was measured by radioimmunoassay.

Results After the nude mice were fed with all-trans retinoic acid, the growth of splenic tumor and its liver metastasis were inhibited and the metastatic rates decreased by 50% (BGC-823) and 33.3% (MKN-45), respectively. The microvessel density in splenic and hepatic tumors reduced by 28.58% and 35.47% (BGC-823), 19.45% and 14.52% (MKN-45), respectively. The concentration of carcinoembryonic antigen decreased by 50.24% (BGC-823) and 48.10% (MKN-45).

Conclusion All-trans retinoic acid may effectively inhibit the growth of xenograft tumor in spleen and its metastasis to liver in nude mice, which can be corroborated by the decrease of carcinoembryonic antigen and microvessel density.

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Retinoic acid displays a wide range of biological activities on cellular differentiation, proliferation, vision, immunity and malignant transformation, and has been shown to suppress carcinogenesis in experimental animals. It also exhibits different degrees of efficacy in chemoprevention and chemotherapy of many types of malignant tumors. The use of all-trans retinoic acid has been found to suppress acute promyelocytic leukemia dramatically in patients, 3 and also has high response rates in patients with cervical cancer and metastatic squamous cancer of the skin. 4 Thus, retinoic acid has been considered a potent cancer chemopreventing agent.

Tumor metastasis is the leading cause of death in cancer patients. Metastasis is the process by which some tumor cells separate from the primary tumor, travel to distant site via the circulatory system, and establish the secondary tumors. Recently, it has been reported that retinoic acid is effective in inhibiting metastatic model in vitro in some steps, including cell adhesion to amnion, degradation of extracellular matrix, formation of cytoskeleton, and so on. 5, 6 In this study, the effect of all-trans retinoic acid on the growth of xenograft tumor and its metastasis in nude mice were investigated. Its effect of all-trans retinoic acid on the microvessel density and carcinoembryonic antigen in vivo was analyzed. Our data demonstrate that feeding with all-trans retinoic acid may suppress the growth of splenic xenograft tumors and their metastasis from spleen to liver. All-trans retinoic acid may contribute to the regulation of carcinoembryonic antigen and inhibition of angiogenesis, which are associated with metastasis and development of tumor.

METHODS

Cell lines

Human gastric cancer cell line BGC-823 was obtained from the Institute of Cell Biology, Shanghai, China.

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MKN-45 cell line from Japan was kept by the Institute of Cell Biology, Shanghai, China. The two cell lines were maintained in RPMI-1640 medium supplemented with 10% FCS, 1 mmol/L glutamine, and 100 U/ml penicillin.

**Athymic nude mice**

Athymic nude mice were obtained from the Cancer Research Center of Xiamen University, housed in a laminar flow under sterilized condition with the temperature maintained at 28°C and light for 12 hours and dark for 12 hours. The mice were fed with autoclaved mouse chow.

**Inoculation of nude mice with cancer cells**

Cells were trypsinized, washed twice with ice-cold phosphate buffered saline. The inoculation dosage was 0.05 ml of cell suspension at the concentration of 2.5×10⁶ cell/ml per mouse.

Before inoculation, each nude mouse was injected with 0.1 ml of 0.5% CCl₄, and 24 hours later, anesthetized with sodium pentothal and subjected to routine surgical operations. Cells were inoculated into spleen subcapsule. The inoculated nude mice were randomly separated into experimental group (6 mice for one group), each of them was orally perfused with 0.7 mg all-trans retinoic acid every other day (all-trans retinoic acid was dissolved in absolute ethanol and DMSO with a ratio of 1:1 under subdued light), and control group (6 mice), which was administered with the corresponding solvent without all-trans retinoic acid. Food consuming and body weight of nude mice were measured weekly. Six weeks later, the nude mice were sacrificed and tumors formed in the spleen and liver were removed, weighed, fixed, and embedded.

