Reduced Growth Rate of Dimethylhydrazine-induced Colon Tumors in Rats

Akira Tsunoda, Miki Shibusawa, Yuko Tsunoda, Naokuni Yasuda, and Tadashi Koike

Department of Surgery, Showa University School of Medicine, 1–5–8 Hatanodai Shinagawa-ku, Tokyo 142, Japan

ABSTRACT

α-Difluoromethylornithine (DFMO) treatment has been shown to modify carcinogenesis in many experimental tumor models, including breast, urinary bladder, and colon. This study was designed to determine whether DFMO treatment can inhibit tumor growth on chemical-induced colon cancer in rats. Effectiveness of DFMO in combination with mitomycin C (MMC) was also evaluated. Forty-two Sprague-Dawley rats received dimethylhydrazine (20 mg/kg) s.c. once weekly for 20 wk to induce colon cancer. Then a double-contrast barium enema was performed, and colon tumors were detected. The animals were divided into four groups that were subjected to the following treatments: none; DFMO alone; MMC alone; and a combination of DFMO plus MMC. After 5 wk of treatment, the barium enema was repeated. For the evaluation of treatment efficacy, tumor doubling time was adopted. The mean tumor doubling time in the control group was 20.7 ± 9.1 days (SD). “Response” was judged as effective when tumor doubling time in treatment groups was more than 38.9 days, calculated from the mean + 2 SDs in the control group. Response rates in the DFMO, MMC, and DFMO plus MMC groups were 40.0%, 10.0%, and 82.3%, respectively. DFMO was a more effective inhibitor of tumor growth than MMC, and DFMO in combination with MMC resulted in a synergic diminution of tumor growth. The double-contrast barium enema is useful to observe sequential tumor growth and may be appropriate for the evaluation of new treatment on experimental colon cancer in rats.

INTRODUCTION

DFMO is an irreversible inhibitor of ornithine decarboxylase, the first and rate-limiting enzyme in the polyamine biosynthetic pathway (1). The polyamines are required for several cellular functions such as DNA replication, protein synthesis, stabilization of macromolecular structures, cell growth, and cell differentiation (2). The ability of DFMO to block cellular growth is the basis for its use as an antitumor agent. Indeed, DFMO treatment has been shown to inhibit carcinogenesis in some experimental tumor models such as breast (3), urinary bladder (4), and colon (5). In experimental colon carcinogenesis, DFMO has been reported to reduce colon cancer incidence when it is given during the induction of tumors (5, 6). Recently, DFMO treatment was also shown to inhibit established autochthonous tumor growth by using endoscopic measurement (7). The formula for the volume of a sphere was adopted in their study; however, the configuration of tumors was reported to tend toward the ellipsoidal (8, 9). The purpose of this study is to find a more reliable method, using our previously described technique (9), of serial radiographic measurement of autochthonous tumors in order to assess directly the effect of DFMO on tumor growth. We extended the study to see the effectiveness of MMC or combination treatment of DFMO plus MMC.

MATERIALS AND METHODS

Animals and Chemicals. Male Sprague-Dawley rats weighing 110 to 120 g (6 wk of age) were obtained from Saitama Laboratory Animals Co., Ltd., and were acclimated for 1 wk. The animals were maintained in an air-conditioned room at 23 ± 5°C with 50 ± 10% humidity under a 12-h light-dark cycle. They had free access to chow diet (Oriental Yeast Co., Ltd.) and drinking water. Body weight was measured once every week.

Double-Contrast Barium Enema Technique. Food was not given for 3 days before examination. Bowel preparation consisted of 40% glycerin and was performed 24 h prior to the barium enema. Barium S100 (Toho Kagaku) diluted with tap water to a 60% weight/volume concentration was used. Under ether anesthesia, the barium enema was performed through a French 12 Foley catheter and usually required 3 to 5 ml of the barium sulfate suspension and 10 to 15 ml of air in order to obtain satisfactory mucosal coating and distention. Films were obtained in both frontal and lateral projections to get three diameters of the tumors detected. The length from the anus to the tumor was measured to identify the same tumor on serial barium examinations. A Hitachi TV-210 overtube was used for examination, and a magnification factor of 1.09 was adopted. The average exposure required a 45 kVp, 200 mA, and an exposure time of 4 ms.

