Chemopreventive effects of ONO-8711, a selective prostaglandin E receptor EP1 antagonist, on breast cancer development

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Introduction

Development of breast cancer is a multiple process involving an interplay between genetic and environmental factors, including diets and hormones. Breast cancer mortality remains the second leading cause of cancer deaths in women among western countries (1). Recently, several epidemiological studies have suggested that there is an inverse association between the risk of breast cancer and intake of non-steroidal anti-inflammatory drugs (NSAIDs) (2,3), which block the prostaglandin (PG) cascade by inhibition of its rate-limiting enzyme, cyclooxygenase (COX). There are two isoforms, COX-1 and COX-2. Accumulating evidence indicates that COX-2, which is inducible and contributes to pathological processes such as inflammation and abnormal cell proliferation, plays a pivotal role in mammary gland (4) and colon (5) cancer development. Several selective inhibitors have in fact already demonstrated inhibitory effects on mammary gland (6,7) and colon (8,9) carcinogenesis in rodent models. In human breast cancers, elevated levels of COX-2 and PGE2 have been widely reported (10–13). Furthermore, levels of COX-2 and PGE2 were elevated in rodent mammary gland tumors (7,14). PGE2 exhibits biological activity through binding to membrane receptors, EP1–4. It was suggested that EP1 receptor was detected by reverse transcription-polymerase chain reaction (RT-PCR) in breast cancers, not in normal tissues. These results suggest that EP1 receptor is associated with breast cancer development and selective PGE receptor EP1 antagonists may possess chemopreventive effects through the induction of apoptosis without any side effects.

Levels of prostaglandin E2 (PGE2) in human and rodent breast cancers are higher than surrounding normal tissues. PGE2 exhibits biological activity through binding to membrane receptors, EP1–4. The present study was designed to investigate the effects of ONO-8711, a newly synthesized selective PGE receptor EP1 antagonist, on 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)-induced breast cancer development. Starting at 7 weeks of age, female Sprague–Dawley (SD) rats were given PhIP (85 mg/kg body weight) by gavage four times weekly for two weeks. Dietary administration of ONO-8711 at 400 or 800 p.p.m. delayed occurrence of breast tumors for 2 or 4 weeks, respectively. At 20 weeks after the last dosing of PhIP, all animals were killed and complete autopsy was made. All breast tumors were diagnosed as invasive ductal adenocarcinomas histopathologically. Administration of ONO-8711 at 800 p.p.m. significantly decreased PhIP-induced breast cancer incidence, multiplicity and volume compared with those of rats fed the control diet (56% versus 79%, P < 0.05, 1.2 versus 2.5, P < 0.05, 0.7 versus 1.4 cm3, P < 0.01, respectively). Apoptosis was significantly increased in breast cancer cells by feeding of ONO-8711 at 800 p.p.m. of 158% (P < 0.05). EP1 receptor was detected by reverse transcription-polymerase chain reaction (RT-PCR) in breast cancers, not in normal tissues. These results suggest that EP1 receptor is associated with breast cancer development and selective PGE receptor EP1 antagonists may possess chemopreventive effects through the induction of apoptosis without any side effects.

Abbreviations: AgNOR, silver-stained nucleolar organizer region protein; COX, cyclooxygenase; ISEL, in situ end-labeling of fragmented DNA; NSAID, non-steroidal anti-inflammatory drug; PG, prostaglandin; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; RT-PCR, reverse transcription-polymerase chain reaction; SD, Sprague–Dawley.

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Materials and methods

Chemicals and animals
PhIP-HCl was purchased from the Nard Institute (Osaka, Japan). The selective EP$_1$ antagonist, ONO-8711 (6-[(2PhIP-HCl was purchased from the Nard Institute (Osaka, Japan). The selective EP$_1$ antagonist, ONO-8711 (6-[(2

Experimental procedure
At 7 weeks of age, all rats except those intended for vehicle (saline) treatment received PhIP (85 mg/kg body weight) by gavage four times weekly for 2 weeks. Rats treated with PhIP were divided into three groups fed the control or experimental diets including 400 or 800 p.p.m. ONO-8711. Those receiving the vehicle were divided into two groups given the control or the experimental diet with ONO-8711 at 800 p.p.m. All animals had ad libitum access to diets and tap water during the study. They were monitored daily for their general health and mammary tumor development. The experiment was terminated 20 weeks after the last dosing of PhIP; all animals being killed under ether euthanasia. At complete autopsy, the numbers and sizes of all mammary tumors were recorded. The (L) width (W) and depth (D) of each lesion were measured with calipers and tumor volume (V) was calculated using the formula $V=L\times W\times D/6$ (23). Half of each tumor was fixed with 10% neutral buffered formalin for evaluation of histopathological diagnosis and the remainder was frozen for subsequent biochemical analyses. For histopathological examination, formalin-fixed tumors were embedded in paraffin and 5 μm sections were prepared and stained with hematoxylin and eosin. Pathological diagnosis of mammary gland tumors was made principally based on the classification of Russo et al. (24). Invasive ductal carcinomas were diagnosed as mammary gland tumors with invasion to surrounding tissues.