**Immunohistochemistry**

Sections from tumor were deparaffinised. Endogenous peroxidase was blocked by 0.3% hydrogen peroxide in methanol for 30 minutes. After washed with phosphate buffered saline, sections were incubated with 10% normal goat serum in phosphate buffered saline for 20 minutes at room temperature to block non-specific binding of the second antibody, then incubated overnight at 4°C with rabbit anti-human factor-VIII-related-antigen antibody (Zymed) 1:100 in phosphate buffered saline containing 1% bovine serum albumin. Rinsed three times in phosphate buffered saline, sections were treated with biotinylated anti-rabbit immunoglobulin for 1 hour at room temperature, then washed again and reacted with streptavidin-biotin system using 0.04%, 3,3′-diaminobenzidine tetrahydrochloride for 1 minute as chromogen. Positive control was also substituted for the primary antibody.

**Carcinoembryonic antigen assay**

Blood was collected from ophthalmic artery before nude mice were sacrificed. After incubation of serum with anti-carcinoembryonic antigen solution at 37°C for 2 hours, 125I-carcinoembryonic antigen (Western Biotechnique Institute, Beijing, China) was added, overnight at 4°C, then treated with separating agent for 15 minutes at room temperature, and centrifugated at 2000 r/min for 10 minutes. The supernatant was discarded, and the sediment was measured for its radioactivity.

**Statistical analysis**

Tukey's procedure was applied to estimate the difference among groups. Statistical significance was defined as P < 0.05.

**RESULTS**

**Effect of all-trans retinoic acid on xenograft tumor in spleen and hepatic metastasis**

When cells were inoculated into spleen subcapsule of nude mice for 6 weeks, xenograft tumors in the spleen were found in all of animals, which grew single, grey-white, unclear in border, and difficult to be separated. The number of spleen tumors in the experimental group was less than that in the control group and the mean weight of the spleen in the experimental group was significantly lighter (Table 1). The metastatic tumors formed in the liver were observed in both experimental and control groups, which were multiple, noncapsuled and mostly located superficially in the liver. In the experimental group, however, by comparison with the control the occurrence of metastatic tumors were obviously decreased by 50% (BGC-823) and 50% (MKN-45), respectively, the number of liver metastatic tumors reduced from 107 to 38 (BGC-823) and from 40 to 10 (MKN-45), respectively, and the mean weight of the liver was also obviously lighter (Table 1).

**Table 1. Effect of all-trans retinoic acid on xenograft tumors and metastatic tumors in nude mice**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Rate of tumor in spleen (%)</th>
<th>No. of tumor in spleen</th>
<th>Mean weight of tumor in spleen (g)</th>
<th>Rate of tumor in liver (%)</th>
<th>No. of tumor in liver</th>
<th>Mean weight of tumor in liver (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BGC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100 (6/6)</td>
<td>18</td>
<td>1.27±0.65</td>
<td>100 (6/6)</td>
<td>107</td>
<td>2.79±1.24</td>
</tr>
<tr>
<td>Experimental</td>
<td>100 (6/6)</td>
<td>8</td>
<td>0.58±0.37</td>
<td>50 (3/6)</td>
<td>38</td>
<td>1.43±0.67</td>
</tr>
<tr>
<td>MKN</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100 (5/5)</td>
<td>10</td>
<td>0.77±0.33</td>
<td>100 (5/5)</td>
<td>40</td>
<td>2.12±0.39</td>
</tr>
<tr>
<td>Experimental</td>
<td>100 (6/6)</td>
<td>6</td>
<td>0.42±0.16</td>
<td>50 (3/6)</td>
<td>10</td>
<td>1.52±0.50</td>
</tr>
</tbody>
</table>

* P < 0.05; ** P < 0.01; * One of the MKN-45 inoculated mice in the control group died at the 40th day because of the development of tumor.

