Suppression of Lung Metastasis by Aspirin but Not Indomethacin in an in vivo Model of Chemically Induced Hepatocellular Carcinoma

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To examine the effect of non-steroidal anti-inflammatory drugs on metastasis formation, aspirin (ASP, 0.5% in diet) and indomethacin (IM, 0.005% in drinking water) were applied to an in vivo highly metastatic rat hepatocellular carcinoma (HCC) model in F344 male rats. Administration for 8 weeks after induction of highly metastatic HCC by sequential treatment with diethylnitrosamine and N-nitrosomorpholine did not cause any significant change in survival rate or body weight. Multiplicity of HCC in the liver increased during ASP or IM treatment without any significant histological alteration. Although absent in the rats killed at the end of the period of carcinogen exposure, lung metastasis at the end of the experiment was found in 100%, 89% and 100% of rats in the control, ASP and IM groups, respectively. Degree of metastasis was classified into three groups according to the number of metastatic nodules, i.e., slight (1–5 nodules), moderate (6–50) and severe (more than 51), which amounted to 0%, 43% and 57% in the control group. ASP significantly reduced the degree of metastasis, the incidences being 33%, 44%, and 11%, respectively, whereas IM was without significant influence. Both agents suppressed cell proliferation in HCCs, without any alteration of pan-cadherin expression. However, expression in HCC of mRNAs for intercellular adhesion molecule-1 and vascular cell adhesion molecule-1, both of which are considered to play key roles in attachment of cancer cells to the endothelium, was significantly suppressed by ASP. Thus, the present study demonstrated that ASP, but not IM, has the potential to inhibit lung metastasis of rat HCC in vivo, possibly via reduced attachment of tumor cells to the vascular endothelium. Moreover, these data indicate this in vivo model for induction of rat highly metastatic HCC to be a useful tool for the assessment of the efficacy of therapeutic treatments to block metastasis formation.

Key words: Aspirin — Indomethacin — Lung metastasis — in vivo lung metastasis model — VCAM-1

We have recently established an in vivo lung metastasis model in which hepatocellular carcinoma (HCC) induced by sequential treatment with two hepatocarcinogens, diethylnitrosamine (DEN) and N-nitrosomorpholine (NMOR), metastasize to the lung very frequently.1 This model has advantages for investigation of the mechanisms of multistep metastasis of malignant tumors and for the assessment of the efficacy of therapeutic treatments against metastasis in vivo.

The metastatic cascade is a continuous process which begins with proliferation of the primary tumor and ends with proliferation of the metastatic foci.2 Thus, interference with cell proliferation might prevent metastasis formation. Nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin (ASP) and indomethacin (IM) are well-known as potential chemopreventive agents through their modulation of levels of prostaglandins, PGE2 and PGF2α, and cyclooxygenase (COX) in the colon,3–5 and also other organs.6–9 In order to evaluate the anti-metastatic potential of NSAIDs, two typical examples, ASP and IM were examined here using our in vivo lung metastasis model. Cell proliferation in HCC and expression of cadherin10–12 as a factor related to the detachment of tumor cells, were also assessed. In addition, expression in HCC of the mRNAs for E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) which are considered to play key roles in attachment of tumor cells to the vascular endothelium, were also evaluated, along with COX-1 and COX-2 expression.

MATERIALS AND METHODS
Chemicals DEN, NMOR, acetylsalicylic acid (ASP), and IM were obtained from Tokyo Kasei Kogyo Co., Ltd.
(Tokyo), pan-cadherin antibody from Sigma Immuno Chemicals (St. Louis, MO), and monoclonal anti-rat proliferating cell nuclear antigen (PCNA) antibody from DAKO Japan Corp. (Tokyo).

**Animals** Five-week-old male F344 rats were obtained from Charles River, Japan, Inc. (Atsugi) and randomly housed, three animals per plastic cage, with hard wood chips as bedding in an air-conditioned room under specific pathogen-free (SPF) conditions at 22±2°C and 55±5% humidity with a 12 h light/dark cycle. Food (Oriental MF, Oriental Yeast Co., Tokyo) and tap water were available *ad libitum*.