Analyses for cell proliferation and apoptosis
Deparaffinized sections underwent silver-stained nucleolar organizer region protein (AgNOR) staining according to Ploton et al. (25) to assess cell proliferation. Briefly, the colloidal silver solution for staining the NORs was prepared by dissolving gelatin in 1% aqueous formalic acid at concentration of 2%. This solution was mixed with 60% aqueous silver nitrate as the working solution, in which sections were incubated for 60 min at room temperature, protected from sunlight, then washed with deionized water. Careful focusing was allowed AgNOR to be visualized in nuclei as black dots under a microscope. For measurement, three photographs of each lesion, at a final magnification of ×400, were prepared and 500 to 750 tumor cells in each photograph were analyzed. Other sections were stained with in situ end-labeling of fragmented DNA (ISEL) using ApoTag in situ detection peroxidase kit (Intergen Co., Purchase, NY) according to the manufacturer’s instructions. Reverse transcription with random 9 mers was used to generate cDNAs from 0.8 μg of total RNA extracts using reverse transcriptase (AMV Reverse Transcriptase XL) and a Takara RNA LA PCR kit (Takara Biomedical Co., Inc., Japan). The following primers were used for PCR amplification of the resulting cDNA (PCR conditions were 94°C for 120 s and then 30 cycles of 98°C for 5 s, 60°C for 30 s, and 72°C for 30 s): EP$_1$ forward primer 5'– TACCGCACATCCAGCG–3' (nucleotides 684–702), and reverse primer 5'– GCAAGCCGCCGCCC AGG–3' (nucleotides 922–939) (26); β-actin (203 bp) forward primer 5’– TCCTCCCTGGGAAGAGCTA–3' (nucleotides 741–760), and reverse primer 5’– CCAGACACGACTGGTGTTGGC–3' (nucleotides 924–943) (27).

Statistical analysis
Body weight, tumor incidence, tumor multiplicity, and volume data, as well as AgNORs count/nucleus, and apoptotic indices were compared between the animals fed the control and ONO-8711 diets. Tumor incidence, expressed as the percentage of tumor-bearing animals, was analyzed by Fisher’s exact probability test, whereas tumor multiplicity, expressed as the mean number of tumors per animal, and the other variables were analyzed by the unpaired Welch’s t-test. Differences were considered statistically significant at $P < 0.05$.

Results and discussion

The body weights of animals throughout the study are indicated in Figure 2A. As expected, body weights of rats treated with saline were higher than those in rats treated with PhIP, but no body weight retardation was evident with ONO-8711 feeding. No toxic symptoms were observed in rats fed ONO-8711 at both doses during the study.

Figure 2B and C indicate time course of changes in incidence and multiplicity of palpable breast tumors, respectively, during

Fig. 1. Structure of ONO-8711.

Fig. 2. Effects of PhIP and ONO-8711 on body weights (A) and palpable mammary tumor development (B, incidence; C, multiplicity) throughout the study. Closed circles, PhIP and control diet; open circles, PhIP and 400 p.p.m. ONO-8711; open squares, PhIP and 800 p.p.m. ONO-8711; closed triangles, vehicle and control diet; open triangles, vehicle and 800 p.p.m. ONO-8711.
the study. At 8 weeks after the last dosing of PhIP, first palpable breast tumors were noticed in rats treated with PhIP and fed the control diet. In rats fed ONO-8711 at 400 or 800 p.p.m., first palpable breast tumors were found at 10 or 12 weeks after the last PhIP treatment, respectively. Thus, administration of ONO-8711 at 400 or 800 p.p.m. delayed occurrence of breast tumors for 2 or 4 weeks, respectively. Incidences of palpable breast tumors in rats treated with PhIP and fed the control diet were slightly higher than in their counterparts receiving the experimental diets, but the difference lacked statistical significance. Multiplicity of palpable breast tumors in rats fed ONO-8711 at 800 p.p.m. was lower than that in rats fed the control diet throughout the study; significant differences between them after 19 weeks were identified. In both incidence and multiplicity of palpable breast tumors, there were no differences between ONO-8711 at 400 p.p.m. and the control diet groups. No breast tumors were observed in rats treated without PhIP initiation during the study.

At 20 weeks after the last dosing of PhIP, all animals were killed and complete autopsy was performed. Rats treated with saline and fed ONO-8711 at 800 p.p.m. did not produce any gross or histological changes in mammary glands, liver, lungs, kidneys, stomach, or intestines which would indicate toxicity. No neoplastic lesions other than breast tumors were found in rats treated with PhIP and fed the control or ONO-8711 at both doses. Histopathological examination revealed all mammary gland tumors induced by PhIP to be diagnosed as invasive ductal carcinomas. Table I summarizes the final data for breast cancers induced by PhIP. In the ONO-8711 at 400 and 800 p.p.m. feeding groups, 62% (18/29) and 56% (18/32) of rats developed breast cancers, respectively, whereas the figure was 79% (26/33) for the controls. ONO-8711 at 800 p.p.m. feeding significantly suppressed cancer incidence compared with the control diet group (P < 0.05). Moreover, administration of ONO-8711 at 800 p.p.m. significantly decreased cancer multiplicity (2.5 versus 1.2, P < 0.05) as well as breast cancer volume (0.7 versus 1.4 cm³, P < 0.01). ONO-8711 at 400 p.p.m. feeding decreased cancer incidence, however, the difference was not statistically significant. The lack of an inhibitory effect of ONO-8711 at 400 p.p.m. may indicate that higher blood levels of this agent are required to achieve adequate mammary gland exposure.

