Abstract. Background: Lung cancer is the leading cause of cancer-related death worldwide. We previously reported that respiration-gated X-ray micro-computed tomography (micro-CT) is a useful tool for analyzing lung tumor development in animal models. Materials and Methods: Lung tumors were induced by a single intraperitoneal injection (250 mg/kg) of urethane in male A/J mice, followed by indomethacin treatment at 5 ppm in the diet. The mice were scanned by micro-CT every 4 weeks from 10 to 26 weeks after urethane administration. Results: Total incidence and multiplicity of lung tumors were not significantly reduced by indomethacin treatment, as compared with untreated mice. However, the incidence of adenocarcinoma tended to be reduced by indomethacin treatment. Moreover, the size of lung tumors, especially adenomas, was suppressed by indomethacin treatment. Micro-CT analysis revealed that indomethacin effectively suppressed tumor development after urethane treatment for 10 weeks. Conclusion: These findings indicate that indomethacin suppresses lung carcinogenesis in mice and micro-CT is a useful non-invasive imaging approach for evaluating the characteristics and suppression of lung tumors in mice treated with cancer chemopreventive agents.

Lung cancer is the leading cause of cancer-related death worldwide (1), and reducing tobacco use and exposure to environmental carcinogens are effective ways to prevent lung carcinogenesis. The recent development of high-resolution computed tomography is able to identify small nodules in the lungs, including focal ground-glass opacities, which need to be followed in cancer check-ups. Thus, development of effective methods for preventing lung carcinogenesis is an urgent task.

Non-steroidal anti-inflammatory drugs (NSAIDs), such as indomethacin and aspirin, are reported to be useful chemopreventive agents for colorectal cancer, as demonstrated by experimental, epidemiological and clinical studies (2-6). NSAIDs, including indomethacin have also been shown to be useful candidate chemopreventive agents for lung tumors (7, 8). It has already been reported that indomethacin reduces the number of urethane-induced lung tumors in A/J mice (9). Indomethacin is a conventional NSAID, which has long been clinically employed to target inflammation. The molecular mechanisms underlying its protective effects are considered to be mainly due to inhibition of the activity of cyclooxygenase-1 (COX-1) and COX-2, key enzymes of prostanoid synthesis (10).

We recently applied X-ray micro-computed tomography (micro-CT) to detect lung space occupied lesions (SOLs) induced by a single intraperitoneal injection (250 mg/kg...
BW) of urethane in male A/J mice, from 10 to 30 weeks after exposure to the carcinogen, and provided evidence that micro-CT is a useful non-invasive imaging approach for evaluating the characteristics and growth of lung tumors in mice (11). Our results also indicated that tumors grew at markedly varying speeds, and reflect histopathological findings after autopsy. Furthermore, these results indicate that micro-CT is also useful for evaluating lung tumor regression, induced by cancer chemopreventive agents.

In the present study, pre-neoplastic and neoplastic lesions (hyperplasia, adenoma and adenocarcinoma) were induced in the lungs of male A/J mice by a single intraperitoneal injection of urethane with or without indomethacin treatment at 5 ppm in the diet to evaluate its chemopreventive effects. In addition, lung SOL development was monitored periodically using respiration-gated micro-CT.

Materials and Methods

Animals. A/J Jms Slc mice, 5-week-old males, were purchased from Japan SLC Inc. (Hamamatsu, Japan) and five mice each were housed in a plastic cage with wood chip bedding in an air-conditioned animal room maintained at 24±2°C and 60±5% relative humidity, with a 12 h light-dark cycle. Basal diet (AIN-76A; CLEA Japan, Inc., Japan) and water were available ad libitum throughout the experiment.

Experimental protocol for A/J mice treated with indomethacin. At 6 weeks of age, mice (n=9) were treated with a single intraperitoneal injection of urethane (250 mg/kg; Sigma, St Louis, MO, USA) in 0.9% NaCl saline. Control mice (n=5) were given a single saline intraperitoneal injection. At the same time administration of indomethacin was started. Indomethacin was purchased from Sigma Chemical Co. (St Louis, MO, USA) and well-mixed at concentrations of 5 ppm with the basal diet. The dosage of indomethacin was determined by a previous report and our experiment (9, 12). The mice were scanned by micro-CT every 4 weeks from 10 to 26 weeks after urethane or control vehicle (0.9% NaCl saline) injection. The experiments were conducted according to the Guidelines for Animal Experiments in the National Cancer Center Hospital from 2006 to 2008. The paraffin-embedded sample stocks were used for integrin immunohistochemical staining. The samples include normal lung tissue (n=1), atypical adenomatous hyperplasia (n=11) and localized tumors with a lepidoic growth pattern and alveolar collapse (Noguchi type B, n=11) (14), which were diagnosed by a pathologist. The study protocol was approved by the Institutional Review Board of the National Cancer Center, Tokyo, Japan.