**Effect of all-trans retinoic acid on microvessel formation**

Immunohistochemical analysis indicated a strong staining for microvessel in all of the tumor sections, including that of the spleen and liver. The microvessels of
hepatic BGC-823 tumors was shown in Fig. 1; the other was the same. The microvessel density in the experimental group was lower than that in the control group. The number of microvessels differed obviously between the two groups, and the inhibition rate of microvessel density in the experimental group was 28.58% (BGC-823), 19.54% (MKN-45) in splenic tumors, and 35.47% (BGC-823), 14.52% (MKN-45) in hepatic tumors, respectively (Table 2).

Table 2. The effect of all-trans retinoic acid on microvessel density.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumor</th>
<th>Microvessel density</th>
<th>Inhibitory rate of MVD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>Experimental</td>
</tr>
<tr>
<td>BGC</td>
<td>Spleen</td>
<td>12.70±2.74</td>
<td>9.07±3.04</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>11.56±2.91</td>
<td>7.46±2.38</td>
</tr>
<tr>
<td>MKN</td>
<td>Spleen</td>
<td>8.70±2.11</td>
<td>7.00±1.89</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>10.40±2.55</td>
<td>8.89±2.23</td>
</tr>
</tbody>
</table>

*p < 0.05

The microvessel density in the experimental group was lower than that in the control group. The number of microvessels differed obviously between the two groups, and the inhibition rate of microvessel density in the experimental group was 28.58% (BGC-823), 19.54% (MKN-45) in splenic tumors, and 35.47% (BGC-823), 14.52% (MKN-45) in hepatic tumors, respectively (Table 2).

Table 3. The effect of all-trans retinoic acid on carcinoembryonic acid in nude mice.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Control (ng/ml)</th>
<th>Experimental (ng/ml)</th>
<th>Inhibitory rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BGC</td>
<td>2.07±2.01</td>
<td>1.03±0.98</td>
<td>50.24</td>
</tr>
<tr>
<td>MKN</td>
<td>2.37±1.54</td>
<td>1.23±0.97</td>
<td>48.10</td>
</tr>
</tbody>
</table>

Side-effects of all-trans retinoic acid on nude mice

The side-effects of all-trans retinoic acid on food-intake, body weight and bone structure were examined during all-trans retinoic acid feeding. In the early 1—2 weeks, the food-intake and body weight of nude mice decreased in the experimental group (Fig. 2), accompanied by a decrease in carcinoembryonic acid. However, the concentration of carcinoembryonic acid decreased by 50.24% (BGC-823) and 48.10% (MKN-45) in the experimental group as compared with that of the control (Table 3).
with other side-effects, such as embubesence of skin and desquamation, and sluggish motion. However, these effects disappeared later. In addition, no bone fracture was found in both experimental and control groups as confirmed by X-ray scanning.

**DISCUSSION**

In this animal model, nude mice were first injected with CCl4 to make their livers impaired; some growth factors were secreted in the course of liver regeneration and they could facilitate tumor cell proliferation and mass formation in the liver. Autopsy and statistical results showed that xenograft tumors of the inoculated BGC-823 and 6 MKN-45 cells formed first in the spleen, then metastasized to the liver. However, the feeding of all-trans retinoic acid for 6 weeks apparently caused the suppression on the growth of xenograft tumors in the spleen and metastatic tumors in the liver. Moreover, tumor metastasis from the spleen to the liver was inhibited in the experimental group, in which 50% individuals with splenic tumor developed the metastasized hepatic tumor, as compared with the 100% metastasis in the control group. These results indicate that all-trans retinoic acid not only inhibits the growth of cancer cells in vitro, including cancer cells of breast, lung and stomach, but also suppresses the growth of tumor in vivo, suggesting that all-trans retinoic acid is a promising agent for tumor prevention and treatment in vivo.

