Suppression of metastasis by nuclear factor-κB inhibitors in an in vivo lung metastasis model of chemically induced hepatocellular carcinoma

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To evaluate the suppressive effects of nuclear factor kappa B (NF-κB) inhibitors on metastasis, three agents, pentoxifylline (PTX, 0.5% in diet), N-acetyl-L-cysteine (NAC, 0.5% in diet), and aspirin (ASP, 0.5% in diet) were applied in an in vivo highly metastatic rat hepatocellular carcinoma (HCC) model in F344 male rats. Administration of NF-κB inhibitors 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. The incidence of HCC was 100% at week 23, regardless of treatment with NF-κB inhibitors. PTX, NAC, and ASP did not exert any significant effect on the development or differentiation of HCCs, although PTX tended to decrease the multiplicity of HCC. Although no lung metastasis was observed in the rats killed at the end of the period of carcinogen exposure, lung metastasis was found in 100% of animals in all the groups at the end of the experiment. Multiplicity of lung metastasis was significantly decreased by PTX and NAC, whereas ASP was without significant influence. The size of metastatic nodules was also significantly reduced in the PTX treatment group. Furthermore, the inhibitory κ-B (IkB) protein level, considered to be a marker for the degree of NF-κB transcription, was significantly suppressed by PTX. mRNA expression in HCC for vascular cell adhesion molecule-1 (VCAM-1), which is considered to play a key role in attachment of cancer cells to the endothelium, was significantly suppressed by PTX. Among the splicing variants of VEGF, VEGF-A120, VEGF-A144, VEGF-A164, and VEGF-A188, suppressed mRNA expression of VEGF-A188 appeared to be correlated with suppression of lung metastasis formation. In conclusion, the present study demonstrated that NF-κB inhibitors have the potential to inhibit lung metastasis from rat HCCs in vivo, and PTX is especially promising. Its mechanism of action may involve suppression of VCAM-1 and VEGF-A188 production.

W e 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), very frequently metastasizes to the lung.15 This model has advantages for investigation of the mechanisms of multistep metastasis by malignant tumors, and for the assessment of the efficacy of therapeutic treatments against metastasis in vivo.

Our previous study demonstrated that aspirin (ASP) has the potential to inhibit lung metastasis by rat HCCs in vivo, although the observed suppressive effect was marginal.20 The mechanism appeared to involve a decrease in the intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), which play important roles in attachment of tumor cells to the vascular endothelium.21

Induction of ICAM-1 and VCAM-1 is mediated by the transcription factor nuclear factor-kappa B (NF-κB).22, 23 ASP has been shown to inhibit NF-κB-dependent transcription weakly.7 Therefore, a stronger inhibitor of NF-κB might be expected to have a stronger inhibitory effect on lung metastasis formation.

In order to evaluate the suppressive effects of NF-κB inhibitors, we examined three examples, pentoxifylline (PTX),8, 9, 10 N-acetyl-L-cysteine (NAC),10, 11 and ASP,7, 12 in our in vivo lung metastasis model. To evaluate the degree of inhibition of NF-κB transcription, inhibitor of κB (IkB) protein levels in HCCs were determined by western blotting. NF-κB may influence metastasis through effects on tumor angiogenesis,12, 13 as well as cell adhesion molecules (CAM).14 For this reason, mRNA expression levels of E-selectin,15 ICAM-1,14, 15 and VCAM-116 were also evaluated, along with those of four splicing variants of VEGF-A, VEGF-A120, VEGF-A144, VEGF-A164, and VEGF-A188.

Materials and Methods

Chemicals and animals. DEN, NMOR, and PTX were obtained from Sigma-Aldrich (Tokyo), and NAC and ASP from Tokyo Kasei Kogyo Co., Ltd. (Tokyo). 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. The experimental protocol is shown in Fig 1. As previously reported,12, 13 6-week-old male F344 rats were given a single i.p. injection of DEN at a dose of 100 mg/kg body weight for initiation of liver carcinogenesis, and then received 120 ppm NMOR in the drinking water for 14 weeks. The rats given the two carcinogens were divided into four groups. Those in groups 2, 3, and 4 were administered 0.5% PTX, 0.5% NAC, and 0.5% ASP in the diet, respectively. The dose was selected on the basis of the results of a previous 2-week study (data not shown). Group 1 served as a control, maintained without further treatment. An interim sacrifice was performed at week 14 to confirm the induction of HCCs, and the absence of lung metastases. All animals were killed under ether anesthesia.