Human lung tissue samples. A total of 23 lung tissue samples were obtained from patients who underwent lobectomy at the National Cancer Center Hospital from 2006 to 2008. The paraffin-embedded sample stocks were used for integrin immunohistochemical staining. The sections used for histopathological examination with the avidin–biotin complex immunoperoxidase technique. Polyclonal goat anti-COX-2 antibody (M-20) and polyclonal rabbit anti-vascular endothelial growth factor (VEGF) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:100 dilution were used. As a secondary antibody, anti-goat IgG, biotinylated and absorbed with rat serum (Vector Laboratories, Burlingame, CA, USA), was employed at 1:200 dilution. Staining was performed using avidin–biotin reagents (Vectastain ABC reagents; Vector Laboratories), 3,3'-diaminobenzidine and hydrogen peroxide, and the sections were counterstained with hematoxylin to facilitate orientation. As negative controls, consecutive sections were immunostained without exposure to the primary antibody.

Statistical analysis. The significance of differences in the multiplicity of urethane-induced mouse lung SOLs was analyzed using the Student’s t-test, and statistical analysis for the number of SOLs which had more than doubled in diameter was performed with the χ² test. Differences were considered to be statistically significant with p-values of less than 0.05.
Results

Incidence and multiplicity of lung SOLs assessed by micro-CT and histopathological analysis. The lung SOLs induced by urethane were easily distinguished from surrounding tissues in the micro-CT images. Reconstructed three-dimensional images were useful to differentiate the masses (globular) and blood vessels (tube structure) in lungs, even though both have a similar X-ray absorption. The smallest detectable SOL was approximately 0.5 mm in diameter. The incidence of SOLs detected by micro-CT was 100%, (10-26 weeks after urethane treatment). The number of SOLs/mouse (multiplicity) detected by micro-CT increased from 10 to 26 weeks after urethane treatment, as shown in Table I. The multiplicity of lung SOLs at 26 weeks after urethane treatment was 10.0±3.0 (mean±SD). With indomethacin treatment, the multiplicity of lung SOLs, determined by micro-CT at the end of the experiment, was reduced by approximately 20% as compared with the untreated mice (Table I).

Table II shows the incidence and multiplicity of lung SOLs at the end of experimental period, as determined by histopathological analysis. The number of total SOLs (10.3±2.6) was similar to that detected by micro-CT. The incidence of hyperplasia (bronchiolo-alveolar hyperplasia) and adenoma (bronchiolo-alveolar adenoma) was 100%, and that of adenocarcinoma (bronchiolo-alveolar adenocarcinoma) was 44%. No spontaneous tumors were observed in the A/J mice without urethane treatment. The actual number of lung SOLs examined by histopathological analysis was: eight adenocarcinomas in the indomethacin-untreated group, and one adenocarcinoma in the indomethacin-treated group. Similar numbers of hyperplasia and adenoma were detected in both indomethacin-treated and untreated groups. The multiplicity of adenocarcinoma averaged 0.9 in the indomethacin-untreated mice, and tended to be reduced to 0.1 (p=0.08) in the indomethacin-treated mice (Table II).

Change of number of lung SOLs by periodic micro-CT analysis. Periodic micro-CT analysis of urethane-induced lung SOLs in living mice revealed that the total number of SOLs started to differ between groups from 18 weeks after urethane injection (Figure 1). Consistent with previous work (9), the percentage of reduction was almost the same throughout the experiment. Thus, the number of newly-developed SOLs within 4 weeks, i.e. newly-detected SOLs by micro-CT, in mice was counted successively, and are presented in Figure 1. Moreover, histopathological analysis revealed that tumors diagnosed as adenocarcinoma at the end of the experiment existed as SOLs in CT images from the early experimental periods: three tumors at 14 weeks and one tumor at 18 weeks. Interestingly, the number of newly-developed lung SOLs in the untreated group started to
Figure 2. Increase of lung adenocarcinoma diameters in A/J mice by axial micro-computed tomography (CT) images and histopathological hematoxylin and eosin (HE) staining. A: Growth curves of nine adenocarcinomas are shown. Each tumor scanned by micro-CT was reconstructed into three-dimensional images (axial, sagittal, coronal and oblique) and maximum diameters were measured periodically. The red line shows the growth of adenocarcinoma in the indomethacin-treated group. The blue line shows that of the untreated group. B: Micro-CT images of the most aggressive lung adenocarcinoma (curve no. 1 in A). C: Micro-CT images of lung adenocarcinoma in the indomethacin-treated group (curve no. 2 in A). D: Histopathology of the lung space occupied lesion (SOL) shown in B. E: Histopathology of tumor shown in C. (D and E: bar=500 μm). Tumors observed in the lung are shown by arrows.