Carcinogenesis Study. A group of 42 Sprague-Dawley rats received 20 weekly s.c. injections of DMH dihydrochloride (Aldrich Chemical Company) at a dosage of 20 mg/kg of body weight. A barium enema was then performed, and colon tumors were detected. According to the longest diameter of the tumor, tumors detected radiographically were assigned to one of three classes: Class A, under 5 mm; Class B, over 5 mm and under 10 mm; or Class C, over 10 mm. Rats with Class A tumors were first distributed into treatment subgroups, irrespective of the association with Class B or Class C tumors. Next, rats with Class B tumors, irrespective of the association with Class C tumors, were allocated, and finally rats with Class C tumors only were assigned, so that those tumors with similar size and number may be equally distributed into four groups. The four treatment groups were as follows: Group A, control; Group B, DFMO (generously supplied by Merrell Dow Research Institute, Cincinnati, OH); Group C, MMC; and Group D, DFMO plus MMC. DFMO was offered ad libitum as a 0.5% exchange resin (CK-10U: Mitsubishi Kasei Co., Ltd.) with Buffers A and B, while MMC was given i.p. at a dosage of 1 mg/kg of body weight/rat/wk 4 days after the final DMH injection. Under ether anesthesia, the barium enema was performed through a French 12 Foley catheter and usually required 3 to 5 ml of the barium sulfate suspension and 10 to 15 ml of air in order to obtain satisfactory mucosal coating and distention. Films were obtained in both frontal and lateral projections to get three diameters of the tumors detected. The length from the anus to the tumor was measured to identify the same tumor on serial barium examinations. A Hitachi TV-210 overtube was used for examination, and a magnification factor of 1.09 was adopted. The average exposure required a 45 kVp, 200 mA, and an exposure time of 4 ms.

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1 To whom requests for reprints should be addressed.

2 The abbreviations used are: DFMO, α-difluoromethylornithine; DMH, dimethylhydrazine; MMC, mitomycin C.
and B. Buffer A (pH 4.9) contained 0.28 M NaOH, 0.89 M NaCl, 10% acetonitrile, and 4% acetic acid. Buffer B (pH 4.9) was the same solution as Buffer A except for the concentration of NaCl, where 2.64 M was adopted. The flow rate was 0.7 ml/min. Detection of polyamines was carried out by fluorescence intensity after reaction of the column effluent with an o-phthal aldehyde solution. The flow rate of the o-phthal aldehyde solution was 0.1 ml/min, and fluorescence was measured at an excitation wavelength of 33 nm and an emission wavelength of 455 nm. Polyamine content was expressed in nmol/g of wet tumor weight.

Tumor Volume and Assessment of Tumor Response. Tumor volume was calculated from:

\[ V = \frac{\pi}{6} abc \]

where \( a, b, \) and \( c \) are diameters of an ellipsoid determined from both frontal and lateral projections after correction for magnification (13). Fig. 1 shows the representative tumor.

For assessment of tumor response, tumor doubling time was used and compared among treatment subgroups, since the growth curve of DMH-induced colon tumors was reported to be almost exponential in our previous study (9). Mathematically, this can be expressed by (14)

\[ T_D = \frac{t \ln 2}{\ln V_t - \ln V_0} \]

where \( T_D \) is tumor doubling time, \( t \) is time at tumor volume measurement, \( V_t \) is tumor volume at time \( t \), and \( V_0 \) is tumor volume at first observation.

Statistical Analysis. Statistical significance values of animal weight, tumor doubling time, and disparity in polyamine levels were determined by Student's \( t \) test.

RESULTS

Weights, Premature Mortality, and DFMO Intake. Table 1 demonstrates animal weights before and after treatment. Treatments were associated with significantly lower final weights in both the MMC group and the DFMO plus MMC group compared with the control group. Six deaths occurred prematurely, where 2 died before treatment, and 4 (2 each from Groups C and D) died during treatment. Thirty-six rats were therefore evaluable. Based on water consumption records, the average intake per rat of DFMO during treatment was 8.0 ± 1.4 and 6.4 ± 1.2 g for the DFMO group and the DFMO plus MMC group, respectively.

Radiological Investigation. Each rat developed multiple tumors, and tumors were variable in size. Although all tumors were not demonstrated with the barium enema, a tumor more than 2 mm in diameter was usually detected. The majority of tumors were polypoid in outline. Invariably, all small tumors had a smooth spherical or ellipsoidal outline. It was only when the tumors reached an appreciable size that irregularity and nodularity developed. Fig. 2 shows the sequential radiograph of a representative tumor in the DFMO group.