The major organs were weighed, then parts of the liver tumors were excised and the liver slices were frozen in liquid nitrogen. The remaining liver tissue and samples from other organs were fixed in 10% buffered formalin. The livers 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 15 to 20 plane sections of the lungs

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were prepared for each rat and processed for production of paraffin sections, which were stained with hematoxylin and eosin.

**Quantitative analysis of lung metastatic nodules.** The method for quantitative analysis of lung metastatic nodules in sections was reported previously. 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).

**Western blot analysis for IgG-κ protein expression.** To examine the Igκ protein expression, HCC tissues cut from frozen liver sections were lysed in radio-immunoprecipitation (RIPA) buffer [20 mM Tris-HCl pH 7.4, 0.1% SDS, 1% Triton X-100, 1% Na deoxycholate]. Aliquots of 50 µg/ml were denatured by boiling in SDS-PAGE sample buffer, resolved on 12% SDS-PAGE gels, and transferred to polyvinylidene fluoride (Immobilon-P PVDF) membranes (Millipore Corp., Marlborough, MA). Equal protein loading was confirmed with anti-β-actin antibody. The membranes were blocked in PBS containing 5% nonfat dry milk and probed with goat anti-Igκ (Santa Cruz) at 1:10,000 dilution. Peroxidase-conjugated anti-goat IgG (Santa Cruz) was used as the secondary antibody, and protein expression was then quantitated with a densitometer detection system (ECL detection KIT from Amersham). Relative protein expression was normalized with the total areas of lung tissues per animal and from metastatic nodules in lung (Table 1). There was no significant difference in the cause of death among groups (Table 1). Although slight changes in the body weight curve were observed for the PTX and NAC treatment groups, no significant alteration was apparent throughout the experimental period (Fig. 3B). Thus, the survival rates and loss of body weight were not improved by the treatment with NF-κB inhibitors. Furthermore, no significant differences were noted in the relative liver and kidney weights (data not shown).

**Histology of primary HCCs.** At week 16, the incidence and the multiplicity of HCCs were 100% and 4.2 (Fig. 4A), respectively, 45% of HCCs were well differentiated, 35% were moderately differentiated (Fig. 4C), and 20% were poorly differentiated (Fig. 4C).

**Results**

Effects of NF-κB inhibitors on survival and body weight change. Survival rates of all rats decreased gradually from week 15 and at the end of the experiment, they were 24%, 29%, 24%, and 14% in the control, PTX, NAC, and ASP groups, respectively (Fig. 3A). The main cause of death was bleeding from HCC in liver or from metastatic nodules in lung (Table 1). There was no significant difference in the cause of death among groups (Table 1). Although slight changes in the body weight curve were observed for the PTX and NAC treatment groups, no significant alteration was apparent throughout the experimental period (Fig. 3B). Thus, the survival rates and loss of body weight were not improved by the treatment with NF-κB inhibitors. Furthermore, no significant differences were noted in the relative liver and kidney weights (data not shown).

**Fig. 1.** Experimental protocol. Six-week-old male F344 rats were given a single i. p. injection of DEN at a dose of 100 mg/kg body weight, and received 120 ppm NMOR in the drinking water for 14 weeks. Then rats were divided into two groups, control group without further treatment and NF-κB inhibitor treatment group (0.5% PTX, 0.5% NAC, or 0.5% ASP in the diet, respectively). \(\uparrow\) DEN injection; \(\downarrow\), NMOR treatment; \(\uparrow\), NF-κB inhibitor treatment.

**Fig. 2.** Structures of splicing variants of the VEGF-A family. The expression of VEGF-A is subject to complex regulation as alternative splicing of the murine VEGF gene results in at least four isoforms containing 120, 164, 144, and 188 amino acids. Besides the common regions (exons 1–5 plus exon 8) that form VEGF-120, inclusion of an additional exon 6a generates VEGF-A144, and an additional exon 6a and 7 generates VEGF-A188. The primers for splicing variants of VEGF-A were designed to obtain specific PCR products.
differentiated. At week 23, regardless of the treatment, the incidence of HCC was 100% (Fig. 4B, Table 2). Although treatment with PEN tended to decrease the multiplicity of HCC, PEN, NAC, and ASP did not exert significant effects on the development or differentiation of HCCs (Table 2). Vessel invasion of HCC was observed in the liver (Fig. 4, D and E), but no significant differences were observed in the incidences of vessel invasion (Table 2).