Figure 3. Virtual in vivo micro-computed tomography (CT) images of lung space occupied lesion (SOL) and histopathological findings. Axial micro-CT images of the thorax of a mouse at the end of the experiment are shown. Histopathology of representative hyperplasia (A), adenoma (B) and adenocarcinoma (C) observed in urethane-treated mouse (all indomethacin-untreated mice); bar=500 μm. Micro-CT images representing hyperplasia (D), adenoma (E) and adenocarcinoma (F). Tumors observed in the lung are shown by arrows.
decrease after 18 weeks, and indomethacin suppressed lung SOL development effectively at that period. Of note, one adenocarcinoma observed in the indomethacin-treated group, which was diagnosed at the end of experiment, existed as an SOL in CT images from 10 weeks.

Size of lung SOLs assessed by micro-CT. The longitudinal diameters of lung SOLs at 26 weeks, assessed by micro-CT, were similar to those measured by digital caliper after sacrifice (Table III). The longitudinal diameter tended to increase with histological changes, i.e. hyperplasia to adenocarcinoma. Indomethacin treatment reduced the size of lung tumors, especially of adenomas (Table III).

Figure 2A shows growth curves for all nine adenocarcinomas developed in A/J mice with or without indomethacin treatment. The adenocarcinoma grew most aggressively, 0.19 mm in diameter in one week (line no.1), and appeared as solid-type nodules with a clear tumor margin on micro-CT images (Figure 2B). Histopathologically, images exhibited an irregular nodular growth pattern without any glandular or tubular formation with little connective tissue. Nuclei were pleomorphic and mitotic figures were also observed (Figure 2D). Representative micro-CT images of hyperplasia, adenoma and other adenocarcinoma (line no.3 of Figure 2A) developed in A/J mice without indomethacin treatment, are shown in Figure 3. On the other hand, hyperplasia and adenoma grew at a slow to moderate speed throughout the experiment. These hyperplasias (Figure 3A) and adenomas (Figures 3B) exhibited clear edges and/or spiked edges in CT images. Figure 3B illustrates a papillary-type adenoma. As seen in adenoma, mitotic rates and degree of cellular pleomorphism were low. One adenocarcinoma

Table III. Size of lung SOLs at 26 weeks, assessed by micro-CT and by digital caliper.

<table>
<thead>
<tr>
<th>Indomethacin</th>
<th>Hyperplasias</th>
<th>Adenomas (Ad)</th>
<th>Adenocarcinomas (Ca)</th>
<th>Total SOLs</th>
<th>Ad+Ca</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 ppm</td>
<td>1.52±0.37</td>
<td>1.82±0.55</td>
<td>2.03±0.33</td>
<td>1.74±0.51</td>
<td>1.85±0.53</td>
</tr>
<tr>
<td>Digital Caliper</td>
<td>1.42±0.55</td>
<td>1.79±0.56</td>
<td>1.94±0.65</td>
<td>1.65±0.60</td>
<td>1.81±0.58</td>
</tr>
<tr>
<td>5 ppm</td>
<td>1.33±0.40</td>
<td>1.49±0.41 **</td>
<td>2.93</td>
<td>1.45±0.44 **</td>
<td>1.52±0.46 **</td>
</tr>
<tr>
<td>Digital Caliper</td>
<td>1.30±0.49</td>
<td>1.52±0.43*</td>
<td>2.65</td>
<td>1.46±0.48*</td>
<td>1.54±0.45*</td>
</tr>
</tbody>
</table>

Data are mean±SD. **p<0.01 vs. 0 ppm. *p<0.05 vs. 0 ppm.
(Figure 2A, red line, no.2), developed in the indomethacin-treated group was a large tumor that grew particularly rapidly, increasing from 0.9 mm to 2.9 mm within 16 weeks. This solid-type nodule with a clear tumor margin on CT images, is illustrated in Figure 2C.

The number of tumors that doubled in size from first detection was significantly reduced upon indomethacin treatment (Table IV). Regardless of indomethacin treatment, all adenocarcinomas more than doubled in diameter.

