The Effect of Meloxicam, a Selective COX-2 Inhibitor, on the Microvasculature of Small Metastatic Liver Tumors in Rats

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Background: COX-2 is involved in tumor angiogenesis and modulation of the production of angiogenic factors by colorectal carcinoma cells. It has been shown that COX-2 inhibitors have inhibitory activities against various types of tumor, including colorectal carcinoma. In this study, we investigated the tumor vessels of small metastatic liver tumors in rats and the effect of meloxicam, a selective COX-2 inhibitor, on their growth and microvasculature.

Methods: The metastatic liver tumors were produced by intraportal inoculation of RCN-H4 cells in male F344/DuCrj rats (n = 40). The microvasculature was examined by scanning electron microscopy and stereomicroscopy. Microvascular casts were produced by perfusion via the abdominal aorta 14 days after tumor inoculation. Four groups (control, groups 1–3) of rats were treated with meloxicam 0, 0.6, 1.0 and 3.0 mg/kg/day, respectively, by oral gavage 5 days/week for two weeks from the day of inoculation of RCN-H4 cells.

Results: The mean number of tumors was significantly decreased in groups 1–3 (5.6 ± 0.8 standard deviation, SD; 3.6 ± 1.1; and 5.5 ± 1.1, respectively) compared with control (11.2 ± 2.7; P = 0.0002, each). Meloxicam also significantly reduced the mean diameter of the tumor: 730 ± 254, 685 ± 212 and 644 ± 139 in groups 1–3, respectively, in comparison with 870 ± 276 in control (P = 0.0025, 0.0011 and <0.0001, respectively).

Conclusions: Meloxicam’s anti-angiogenic activity interferes with the growth of metastatic liver tumors. Meloxicam might have therapeutic potential for liver metastasis of colorectal carcinoma.

Key words: COX-2 – colorectal carcinoma – liver metastasis – microvasculature

INTRODUCTION

Colorectal carcinoma is widespread and frequently fatal in the West (1) and its incidence in Japan is also increasing (2). The liver is one of the major targets for colorectal carcinoma metastases and liver metastases indicate a poor prognosis. Therefore, effective therapeutic agents against liver metastasis would be of high clinical importance.

Epidemiological studies have suggested that non-steroidal anti-inflammatory drugs (NSAIDs) might reduce the risk of colorectal carcinoma (3–7) and decrease the number and size of polyps in patients with familial adenomatous polyposis (8–10). These studies imply that NSAIDs could modulate carcinogenesis and the development of colorectal carcinoma. NSAIDs are known to inhibit cyclooxygenase (COX), the key enzyme in the conversion of arachidonic acid to prostaglandins. Two isoforms of COX, COX-1 and COX-2, are recognized (11). COX-1 is constitutively expressed in many normal tissues to regulate and maintain normal cellular functions. In contrast, COX-2 is induced by several inflammatory stimuli, such as cytokines, growth factors and tumor promoters (11), and expressed in colorectal carcinoma (12,13). COX-2 is thought to influence carcinogenesis and the development of colorectal carcinoma. Recent studies on clinical materials have shown that COX-2 levels are increased in approximately 85% of colorectal carcinoma (12,14–16), indicating that it might play an important role in colon carcinogenesis (17). Tsujii et al. reported that COX-2 is involved in tumor angiogenesis and modulates the production of angiogenic factors by colon carcinoma.
The mechanism of liver metastasis of colorectal carcinoma consists of multiple steps (21). Angiogenesis is known to be essential for the growth of both primary and metastatic tumors: growth beyond 1–2 mm³ requires an adequate blood supply (22). Angiogenic activity is one of several requirements for metastasis; as neovascularization appears to be necessary for cells to escape from the primary tumor and may also be necessary for growth of a metastatic implant (23), angiogenesis is a crucial factor at the initial and final stages of the metastatic sequence (24–26).

Angiogenesis has been studied using various methods. The microvascularization of liver and lung metastatic tumors of colorectal carcinoma has been studied in rats by a resin corrosion technique and a stereomicroscope (27,28). This technique allows visualization of the three-dimensional microvasculature of metastatic liver tumors, which cannot be observed by cross-sectional techniques.

The aim of our study was to examine the effect of meloxicam, a selective COX-2 inhibitor, on the growth and microvascularization of liver metastatic tumors in rats, using scanning electron microscopy (SEM).