**Treatment** As previously reported,1) 6-week-old male F344 rats were given a single i.p. injection of DEN at a dose of 100 mg/kg body weight as an initiation of liver carcinogenesis, and then received 120 ppm NMOR in the drinking water for 16 weeks. The rats given the two carcinogens were then divided into three groups. Those in groups 2 and 3 were administered 0.5% ASP in the diet, and 0.005% IM in the drinking water, respectively. The doses were selected on the basis of a previous chemoprevention experiment as the highest doses without side effects, including ulceration.8) Group 1 served as a control, which was maintained without further treatment. Although the total experimental period was originally planned to be 24 weeks, the experiment was terminated at week 22 due to poor survival. An interim sacrifice was performed at week 16 to confirm the induction of HCC but no formation of lung metastases. All animals were killed under ether anesthesia.

The major organs were weighed, then parts of liver tumors were excised and one of the liver slices was fixed in cold acetone. Samples were also frozen in liquid nitrogen and remaining liver tissue and samples from other organs were fixed in 10% buffered formalin. The lungs were inflated with 10% neutral buffered formalin injected through the trachea and each was separated into three right lobes and one left lobe. Step sections of each lobe were made with an interval of at least 0.3–0.4 cm and a total of 15 to 20 plane sections of the lungs were prepared for each rat, processed for production of paraffin sections, and stained with hematoxylin and eosin. The acetone-fixed liver sections were immunohistochemically stained for binding of monoclonal pan-cadherin antibody and monoclonal anti-rat PCNA antibody using the avidin-biotin-peroxidase complex method (Vectastain ABC kit, Vector Lab., Inc., Burlingame, CA). After deparaffinization, sections were sequentially treated with 0.5% H2O2 in methanol for 30 min, 0.05% Tween 20 in phosphate-buffered saline (PBS) for 3 min twice at room temperature, 2.5 N HCl for 20 min, 0.1% trypsin for 15 min at 37°C, and then 5% skim milk in PBS for 1 h. The sections were then incubated with diluted anti-PCNA antibody (1:50) for 2 h at 4°C, or anti-pan-cadherin antibody (1:500) overnight at 4°C, followed by sequential exposure to biotin-labeled goat anti-rabbit IgG and ABC. The sites of peroxidase binding were demonstrated with diaminobenzidine. Step sections of livers were processed routinely for hematoxylin and eosin staining for identification of liver lesions.

**Quantitative analysis**

**Lung metastatic nodules:** The method for quantitative analysis of lung metastatic nodules was reported previously.3) Lesions were counted under a light microscope and the total areas of lung tissues per animal were measured with the assistance of an image analyzer (VIP-21C, Olympus-Ikegami Tsushin Co., Tokyo).

**Cadherin staining:** Pan-cadherin immunohistochemistry was examined in HCC, adenomas and surrounding normal tissue. Immunostained sections, prepared as previously reported,1) were examined under a light microscope connected to an image analysis system, Image Processor for Analytical Pathology (IPAP, Sumika Technos Corp., Osaka).13) Binary digitized images of liver lesions were obtained automatically by the programmed segmentation procedure. The length of cell surfaces positive for pan-
cadherin staining was measured at a magnification of 600 (at least 10 fields) for each lesion. Here we use the term “staining index” to refer to the parameters expressed as the average positive length per unit area for each immunostained section.