Expression of COX-2 and VEGF in lung SOLs. COX-2 immunostaining was performed to confirm the existence of molecules targeted by indomethacin (Figures 4A-C). The data were obtained from serial sections used for Figure 3. COX-2 was up-regulated in eight examined hyperplastic lesions. COX-2 was up-regulated in 10 out of 11 adenomas. Interestingly, COX-2 was down-regulated in all examined adenocarcinomas. Further details are summarized in Table V, in which COX-2 expression levels are classified into four groups (–, ±, + and ++) by staining strength. Moreover, VEGF immunostaining revealed that VEGF was observed in hyperplasia (6/8) and adenomas (8/11) but not in adenocarcinomas (Figures 4D-F).

COX-2 expression was also observed in macrophage and non-ciliated bronchiolar epithelial cells (clara cells) of human normal lung tissue, epithelial cells of atypical adenomatous hyperplasia and tumor cells of human lung tumors (Noguchi type B).

Discussion

In the present study, micro-CT with a respiratory gating system was shown to be a useful non-invasive tool for evaluating the effects of indomethacin on urethane-induced lung SOL development in mice. This study provides evidence that indomethacin is able to suppress lung tumorigenesis at multiple stages, especially the adenoma-to-adenocarcinoma stage (Table II). Moreover, indomethacin treatment effectively suppressed the size of urethane-induced lung tumors (Table III).

In this urethane-induced lung tumor model, DNA mutations in lung epithelial cells are reported. Several tumor-susceptibility genes, such as cell-cycle-related genes (Brca1, Cdkn2a/b) and cell growth and angiogenesis-related genes (Fos, Jun, Kras, Pkcn and Tnfa) are mutated (15-20). Indomethacin has been reported to inhibit only the activity of β-catenin and COX, but not of cyclin-dependent kinase, activator protein 1 (AP-1) (Fos/Jun) and nuclear factor-kappa B (NF-κB) (21, 22). Thus, one can speculate that indomethacin inhibits lung tumor growth (Table IV) through direct inhibition of COX-1 and COX-2, which might be induced by Fos, Jun, Kras, Pkcn and Tnfa mutation.

Prostaglandin E2 is reported to induce VEGF and increase the angiogenic response (23, 24). Moreover, indomethacin is reported to inhibit blood vessel formation in tumor-induced in vivo angiogenesis assays using C3H/HeJ mice (25). As shown in this study, expression of COX-2 was confirmed in hyperplasia and adenoma in the mice by immunohistochemical assay (Figure 4 and Table V). Expression of COX-2 was also confirmed in the early-stage of human lung tumors (Figure 5). Interestingly, COX-2 was down-regulated in adenocarcinoma, which might be the result of gene instability, as observed in the progressive stage of lung cancer (7). These data suggest that COX-2 inhibitor may be useful in preventing human lung tumor development. Furthermore, VEGF expression in adenoma may be related to the progression of adenoma to adenocarcinoma (Figure 4), and indomethacin may inhibit this angiogenic response.

Our data also propose candidate factors related to malignant transformation, including well-known factors. Proposed factors could be tumor size, tumor growth speed,
characteristics of nodules on CT image, and an allowed time span for tumor growth. Table III demonstrates the possibility that tumor size could correlate with histopathological type, but correlation seems weak. Tumor growth speed could be a strong candidate. However, our present and previous data showed that adenocarcinoma grew from slow to fast (Figure 2A), and we concluded that growth speed might be a susceptibility factor. Among the fast growing nodules, a smooth surface of the nodule (Figures 2B-E) is one of the characteristics of adenocarcinoma. The characteristics of lung adenocarcinoma in CT images need to be further investigated in detail. Finally, we concluded that the time span allowed for tumorigenesis is the most important factor for malignant transformation because our data (Figure 2B) demonstrate that all nodules which were finally diagnosed as adenocarcinoma at the end of the experiment existed at the early-stage of this experiment. These results indicate that the whole span of administration of indomethacin is not required to achieve similar chemopreventive effects of indomethacin, and experiments with several administration time spans are desired to obtain informative data.

In conclusion, our results provide evidence that respiratory-gated micro-CT scanning of live mice has the potential to evaluate the effects of cancer chemopreventive agents on lung tumorigenesis. Using this method, indomethacin revealed chemopreventive effects on lung tumorigenesis. Thus, this novel approach could be used as a screening technique for chemopreventive agents using a reduced number of sacrificed animals compared to other methods. Moreover, this novel approach may also have an impact on the study of natural lung tumor regression and of cancer therapeutic agents.

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References


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