Antitumor Effects of Lipoxygenase Inhibitors on Murine Bladder Cancer Cell Line (MBT-2)

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Arachidonic acid (AA) metabolites derived from both the lipoxygenase (LOX) and cyclooxygenase (COX) pathways transduce a variety of signals related to cell growth. Selective blockade of the different metabolic pathways of AA (using a general LOX inhibitor NDGA, a 5-LOX inhibitor AA861, a 12-LOX inhibitor baicalein and a general COX inhibitor ibuprofen) revealed that murine bladder cancer cell line (MBT-2) cell proliferation was inhibited by the LOX inhibitors concentration-dependently, but not by the COX inhibitor. Among the LOX inhibitors, baicalein showed the strongest inhibition and induced apoptosis of MBT-2. Proliferation of MBT-2 was also significantly inhibited by 12-LOX antisense oligonucleotides. In an in vivo experiment, the antitumor effects of baicalein administration on C3H/HeN mice implanted with MBT-2 were recognized. These results suggested that LOX inhibition may be significant in the treatment of bladder cancer.

Materials and Methods

Cells and culture. The murine bladder cancer cell line (MBT-2) was kindly provided by Mark S. Soloway. The cells were cultured as monolayers in RPMI1640 (Nikken Bio Medical Laboratory, Japan) supplemented with 10% fetal calf serum (FCS) (Equitech-Bio, Inc., Ingram, TX, USA), 100 units/ml penicillin and 100 μg/ml streptomycin.

Agents. NDGA (nordihydroguaiaretic acid), a non-specific LOX inhibitor, baicalein (5,6,7-trihydroxyflavone), a specific 12-LOX inhibitor, AA861 (2(12-hydroxydodeca-5-10-dinyl)-3,5,6-trimethyl-1-4-benzoquinone), a specific 5-LOX inhibitor and ibuprofen, a non-specific COX inhibitor, were purchased from Sigma (St Louis, MO, USA).

Assay for antitumor activity. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay was performed by the modified method of Mosman (14). Cell cultures were done in triplicate in 96-well microtiter plates (Coster, Cambridge, MA, USA). Each well contained 5x10³ cells in a total volume of 90 μl in RPMI1640 medium supplemented with 10% FCS. After a 4-hour incubation at 37°C with 5% carbon dioxide and 100% relative humidity to remove any impairment of cell adherence to the plates, 10 μl of various concentrations of agents were added. After 96 hours, 10 μl of a 5 mg/ml solution of MTT in phosphate-buffered saline was added to each well and the plates were incubated at 37°C for 6 hours. Acid isopropanol (100 μl of 0.04 N HCl in isopropanol) was added to each well and the wells were mixed thoroughly to dissolve the dark blue crystals. The plates were read on a BIORAD Model 2550 EIA reader, using a test wavelength of 570 nm. The percentage of cell survival of each well was calculated from the following equation:

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\text{Percent of cell survival} = \frac{\text{absorbance of test - absorbance of blank}}{\text{absorbance of control - absorbance of blank}} \times 100
\]

12-LOX antisense oligonucleotide transfection. Oligonucleotides were synthesized by Sawady Technology (Tokyo, Japan) with HPLC purification. Sequences of the 12-LOX antisense and sense oligonucleotides were as follows: antisense, 5'-CAGGTCGT CAGGAGGACA-3'; sense, 5'-TGTCTCCTCGACGACTTG-3' (4).
Cells (1x10^5) cultured in 6-well plates for 24 hours were transfected with oligonucleotides in 10 ìg/ml lipofectin (GIBCO BRL) in serum-free RPMI1640 medium for 4 hours after which oligonucleotides were washed off and cells were cultured in RPMI1640 with 10% FCS for up to 48 hours. Treatment was then repeated for another 24 hours.

Acridine orange stain. Single cell suspensions were fixed in 1% formalin/PBS and stained with acridine orange at a final concentration of 1 ìg/ml. One drop (about 15 ìl) of the stained cell suspension was plated on a microscope slide. Cells were visualized under a UV fluorescence microscope with a blue-green filter. Apoptotic cells were defined as cells showing nuclear fragmentation and chromatin condensation morphologically.

Animals. Male C3H/HeN mice, 8 weeks old, were purchased from Nippon Clea (Kyoto, Japan). They were housed 5 mice per cage in the Animal Care Faculty of Osaka City University Graduate School of Medicine, Japan. The mice were subcutaneously injected in the right thigh with 100 ìl of the suspension containing 5x10^5 viable MBT-2 cells. Baicalein was subcutaneously administered at a dose of 0.05 mg or 0.1 mg per mouse once daily for 10 days from day 11 to day 20. Tumor volume was calculated using the following formula: V=0.5LxW^2, where L is length (long axis) and W is width (short axis).

Statistical analysis. All values are expressed as the mean±SD. Statistical analysis was performed using Student’s t-test.