To elucidate the mechanisms of inhibition by ONO-8711 on PhIP-induced breast cancer development, cell proliferation and apoptotic index were investigated (Table II). Neither dose of ONO-8711 showed any effects on AgNORs count/nucleus in breast cancers. However, the apoptotic index in cancers was significantly elevated by administration of ONO-8711 at 800 p.p.m. compared with that in the control diet (0.43 versus 1.11, P < 0.05). Apoptosis is a programmed process of active cellular self-destruction, which requires the expression of a number of genes and usually affects single cells surrounded by viable neighbors (28). By using ISEL on tissue sections, early stages of apoptosis, not detectable by routine H&E stains, can be identified (29). The net growth rate of a tumor mass reflects the balance of cell gain and loss and in the present study appeared to exert its effects mainly on the latter. Using RT-PCR method, EP1 receptor was detected in breast cancers, not in normal mammary gland tissue. Left lane, marker; lanes 1–8, breast cancers induced by PhIP; lanes 9–16, normal mammary gland tissues.

**Table I. Effects of ONO-8711 on PhIP-induced breast cancers**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats examined</th>
<th>Incidence (% rats with cancers)</th>
<th>Multiplicity (no. of cancers/rat)</th>
<th>Cancer volume (cm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PhIP-treated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control diet</td>
<td>33</td>
<td>26/33 (79)a</td>
<td>2.5 ± 2.5b</td>
<td>1.4 ± 2.0b</td>
</tr>
<tr>
<td>400 p.p.m. ONO-8711</td>
<td>29</td>
<td>18/29 (62)</td>
<td>2.4 ± 3.9</td>
<td>1.2 ± 1.6</td>
</tr>
<tr>
<td>800 p.p.m. ONO-8711</td>
<td>32</td>
<td>18/32 (56)c</td>
<td>1.2 ± 1.7c</td>
<td>0.7 ± 0.6d</td>
</tr>
</tbody>
</table>

*a*Values in parentheses indicate percentage rats with breast cancers.

*b*Mean ± SD.

*Significantly different from control diet group by Fisher’s exact probability test (P < 0.05).

*d*Significantly different from control diet group by Welch’s t-test (*P* < 0.05 and *e* *P* < 0.01).

**Table II. Effects of ONO-8711 on cell proliferation and apoptosis in PhIP-induced breast cancers**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AgNORs count/nucleus</th>
<th>Apoptotic index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PhIP-treated</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control diet</td>
<td>1.39 ± 0.31a</td>
<td>0.43 ± 0.44a</td>
</tr>
<tr>
<td>400 p.p.m. ONO-8711</td>
<td>1.41 ± 0.07</td>
<td>0.42 ± 0.37</td>
</tr>
<tr>
<td>800 p.p.m. ONO-8711</td>
<td>1.46 ± 0.18</td>
<td>1.11 ± 0.92b</td>
</tr>
</tbody>
</table>

*a*Mean ± SD.

*Significantly different from control diet group by Welch’s t-test (P < 0.05).
express the EP1 receptor and the results point to a physiological importance. The expression of EP1 receptor may be crucial to progress mammary gland carcinogenesis, and ONO-8711 blocks EP1 receptor resulting in inhibition of breast cancer development. In PhIP-induced breast cancers, COX-2 protein was overexpressed (7) and that may contribute to overload of PGE2. COX-2 and PGE2 are closely associated with estrogen biosynthesis through aromatase gene (CYP 19) and these three members may be involved in mammary gland carcinogenesis through EP1 receptor. Recently, Liu et al. (30) reported that enhanced COX-2 expression is sufficient to induce mammary gland tumorigenesis. COX-2 can act as an oncogene under certain circumstances, leading to the production of PGE2 which could then act in an autocrine or paracrine way to induce signaling via EP receptors, at least EP1 receptor.

In conclusion, ONO-8711, a selective PGE receptor EP1 antagonist, significantly inhibited breast cancer development, presumably through the induction of apoptosis. Also, we found EP1 receptor expression in PhIP-induced breast cancers, but not in normal tissues. Moreover, it is noteworthy that ONO-8711 at 800 p.p.m. did not induce any symptoms of toxicity. These findings suggest that EP1 receptor expression is involved in mammary gland carcinogenesis and EP1 receptor antagonists may possess chemopreventive activity through the induction of apoptosis.

Acknowledgements

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References


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