**Competitive RT-PCR:** Immediately after the animal experiment was terminated, total RNAs from primary HCC were extracted using ISOGEN (Nippon Gene, Tokyo). After DNase treatment, 1 µg of the RNA was converted to cDNA with avian myoblastosis virus reverse transcriptase (TaKaRa, Ohtsu) in 20 µl of reaction mixture. Aliquots of 2 µl of cDNA samples were then subjected to quantitative PCR in 20 µl reaction mixtures using FastStart DNA Master SYBR Green I and a Light Cycler apparatus (Roche Diagnostics, Mannheim, Germany). Primers used for COX-1 were 5′-CTGGCAGCTGCTCATCCATCTA-3′ and 5′-CAGTATCCGTGTGTCAGCAAGA-3′; for COX-2, 5′-TATCAGGTGCATGCGAGGAAGG-3′ and 5′-ATTGAGGCAGTCGGGTCTC-3′; for E-selectin, 5′-CAGGAAACAAATGCATCATGG-3′ and 5′-GCTGTTTCTGTCACCATTCCA-3′; for ICAM-1, 5′-GAACTGCTCTTCCTGATCGAA-3′ and 5′-TTTTCCTGTCACCATTCCA-3′; for VCAM-1, 5′-CAAGGACTATTTTTCGCCC-GA-3′ and 5′-GTCTGAATGCATGGCTTGGTTT-3′; for GAPDH, 5′-TGATTCTACCCACGGCAAGTTC-3′ and 5′-TTTTCCTGTCACCATTCCA-3′. Initial denaturation at 95°C for 10 min was followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 5 s, and elongation at 72°C for 30 s. The fluorescence intensity of the double-strand-specific SYBR Green I, reflecting the amount of formed PCR product, was monitored at the end of each elongation step. GAPDH mRNA levels were used to normalize the sample cDNA content.

**Statistical analysis** Statistical analysis of intergroup differences in means and incidence was carried out using analysis of variance (ANOVA), and the Kruskal-Wallis test, respectively. When a positive result was obtained, Scheffe’s multiple comparison test was applied to evaluate statistical significance between treatment subgroups.

**RESULTS**

Survival rates of all rats decreased gradually from week 15, and at the end of experiment, they were 34%, 43%, and 33% in the control, ASP and IM group, respectively (Fig. 1a). Although a slight change in the body weight curve was observed for the IM treatment group, no significant alteration was apparent throughout the experimental period (Fig. 1b). Thus, the survival rates and loss of body weight were not improved by the ASP or IM treatment. Furthermore, no significant differences were noted in the relative liver and kidney weights (Table I). There was no evidence of toxic effects such as ulceration in the gastrointestinal tract caused by the ASP or IM (data not shown).

The incidence of HCC was 100% in all groups at week 22 (Table II). The multiplicity in the control group significantly increased from 6.8±1.9 at week 16 to 15.4±4.5 at

Table I. Body Weight and Relative Organ Weights

| Experimental weeks | Treatment  | No. of rats | Body weight (g) | Liver weight (%) | Kidney weight (%) |
|--------------------|------------|-------------|-----------------|------------------|-------------------|
| 16                 | Control    | 10          | 252.7±13.3      | 8.7±0.8          | 0.6±0.0           |
| 22                 | Control    | 7           | 262.9±14.6      | 10.3±1.5         | 0.7±0.0           |
| 22                 | Aspirin    | 9           | 265.1±9.6       | 10.2±0.8         | 0.7±0.0           |
| 22                 | Indomethacin| 7          | 251.1±18.9      | 9.9±1.1          | 0.8±0.1           |

No significant difference.

Table II. Effects of Aspirin and Indomethacin on Development and Differentiation of Hepatocellular Carcinoma (HCC)

| Experimental weeks | Treatment | No. of rats | Incidence (%) | Multiplicity (No./rat) | Differentiation (%) |
|--------------------|-----------|-------------|---------------|------------------------|---------------------|
|                    |           |             | HCC in the liver |                        | Well | Moderate | Poor |
| 16                 | Control   | 10          | 10 (100)      | 6.8±1.9                | 45   | 35       | 20   |
| 22                 | Control   | 7           | 7 (100)       | 15.4±4.5               | 24   | 48       | 28   |
| 22                 | Aspirin   | 9           | 9 (100)       | 17.3±5.3               | 18   | 61       | 21   |
| 22                 | Indomethacin| 7          | 7 (100)       | 15.0±5.1               | 20   | 57       | 23   |

a) P<0.001 vs. control group at week 22.
week 22. ASP and IM did not exert any significant effect on the development or differentiation of HCCs (Table II).