Tumors. The incidence of colonic tumor in treatment groups is shown in Table 2. A total of 260 tumors were found on necropsy, and the mean tumor incidence per rat in each treatment group was 10.0, 6.7, 5.9, and 4.9 in Groups A, B, C, and D, respectively. Of the 260 tumors, 96 (37%) were evaluated, for which tumor volumes both before and after treatment were available. Of the 96 tumors, 24, 35, 20, and 17 tumors were allocated to Groups A, B, C, and D, respectively. Eighty of 96 evaluated tumors were examined histologically and confirmed to be malignant. The incidence of well-, moderately, poorly differentiated and mucinous adenocarcinoma was 50 (63%), 18 (23%), 8 (10%), and 4 (5%), respectively. The majority showed well-differentiated adenocarcinoma. Tumor size correlation was shown in Table 3. Twenty individual measurable tumors in five rats from the DFMO group were selected and the radiographic volume obtained with the second barium enema was compared with the calculated autopsy volume. At autopsy it was difficult to measure the intraluminal height of the tumor accurately. This may explain some discrepancy in the tumor volume between the two methods.

Assessment of Tumor Response. Tumor volume before and after treatment was shown in a semilogarithmic graph (Fig. 3). The tumor growth rate, which was seen as the slope of the line, was revealed clearly for each tumor. Tumor doubling time was shown in Fig. 4. The mean doubling time in the control group was 20.7 ± 9.1 (SD) days. Response to a treatment was judged as effective when the doubling time was more than 38.9 days, calculated from the mean + 2 SD in the control group. The response rate of each treatment was as follows: DFMO, 40.0%; MMC, 10%; and DFMO plus MMC, 82.3%. No correlation was found between the differentiation of colon cancer and tumor doubling time in each treatment group, although the number was small.

Polyamine Content. Polyamine levels in colon tumor from control and DFMO groups were shown in Table 4. Putrescine levels in the DFMO group were significantly suppressed compared with those in the control group (\( P < 0.001 \)), whereas spermidine and spermine levels in the DFMO group were not significantly different from those in the control group.

DISCUSSION

Chemical-induced autochthonous tumors of different organotropism have increasingly been introduced into experimental

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**Table 1** Mean body weight before and after treatment

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>20 wk (g)</th>
<th>25 wk (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (control)</td>
<td>510 ± 43a</td>
<td>543 ± 73</td>
</tr>
<tr>
<td>B (DFMO)</td>
<td>496 ± 50</td>
<td>481 ± 54</td>
</tr>
<tr>
<td>C (MMC)</td>
<td>463 ± 67</td>
<td>437 ± 72a</td>
</tr>
<tr>
<td>D (DFMO + MMC)</td>
<td>476 ± 52</td>
<td>423 ± 51c</td>
</tr>
</tbody>
</table>

* Mean ± SD.  
\* P < 0.01, Group C versus Group A, 25 wk.  
\* P < 0.001, Group D versus Group A, 25 wk.
Fig. 2. Radiograph of the same tumor before (a) and after (b) treatment in the DFMO group.

### Table 2: Incidence of colon tumor

Response to a treatment was evaluated when tumor volume before and after treatment was obtained.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. of rats before treatment</th>
<th>No. of rats after treatment</th>
<th>No. of total tumors</th>
<th>No. of evaluable tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (control)</td>
<td>10</td>
<td>10</td>
<td>107 (10.0)*</td>
<td>24</td>
</tr>
<tr>
<td>B (DFMO)</td>
<td>10</td>
<td>8</td>
<td>67 (6.7)</td>
<td>35</td>
</tr>
<tr>
<td>C (MMC)</td>
<td>10</td>
<td>8</td>
<td>47 (5.9)</td>
<td>20</td>
</tr>
<tr>
<td>D (DFMO + MMC)</td>
<td>10</td>
<td>8</td>
<td>39 (4.9)</td>
<td>17</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>36</td>
<td>260 (7.2)</td>
<td>96 (24)*</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, number of tumors/rat.
* Numbers in brackets, percentage.

### Table 3: Tumor size correlation

<table>
<thead>
<tr>
<th>Rat</th>
<th>Tumor</th>
<th>Estimated radiographic volume (mm³)</th>
<th>Calculated autopsy volume (mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>77</td>
<td>68</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>29</td>
<td>42</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>24</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>117</td>
<td>139</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>64</td>
<td>52</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>15</td>
<td>18</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>14</td>
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</tr>
<tr>
<td>2</td>
<td>2</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>87</td>
<td>64</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>285</td>
<td>239</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>77</td>
<td>40</td>
</tr>
</tbody>
</table>

Chemotherapy studies (15, 16), since the predominantly used transplanted tumor models alone have proved to be inadequate for predicting the therapeutic value of drugs for clinical use. DMH-induced colon cancer in rats has been reported to be responsive to chemotherapy with agents used clinically in humans (17, 18).