**Suppression of lung metastasis formation.** Although no lung metastasis was evident in the rats killed at week 14, the incidences of lung metastasis were 100% in all groups at week 23 (Fig. 4, F, G, and H). Regarding the number of lung metastases, significant suppression in the PTX group and a tendency for inhibition in the NAC group were observed, but no suppressive effect was apparent in the ASP treatment group (Fig. 5A). Significant reduction of the size of metastatic nodules was noted with PTX and NAC, but not ASP (Fig. 5B).

**PTX, NAC, and ASP suppression of iκB protein levels as determined by western blotting.** Western blot analysis was performed to examine the effect of NF-κB inhibitors, PTX, NAC, and ASP, on iκB protein levels in HCCs. Fig. 6A shows iκB and β-actin protein expression levels in one HCC from an animal treated with PTX, NAC, or ASP. Fig. 6B shows relative iκB protein levels in HCC. Tendencies for decrease were observed in the order from PTX, NAC to ASP, attaining significance with the PTX treatment group.

**Suppression of expression of CAMs.** Expression of VCAM-1, ICAM-1, and E-selectin in HCC was investigated by quantitative RT-PCR (Fig. 7). PTX treatment significantly decreased expression of VCAM-1. NAC also reduced VCAM-1 expression while ASP decreased expression of VCAM-1 and ICAM-1, but without significance (Fig. 7). Treatment of NF-κB inhibitors suppressed mRNA expression of VEGF splicing variants, VEGF-A188, A164, A144, and A120. Expression of VEGF-A188 and A144 was suppressed in the order from PTX, NAC, and ASP. PTX treatment significantly suppressed expression of VEGF-A188, A164, and A144. On the other hand, NAC treatment significantly suppressed expression of VEGF-A164 and A120 (Fig. 8).

### Table 1. Survival rates and main cause of death

| Treatment | Initial No. of rats | Survival rate (%) | Main cause of death (%) |
|-----------|---------------------|-------------------|-------------------------|
|           |                     |                   | Bleeding from | Unknown |
|           |                     |                   | Liver   | Lung   |   |
| Control   | 19                  | 4 (21)            | 5 (26)   | 8 (42) | 2 (11) |
| PTX       | 21                  | 6 (29)            | 10 (48)  | 5 (24) | 0    |
| NAC       | 21                  | 5 (24)            | 10 (48)  | 6 (29) | 0    |
| ASP       | 20                  | 3 (15)            | 11 (55)  | 6 (30) | 0    |

1) Bleeding from HCC in liver.
2) Bleeding from metastatic nodules.
No significant difference.

### Table 2. Effects of NF-κB inhibitors on histology of primary HCC

| Treatment | No. of rats | HCC found in the liver | Vessel invasion (%) |
|-----------|-------------|------------------------|---------------------|
|           |             | Differentiation (No./rat) | well | moderate | poor | |
| Control   | 4           | 4 (100) | 9.2±3.6 | 20%  | 22%  | 59%  | 3 (75) |
| PTX       | 6           | 6 (100) | 6.8±1.6 | 7%   | 39%  | 54%  | 6 (100) |
| NAC       | 5           | 5 (100) | 8.4±2.6 | 7%   | 36%  | 57%  | 4 (80) |
| ASP       | 3           | 3 (100) | 9.0±3.6 | 15%  | 26%  | 59%  | 3 (100) |

No significant difference.

**Discussion**

The NF-κB transcription factor, composed of two proteins, p50 and p65, is a pleiotropic activator that participates in the induction of a wide variety of cellular genes. The contribution of NF-κB to the process of metastasis has been explored in relation to CAMs, and recently, VEGF expression was found to be significantly suppressed by NF-κB signaling blockade, and promoted by coactivation of NF-κB.