Carcinoembryonic antigen belongs to immunoglobulin superfamily of intercellular adhesive molecule. It participates in intercellular interaction and is associated with tumor metastasis. The concentration of serum carcinoembryonic antigen is related to the postoperative survival period of cancer patients. Generally, carcinoembryonic antigen is carried through the body fluid circulation to the liver and degraded and eliminated. Intravenous injection of carcinoembryonic antigen into nude mice and getting saturated in liver cells will obviously increase the number of metastatic tumor colonies. Our observation that the concentration of serum carcinoembryonic antigen was lower in the experimental group suggests that all-trans retinoic acid may modulate carcinoembryonic antigen secretion, resulting in the inhibition of tumor metastasis from the spleen to the liver.

Angiogenesis facilitates the infiltration of reparative cells, enhances the delivery of oxygen, nutrients, growth factors and cytokines, and allows the removal of waste products. Angiogenesis has been shown to be critical for tumor growth, because primary metastatic tumors will not grow beyond 2 mm in diameter without an enhanced vasculature, and the newly formed blood vessels are more penetrative to tumor mass and may contribute to metastases. A negative correlation was observed between patient survival and the microvessel density on several tumor types, including those of breast, prostate, esophagus, and melanoma. Thus, the microvessel density is valuable in guiding further therapy and anti-angiogenesis has been conducted as one of the major methods for tumor treatment. We also found that all-trans retinoic acid could affect the angiogenesis of splenic xenograft tumor and hepatic metastasized tumor in nude mice (Fig. 1, Table 2), which is consistent with that observed in breast and vagina cancer cells in vitro. It is indicated that all-trans retinoic acid may be one of the valuable candidates clinically used for anti-angiogenesis.

Side-effects such as weight loss, bone fracture and less food-intake were often observed in animals treated by some anti-cancer agents including all-trans retinoic acid. Weight loss may be attributed to the gastrointestinal irritation, which results in the decrement of food-intake. The fact that retinoic acid induces bone fracture may be due to its activation of bone remodeling processes, in which longitudinal bone growth is greater than circumferential bone growth. We found that all-trans retinoic acid feeding led to some of side effects in nude mice, such as embubesence of skin and desquamation, and sluggish motion in early stage. In addition, no bone fracture was found in both experimental and control groups. These data suggest that the feeding dose adopted here is not only effective but also rather safe, and the animal model applied in this study is useful for evaluating the action of anti-cancer agent in vivo.

**REFERENCES**

Clinical observation of oral adenosine triphosphate in treating rhinitis medicamentosa

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Objective To observe the effect on the patients with rhinitis medicamentosa treated by oral adenosine triphosphate (ATP).

Methods There were 150 cases with rhinitis medicamentosa who had prolonged use of nasal drops of vasoconstrictors, such as privine for one month to more than ten years. All cases were due to chronic tonsillitis caused by bacterial hypothyroid "coryza", allergic rhinitis, deviation of nasal septum, polyp of sinuses and sinusitis, and vasoconstrictor rhinitis. Of them, 87 were males and 63 females, aged 5–53 years. After original disease treated, 103 patients were given oral ATP tablets 40 mg three times a day for three days or one week at most, and then compared with the rest 47 patients (controls) treated by nasal drops of dexamethazone of 0.25 mg/ml dilution for 1–2 weeks then given normal saline only. The effects on the experimental group of taking ATP and contrast group of using dexamethazone were determined subjectively by patients’ symptoms and objectively by rhinomanometer of Mastre PF-2001. The investigation lasted eight weeks.

Results Patients in the experimental group showed improvement at the beginning several hours to three days after the treatment. Rhinomanometer studies indicated the nasal resistance in 94 cases out of the 103 dropped to more than 50%, with an improved rate of 91.2%. The swollen inferior turbinate became shrunken. The color of the mucosa turned from dark purple to pink. While the time of improvement in the contrast group began at least one week after the treatment. The obvious improvement was shown after 2–3 weeks, and disappearance of symptoms after 4–8 weeks. The improvement rate was 81%.

Conclusion The symptoms of rhinitis medicamentosa can be quickly and effectively improved by taking ATP.