In their investigations, the laparotomy staging system was adopted for primary colon cancer, and overall survival was compared between treated and untreated animals with the same stage of disease. While overall survival is appropriate for the evaluation of treatment, the sequential observation of tumor growth is also suitable and more practical, since the improved survival is likely to be attributable to the reduced growth of colon tumors. Double-contrast barium examination was introduced in the present study. This technique is simpler and safer than laparotomy and can obtain sequential tumor volumes. Although colonoscopy has been used for tumor diagnosis in the animal model (16, 19), exact determination of the tumor size cannot be achieved.

In the present study, less than 40% of the total tumors found on necropsy were evaluated for the assessment of tumor doubling time. Possible reasons for this discrepancy are that rats with DMH-induced colon tumor characteristically develop multiple tumors and that the number of tumors increased with time, so that a considerable number of tumors should have developed during the 5 wk of treatment. Moreover, all tumors were not detectable with the first barium examination, especially those less than 2 mm in diameter. Skucas et al. (8) presented the accuracy of radiographic tumor size determination, which was correlated with a precise volumetric displacement technique. However, there may be a couple of disadvan-
Fig. 3. Tumor volume before and after treatment.

Fig. 4. Tumor doubling time. Response to a treatment was judged as effective when the doubling time was more than 38.9 days, calculated from the mean + 2 SDs in the control group. Doubling time was calculated as \( \infty \) when the tumor volume after treatment was the same as that before treatment.

Table 4 Polyamine content in colon tumor

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Polyamine level (nmol/g of wet tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Putrescine</td>
</tr>
<tr>
<td>A (control)</td>
<td>98.0 ± 41.9*</td>
</tr>
<tr>
<td>B (DFMO)</td>
<td>31.3 ± 30.2</td>
</tr>
</tbody>
</table>

* Mean ± SD.
* P < 0.001, Group A versus Group B.

tages with barium examination. (a) As they mentioned, adequate radiographic measurement could not be obtained for some of the larger tumors. (b) We found difficulty in measuring the height of tumors accurately when they were in the proximal colon, since this part of the colon is not anatomically fixed to the retroperitoneum, and it is especially difficult to obtain the film of tumors in the lateral projection. Therefore, barium enema should be used earlier than in the present study for detecting smaller tumors. For the evaluation of tumor response, the tumor in the proximal colon may be excluded, since the majority of DMH-induced colon tumors in Sprague-Dawley rats tend to develop in the distal half of the colon (9).

DFMO has been reported to reduce colon cancer incidence when it is given during the induction of tumors (5, 6) or after completion of the carcinogen injections (20). Tumor incidence in the DFMO group was almost half that in the control group in our study, and this result was consistent with that of the investigation by Tempero et al. (20).

While chemopreventive efficacy by DFMO has been demonstrated, Zhang et al. (7) recently showed by using serial colonoscopic measurement that DFMO can suppress growth of established autochthonous tumors. The formula for the volume of a sphere was used in their study; however, the configuration of autochthonous tumors was reported to tend toward the ellipsoidal (8, 9). In particular, the height of the tumor was variable as in our investigation. The formula for the volume of an ellipsoid should therefore be used in autochthonous tumors. In this respect, radiographic measurement would be a more reliable method than endoscopic measurement. The barium enema technique was effectively applied as a noninvasive methodology for monitoring autochthonous colon tumors, and DFMO-induced inhibition of growth was demonstrated in the present study.

It has been reported that the chemical induction of colorectal tumors in mice is almost wholly dependent on continuous putrescine synthesis in colonic mucosa (5) and that the growth of established transplanted mouse colon cancers is almost putrescine dependent (21). In our study, tumor putrescine levels from the DFMO group were suppressed and correlated with the reduction in tumor growth, suggesting that the inhibition of tumor doubling time by DFMO may be due to its ability to reduce putrescine levels in colon cancer. It has been recently reported that the prevention of the utilization of polyamines from the gastrointestinal tract, in addition to their endogenous formation, resulted in considerable retardation of tumor growth (22, 23).

The 0.5% solution of DFMO used in the previous investigation was reported to exert no systemic effects when administered continuously (24). In the present study, treatment with
0.5% DFMO, which was limited to last 5 wk of the experiment, was not associated with a significant lower final weight compared with that of the control group, whereas treatment with MMC alone or in combination with DFMO produced lethal toxicity and killed 20% of the animals during treatment, with the surviving animals having a significant weight loss.

MMC is widely used as an antimutagen agent. Coadministration of MMC and DFMO has been reported effective on human colorectal cancer xenografts in nude mice (25).

A synergic effect was almost demonstrated by this combined treatment on autochthonous colon cancer in the rats in our study. This combined treatment with DFMO plus MMC may have potential in the chemotherapy of human colorectal cancer, although a Phage II trial of DFMO (26) produced discouraging results.

REFERENCES

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