Results

Antitumor effects of LOX and COX inhibitors on MBT-2. The antitumor effects of LOX and COX inhibitors on MBT-2 are shown in Figure 1. All LOX inhibitors, the general LOX inhibitor NDGA, the specific 5-LOX inhibitor AA861 and the specific 12-LOX inhibitor baicalein, inhibited cell proliferation in a dose-dependent manner. The concentration of each LOX inhibitor necessary to obtain 50% inhibition (IC50) was as follows: NDGA 5.8 ìM, AA861 8.2 ìM and baicalein 0.43 ìM. The general COX inhibitor ibuprofen showed little growth inhibition. Baicalein, a selective inhibitor of 12-LOX, showed the strongest inhibition.

To clarify the mechanism of growth inhibition induced by baicalein, apoptosis of cells was evaluated. Acridine orange staining showed that cells underwent typical apoptotic morphological changes upon treatment with 1 ìM baicalein at 48 hours. Nuclear fragmentation and condensation of chromatin were apparent 48 hours after baicalein treatment (Figure 2).

12-LOX antisense oligonucleotide-induced growth inhibition in MBT-2. Proliferation of MBT-2 was significantly inhibited by 12-LOX antisense oligonucleotides (p<0.01) but not the control 12-LOX sense oligonucleotides (Figure 3).
Antitumor effects of baicalein administration on C3H/HeN mice implanted with MBT-2. The antitumor effects of baicalein administration on C3H/HeN mice implanted with MBT-2 are shown in Table I. All the control mice showed a progressive increase in tumor volume, reaching 2.23±0.32 cm³ on day 20 and 5.03±1.22 cm³ on day 30 (group 1). When 0.05 mg of baicalein was subcutaneously administered for a total of 10 times from day 11 to day 20, the mean tumor volume was 1.6±0.44 cm³ on day 20 and 3.7±1.55 cm³ on day 30 (group 2). A significant difference was observed between groups 1 and 2 only on day 20. When 0.1 mg of baicalein was subcutaneously administered for a total of 10 times from day 11 to day 20, the mean tumor volume was 1.56±0.38 cm³ on day 20 and 3.38±0.8 cm³ on day 30, and tumor growth was significantly inhibited (group 3). The difference between group 3 and group 1 on both day 20 and day 30 was significant (p<0.01).

Discussion

AA is cleaved from membrane phospholipids by phospholipases. The metabolism of AA can be catalyzed by one of two enzyme families, COX or LOX. Several lines of evidence have shown the importance of the LOX pathway in regulating the growth of cancer cells. With regard to 5-LOX, Ghosh et al. (6) reported that, in human prostate cancer cell lines, the 5-LOX pathway is important in growth regulation and that inhibition of 5-LOX by a specific inhibitor MK886 can trigger massive apoptosis. Gupta et al. (9) showed that 5-LOX is up-regulated in human prostate cancer tissues and that 5-LOX inhibition reduces human prostate cancer cell growth. Avis et al. (10) also reported that the 5-LOX pathway is involved in a variety of lung cancer cell growth, and that the 5-LOX metabolic product 5-HETE can stimulate proliferation of small cell lung cancer cell lines and block growth inhibition caused by exposure of lung cancer cell lines to specific 5-LOX inhibitors. In addition, they revealed that tumor growth was significantly suppressed in an in vivo experiment using NDGA in nude mice bearing heterotransplants of the lung cancer cell line NCI-H209.

With regard to 12-LOX, Wong et al. (11) showed that 12-LOX mRNA and protein are expressed in gastric cancer cell lines, that the 12-LOX inhibitor baicalein and 12-LOX antisense induce apoptosis of gastric cancer cell lines and that the biochemical characteristics of apoptosis are p53-independent combined with a decrease in bcl-2 expression. Ding et al. (12) reported that inhibition of the 12-LOX pathway abolishes proliferation and induces apoptosis in several pancreatic cancer cell lines, that the 12-LOX product 12(S)-HETE stimulates pancreatic cancer cell proliferation and reverses 12-LOX inhibitor-induced growth inhibition and that several intracellular signal transduction pathways are activated upon stimulation with 12-HETE. We also found that, in a murine bladder cancer cell line (MBT-2), all of the LOX inhibitors, the general

Figure 2. Apoptosis in MBT-2 cells treated with baicalein is shown. Cells were treated with acridine orange and examined under a fluorescent microscope. A: nontreated, B: treated with 1 μM baicalein for 48h.

Figure 3. 12-LOX antisense oligonucleotide-induced growth inhibition in MBT-2. **p<0.01 versus corresponding control.
References


Table I. Antitumor activity of baicalein on MBT-2-bearing mice.

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<tr>
<th>Groups and treatment</th>
<th>Tumor volume, cm³</th>
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<tr>
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<tr>
<td>Group 1: nontreated</td>
<td>0.75±0.17</td>
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<tr>
<td>Group 2: baicalein11-20 (0.05mg) day</td>
<td>0.76±0.18</td>
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<tr>
<td>Group 3: baicalein (0.1mg) day11-20</td>
<td>0.77±0.19</td>
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Tumor volume is expressed as mean±SD. **p<0.01, versus control (nontreated)