Although no lung metastasis was evident in the rats killed at week 16, almost 100% incidence was noted in all groups at week 22. Average numbers of metastatic nodules per rat were 71.7±56.1, 22.9±23.0, and 76.5±78.6 in the control, ASP and IM group, respectively. Although there was a tendency of suppression by ASP, it was not significant. Fig. 2 shows the distributions of actual number of metastatic foci in the lung. Each dot represents the total number of metastatic nodules observed in one rat, ranging between 14 and 174, 0 and 56, and 3 and 211, in the control, ASP and IM groups, respectively.

Degree of metastasis was classified into three groups according to the number of metastatic lesions per lung; slight (1–5 lesions), moderate (6–50) and severe (more than 51). As shown in Table III, ASP but not IM significantly reduced the degree of metastasis. The values of the PCNA indices demonstrated that cell kinetics in non-tumorous liver tissue were not altered by either ASP or IM, but in HCCs significant suppression was evident with ASP and a marginal decrease with IM (Table IV).

Pan-cadherin expression was quantitatively assessed on immunohistochemically stained sections. Although the staining pattern was rather homogeneous in the cell surface in normal-appearing tissue, it was heterogeneous in HCC, with a reduction in the staining indices (Table V). No significant alteration of pan-cadherin expression was observed with ASP or IM treatment on either HCC or surrounding non-tumorous tissues.

Expressions of E-selectin, ICAM-1 and VCAM-1, in addition to COX-1 and COX-2, in HCC were investigated by quantitative RT-PCR (Fig. 3). Expressions of COX-1 and -2 in HCC were significantly decreased by treatment

Fig. 2. Distribution of numbers of lung metastases. Each dot represents data for a single rat.

| Table III. Effects of Aspirin and Indomethacin on Degree of Lung Metastasis |
|------------------------|----------------|----------------|----------------|
| Treatment              | No. of rats | Degree of lung metastasis (%) | p |
|                        |             | (-) | Slight | Moderate | Severe |
| Control                | 7           | 0   | 0      | 3 (43)   | 4 (57)  |
| Aspirin                | 9           | 1 (11)| 3 (33) | 4 (44)   | 1 (11)  | 0.047   |
| Indomethacin           | 7           | 0   | 1 (14) | 3 (43)   | 3 (43)  | 0.780   |

a) Degree of lung metastasis was classified according to the number of metastatic nodules; slight (1–5 nodules), moderate (6–50) and severe (more than 51).

b) P values were calculated by means of Scheffe’s test after confirming significance with the Kruskal-Wallis test (P=0.049).

| Table IV. Effects of Aspirin and Indomethacin on Cell Proliferation in the Liver |
|-------------------------------|----------------|----------------|----------------|
| Treatment                     | Non-tumorous tissues | HCC |
|                               | No. of regions examined | Labeling index (%) | No. of HCC examined | Labeling index (%) |
| Control                       | 5           | 2.2±1.4         | 7               | 35.9±9.5          |
| Aspirin                       | 9           | 2.0±1.5         | 11              | 23.1±6.4**        |
| Indomethacin                  | 6           | 2.2±1.1         | 8               | 27.8±7.9          |

** P<0.01 vs. control group.
with NSAIDs. Although expression of E-selectin was not altered, expressions of ICAM-1 and VCAM-1 were significantly decreased by ASP (Fig. 3).