MATERIALS AND METHODS

COX-2 INHIBITOR

Meloxicam was suspended in 0.5% methyl cellulose. The dosing volume was kept constant (0.5 ml/rat), and the concentration was adjusted twice weekly based on body weight. Meloxicam has a COX-1 IC₅₀ of 3.27 μM and a COX-2 IC₅₀ of 0.25 μM; i.e. it is 13.1 times more inhibitory for COX-2 (29).

ANIMALS

A total of 40 male F344/DuCrj rats, 5 weeks old and weighing 100–120 g, were purchased from Japan SLC (Shizuoka, Japan). The rats were housed in polycarbonate cages on wood-chip bedding in an animal room under controlled conditions: a 12 h light/12 h dark cycle, 45 ± 5% humidity and 23 ± 1°C room temperature, with free access to tap water and standard rodent chow (CE-2, Nihon Clea, Tokyo, Japan).

TUMOR CELLS

We used the highly metastatic rat colon carcinoma cell line RCN-H4 (30). RCN-H4 is a subclone established by Inoue (31) according to Fidler’s method; it has a high potency for forming experimental liver metastatic tumors. The RIKEN Cell Bank kindly donated the RCN-H4 line, and the cells were stored at −80°C. Frozen tumor cells were washed in phosphate-buffered saline (PBS) then seeded in 10 cm culture dishes (Falcon, Lincoln Park, NJ, USA) and cultured in 10 ml RPMI 1640 medium (Sigma Chemicals, St Louis, MO, USA) containing 10% fetal bovine serum (FBS; Sigma) and 0.05% penicillin–streptomycin solution (Sigma) at 37°C, 0.5% CO₂, for 7 days until they became semi-confluent on the culture dish.

EXPERIMENTAL PROCEDURES

Rats at 6 weeks of age, after 1 week of acclimatization, were divided randomly into four groups of 10. The rats in groups 1–3 were treated with meloxicam by oral gavage at 0.6, 1.0 and 3.0 mg/kg/day, respectively, five times weekly from the day of inoculation of RCN-H4 cells to the end of the experiment for two weeks. The control group received the same volume of vehicle in the same manner. Body weight, water and food consumption were measured weekly during the experiment.

FORMATION OF METASTATIC LIVER TUMORS

Under ether anesthesia, rats underwent laparotomy through a midline abdominal incision and were inoculated intraportally with a tumor suspension containing 5 × 10⁶ RCN-H4 cells in 0.5 ml PBS using a 30-gage needle and a 1 ml syringe. A small fragment of gelatin sponge was applied to the site of inoculation to prevent bleeding and peritoneal dissemination.

PREPARATION OF VASCULAR CASTS

Microvascular casts were prepared according to the method of Murakami (32). All rats were sacrificed under ether anesthesia 2 weeks after the start of the experiment, and, for arterially perfused casts, the abdominal aorta was cannulated in a retrograde manner using an 18-gage catheter; the tip of which was placed just rostral to the renal arteries. A mixture of resin, Mercox (Oken Shoji, Tokyo, Japan) and methyl methacrylate (20 ml) was injected through the catheter until the inferior vena cava was filled with injected resin. Immediately after resin injection, each liver was removed and placed in a water bath at room temperature, and then subjected to corrosion overnight in a 20% solution of KOH. The specimen was then washed in tap water and the number of tumors appearing on the surface of the liver of each rat counted and added up in each group.

SCANNING ELECTRON MICROSCOPY

The vascular samples were trimmed into suitable blocks with a hand saw and razor blades under a stereoscope, coated with a thin layer of gold by an evaporation method, and observed under a scanning electron microscope (SEM; S-4500; Hitachi, Tokyo, Japan). The accelerating voltage was 15 kV and the working distance was 15 mm. In addition to identifying each component of the intrahepatic microvasculature, the maximum diameters of tumors were...
measured using a scale displayed in the monitor of the scanning electron microscope.

All the metastatic foci which were on the surface of liver were observed using a scanning electron microscope, and the image data of those SEM were input into the personal computer and analyzed. The analysis of the area was performed on a Macintosh computer using the public domain NIH Image program (developed at the US National Institutes of Health and available from the Internet by anonymous FTP from zippy.nimh.nih.gov or on floppy disk from the National Technical Information Service, Springfield, Virginia, part number PB95-500195GE1). The tumor vascular density (TVD) was defined as the ratio of tumor vessel area to whole tumor area.