**DISCUSSION**

In the present study, ASP but not IM significantly reduced the severity of lung metastasis, but not the average number. This indicates that the effect of ASP was marginal. It has been reported that suppression of cell proliferation of tumor cells is associated with inhibition of metastasis formation. However, this might not be a causal relationship but rather secondarily associated with some other factors, such as angiogenesis inhibition, because the present study revealed that although ASP and IM exerted inhibitory effects on cell proliferation of HCCs, only ASP suppressed lung metastasis formation. Thus, it is suggested that inhibition of cell proliferation per se may not be involved in the mechanism of inhibition of lung metastasis by ASP.

Epidemiological studies revealed that NSAIDs, such as ASP and IM, which suppress COX activity, possess considerable potential as chemopreventive agents for colorectal cancer. Constitutive expression of COX-2 has been demonstrated to lead to phenotypic changes that alter the metastatic potential of colorectal cancer cells, and a selective COX-2 inhibitor (JTE-522) was found to exert inhibitory effects on experimental hematogenous metastasis of colon cancer. However, the present data demonstrated that IM did not suppress lung metastasis formation in spite of down-regulation of COX-2, indicating no direct involvement of this enzyme in the inhibitory effect on HCC metastasis.

Change in the expression of cadherin has been implicated in the detachment of tumor cells in the primary site, a phenomenon which is considered as the first step of the metastatic process, and subsequently tumor cells can be transported to a target organ via lymphatic or blood vessels, followed by arrest and growth as metastatic nodules. Loss of cadherin expression is frequent in human and murine high-grade epithelial cancers, and re-establishing functional cadherin complexes in tumor cell lines results in reversion from an invasive to a benign epithelial phenotype. In the present study, however, neither ASP nor IM exerted any apparent influence on cadherin expression within HCC. Therefore, the mechanism of inhibition by ASP might be mainly in a stage of the metastatic cascade after the primary site, such as attachment to the vascular endothelium or re-invasion or re-proliferation in the lung.

The attachment of a cancer cell to the vascular endothelium is a complex phenomenon involving a number of cell adhesion molecules (CAMs). Among these latter, E-selectin, ICAM-1 and VCAM-1 are considered to play primary roles in hematogenous metastasis. Induction of E-selectin, ICAM-1 and VCAM-1 is mediated by the transcription factor nuclear factor-kappa B (NF-kB). ASP has been shown to inhibit NF-kB dependent transcription, and these transcriptions appear not to be related to the inhibition of COX activity, since IM was ineffective.

In the present study, ASP significantly suppressed the expressions of ICAM-1 and VCAM-1, indicating a proba-

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**Table V. Immunohistochemical Evaluation of Cadherin Stainings in Liver**

| Treatment      | No. of regions examined | Positive lengtha | Non-tumorous tissues | HCC |
|----------------|------------------------|------------------|----------------------|-----|
| Control        | 7                      | 2.7±0.4          | 0.3±0.2              |
| Aspirin        | 9                      | 2.3±0.6          | 0.3±0.1              |
| Indomethacin   | 7                      | 2.4±0.6          | 0.2±0.1              |

No significant difference.

a) Positive length=positive length of cell membrane/unit examined area (mm²).

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**Fig. 3. Results of quantitative RT-PCR for COX-1, COX-2, ICAM-1, VCAM-1 and E-selectin in HCCs with or without treatment with aspirin or indomethacin. Y axis, mean±SE of relative expression values (eight HCCs in each group). *, **, *** P<0.05, P<0.01, P<0.001, significantly different from control value.
ble role of inhibition of attachment of tumor cells to the vascular endothelium.

In conclusion, the present study demonstrated that ASP, but not IM, has the potential to inhibit lung metastasis by rat HCC in vivo, the mechanism apparently involving neither inhibition of cell proliferation nor detachment from primary tumors. Inhibition of attachment to the vascular endothelium in the lung is more likely to be the mechanism responsible for the suppression of lung metastasis formation by ASP. This in vivo model for induction of rat highly metastatic hepatocellular carcinomas is clearly a useful tool for the assessment of the efficacy of therapeutic treatments for metastasis formation and for analysis of individual steps in the metastatic process.

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