**SCANNING STEREOMICROSCOPY**

We used the stereomicroscopy (SZX-12; Olympus Optical, Tokyo, Japan) for a diagnosis of liver metastasis. We counted the number of all the metastatic tumors on the surface of the liver by stereomicroscope. We diagnosed a part with the formation of an irregular tumor vessel as metastasis in the part which the sinusoid structure came out of.

**STATISTICAL ANALYSIS**

All data are expressed as mean ± standard deviation. The Kruskal–Wallis test analyzed the effect of COX-2 inhibitor on the diameter and TVD of metastatic tumors. The Mann–Whitney test was used to compare two groups. $P < 0.05$ was considered to be significant.

**RESULTS**

**ARTERIALLY PERFUSED CASTS**

In arterial perfusion casts from normal rats, not only the hepatic arteries and sinusoids but also the portal veins were filled with resin. The vascular beds were formed by the network of sinusoids, which were partitioned by the portal canals conducting the portal veins into individual lobules, where they converged to the central vein and, in turn, the hepatic vein (Fig. 1).

**MICROVASCULATURE OF METASTATIC TUMORS (CONTROL)**

Arterially perfused metastatic tumors appeared by SEM as a blank space surrounded by newly developed vessels (Fig. 2). The tumors were almost round and the lesions were surrounded by a normal sinusoidal pattern. The metastatic tumors appeared as a blank space with a network of newly developed vessels. The diameter of the vessels was larger than sinusoidal vessels, but irregular and with narrow parts.

**INHIBITORY EFFECT OF MELOXICAM ON LIVER METASTASIS**

Final body weights of rats were $148 \pm 11.4$, $138 \pm 20.8$, $135.2 \pm 10.6$ and $129.2 \pm 13.3$ g in the control group and groups 1–3, respectively. Slightly reduced final body weights were observed in the groups with Meloxicam but the decrease was not statistically significant ($P = 0.1634$). The mean number of tumors in each rat was significantly decreased in groups 1–3 (5.6 ± 0.8 standard deviation, SD; 3.6 ± 1.1; and 5.5 ± 1.1, respectively) compared with the control (11.2 ± 2.7; $P = 0.0002$, each). The mean diameter of liver metastatic tumors in the control group was $870 \pm 276$ (ranging from 360 to 1744) μm (Tables 1 and 2).

The number of metastatic tumors in group 1 was approximately half of that of the control group ($P = 0.0002$). The number of tumors was also significantly decreased in groups 2 and 3 compared with the control group. There were no significant differences in number of tumors between the three meloxicam-treated groups.

Meloxicam significantly reduced the diameter of metastatic tumors compared with the control ($P < 0.0001$; Fig. 3). The mean diameter of metastatic tumors in the meloxicam-treated groups was $688 \pm 209$ (ranging from 316 to 1640) μm. The mean tumor size was $734 \pm 254$ (ranging
Table 1. The effect of meloxicam on liver metastasis in rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Tumor diameter (µm)</th>
<th>P-value</th>
<th>Tumor vascular density (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>870 ± 276</td>
<td></td>
<td>32.9 ± 10.5</td>
<td></td>
</tr>
<tr>
<td>Group 1 (0.6 mg/kg meloxicam)</td>
<td>734 ± 254</td>
<td>0.0025</td>
<td>30.2 ± 8.80</td>
<td>0.1107</td>
</tr>
<tr>
<td>Group 2 (1.0 mg/kg meloxicam)</td>
<td>685 ± 212</td>
<td>0.0011</td>
<td>20.1 ± 5.30</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Group 3 (3.0 mg/kg meloxicam)</td>
<td>644 ± 139</td>
<td>&lt;0.0001</td>
<td>16.6 ± 7.10</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

P-value was estimated between control and each group.

Table 2. The number of liver metastases in each rat

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of liver metastases</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.2 ± 2.7</td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td>5.6 ± 0.8</td>
<td>0.0002</td>
</tr>
<tr>
<td>Group 2</td>
<td>3.6 ± 1.1</td>
<td>0.0002</td>
</tr>
<tr>
<td>Group 3</td>
<td>5.5 ± 1.1</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

P-value was estimated between control and each group.

from 316 to 1640) µm in group 1, 685 ± 212 (ranging from 328 to 1149) µm in group 2 and 644 ± 139 (ranging from 422 to 934) µm in group 3. However, the differences in diameters between groups 1 and 2 (P = 0.6686) and groups 2 and 3 (P = 0.2425) were not significant.

Meloxicam also reduced the TVD of metastatic tumors. The mean TVD of metastatic tumors in the control group was 32.9 ± 10.5% (ranging from 3.9 to 66.3%). The mean TVD of metastatic tumors in groups 1–3 was 30.2 ± 8.80, 20.1 ± 5.30 and 16.6 ± 7.10% (ranging from 12.7 to 51.1%, from 6.4 to 34.3% and from 1.7 to 31.4%, respectively). TVD was significantly decreased in groups 2 and 3 compared with the control (P < 0.0001, P < 0.0001), while the difference between the control and group 1 (P = 0.1107) was not significant (Fig. 4).

The morphologic characteristics of each tumor and tumor vessel in the groups treated with meloxicam were similar (group 3 shown in Fig. 5). Tumor vessels in meloxicam-treated groups were sparse and did not fill the blank space.

**DISCUSSION**

We have shown that meloxicam inhibits the growth of metastatic liver tumors. The size of the metastatic tumors decreased in the meloxicam-treated groups, as shown by SEM with a resin corrosion technique and there was
difference in TVD between control group and group 2 or 3. The number of metastatic tumors was also smaller in the meloxicam-treated groups.

A great deal of evidence supports the view that COX-2 contributes to tumorigenesis and that its inhibition might be useful in the prevention of intestinal polyposis and colorectal carcinoma (10,33,34). Sheehan et al. detected no COX-2 staining in normal colons, weak staining in normal mucosa adjacent to COX-2 positive tumors and varying degrees of COX-2 staining in tumor cells (35). We have previously reported a positive relationship between COX-2 expression and tumor growth in colorectal adenoma and adenocarcinoma (15,36,37). Specific COX-2 inhibition, either by targeted knockout of the COX-2 gene or by pharmacological intervention, has been shown to effectively decrease the growth of murine intestinal adenomas (33,38,39).

The COX-2 inhibitor celecoxib reduces the incidence and multiplicity of colon tumors in rats by approximately 93 and 97%, respectively (40), while rofecoxib attenuates the growth and metastatic potential of colorectal carcinoma in mice (39,41). As with other COX-2 inhibitors, meloxicam has been shown to inhibit the growth of HCA-7 colorectal tumors in nude mice (42), and the growth of transplantable colon adenocarcinoma in a murine model (43).

Cancer metastasis consists of multiple interdependent processes. To metastasize, tumor cells must invade, embolize, survive in the circulation, settle in distant capillary beds, and extravasate into and multiply in the organ parenchyma (21). Ishida et al. have reported that tumor emboli were seen in the interlobular portal venules, inlet venules and sinusoids within 2 h of AH60C injection (44): we gave the rats meloxicam 4–6 h after the injection of cancer cells. Our results, namely the significantly decreased number of metastatic tumors in meloxicam-treated groups, demonstrate some influence of meloxicam to restrain the process after their entrapment in the capillary beds of metastatic site.

We have also shown that meloxicam decreased the diameter of metastatic tumors. Several previous studies have shown that COX-2 inhibitors interfere with the growth of metastatic tumors (28,45). The mechanisms of action of COX-2 inhibitors have been postulated to include their antiangiogenic effects (18), suppression of matrix metalloproteinase (MMP) production, induction of apoptosis (46,47), inhibition of cellular proliferation and adhesion, and others (19,48). In this study, there was difference in TVD between control group and group 2 or 3. This might indicate that meloxicam restricts the growth of tumors mainly by interfering with the growth of tumor vessels to reduce blood flow.

In order to investigate the angiogenesis of liver metastasis, many studies have used vascular endothelial growth factor, MMP and microvessel density (19,49–51). We previously reported that the neovascularature of metastatic liver and lung tumors in rats can be examined using a resin cast (27,28). Kobayashi et al. evaluated the effect of a COX-2 inhibitor on neovascularization of metastatic lung tumors by using resin casts to measure the diameter of tumor vessels and the three-dimensional architecture of vascularity (28). They suggested that the COX-2 inhibitor reduced the growth rate of the tumors through poor tumor vessel formation. Here, we have introduced a new concept, TVD (the ratio of tumor vessel area to tumor area), which gives an objective evaluation of vascularity in tumors.

In conclusion, we have demonstrated that meloxicam decreases the number, size and TVD of metastatic liver tumors in rats. Its therapeutic potential for liver metastases of colorectal carcinoma should further be investigated.

Acknowledgments

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Conflict of interest statement

None declared.

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