In the present study, the NF-κB inhibitors PTX, NAC, and ASP showed protective effects in our in vivo lung metastasis model. PTX, widely used as a hemorheological agent in the treatment of peripheral vascular disease, was earlier shown to suppress lung metastasis formation by B16F10 melanoma and NAC, a chemopreventive agent that acts through a variety of mechanisms, inhibits VEGF production in human melanoma cell lines, and invasion of endothelial cells, and invasion of human bladder cancer cells through the suppression of MMP-9. ASP has been demonstrated to inhibit angiogenesis, invasion and metastasis of EBV-associated tumors, and HGF-induced invasiveness of HepG2 human hepatoma cells.
Our previous study demonstrated that ASP has the potential to inhibit lung metastasis by rat HCC in vivo. Among the NF-κB inhibitors evaluated in the present study, PTX exerted the strongest effects on lung metastasis formation and NAC had rather less influence, while ASP did not significantly reduce lung metastasis. Although PTX and NAC suppressed lung metastasis, they did not improve the survival rates in this experiment. This is mainly because the increase in the mortality rates owing to bleeding from primary HCC diminished the decrease that resulted from suppression of lung metastasis. However, the increase and decrease were not significant, and treatment with NF-κB inhibitors did not affect the incidences and multiplicities of HCCs in liver. Therefore, further studies are necessary to elucidate the reasons why PTX and NAC did not affect the survival rates.

ASP was demonstrated to inhibit lung metastasis in our previous study. However, in the present study, we did not find a significant effect. This is mainly because fewer rats could be examined in both the control and ASP groups due to the lower survival rates in the present study. Therefore, the marginal effects on lung metastasis in the previous study were not apparent in the present study.

The IκB family has been shown to control the function of NF-κB complexes, and IκB protein has been shown to activate NF-κB when it is phosphorylated or cleaved by proteasomes through a ubiquitine-dependent pathway. Increased nuclear basal NF-κB activity (p50/p60) results from an in-

Fig. 4. Histology of liver HCC and metastatic nodules. At week 16, HCCs were observed (A) at the incidence of 100%. At week 23, regardless of treatment, 100% incidence was also observed and the number of HCC was increased (D). About 40% were moderately differentiated HCC (C). Vessel invasion of HCC was observed in the liver (D and E). Although no lung metastasis was evident in the rats killed at week 14, the incidences of lung metastasis were 100% in all groups at week 23 (F). Microscopically, small (G) and large (H) metastatic nodules were observed.
creased cytoplasmic IκB-α degradation in human melanoma cell lines. In the present study, IκB protein expression was suppressed by test compounds in the order of PTX, NAC, and ASP, in accordance with previous findings, and the present data. Therefore, these results suggest that the mechanism of reduction of lung metastasis formation observed in this study may involve inhibition of NF-κB transcription.

ICAM-1, VCAM-1, and E-selectin are induced through the transcription of NF-κB. In the present study, ASP treatment reduced mRNA expression of both ICAM-1 and VCAM-1, consistent with our previous findings. However, PTX and NAC reduced only VCAM-1 expression, suggesting a key role for this molecule. To clarify which type of cell is the target of the agents, we conducted immunohistochemical staining for ICAM-1 or VCAM-1 in liver and lung, but obtained no specific positive findings in either organ.

Recently, expression of VEGF, the most potent growth factor for tumor neovascularulation, has been shown to be suppressed by NF-κB signaling blockade, and promoted by coactivation of NF-κB. The expression of VEGF-A is subject to complex regulation, as alternative splicing of the human VEGF gene results in at least five isoforms containing 121, 145, 165, 189, or 206 amino acids (the corresponding murine isoforms are one residue shorter). Besides the common regions (exons 1–5 plus exon 8) that make up VEGF-121, inclusion of an additional exon 6a generates VEGF-A145, and an additional exon 6a and 7 generates VEGF-A189. Exon 7 contains a heparin and heparin-sulfate binding domain, and exon 6 possesses a second heparin-binding and extracellular matrix-binding domain, and the splice variant VEGF-A189 binds to extracellular matrix proteoglycans. In the present study, PTX significantly suppressed expression of VEGF-A splicing variants with heparin- and heparin-sulfate-binding domains.

These results suggest that the mechanism of the suppression of lung metastasis by PTX involves suppression of VEGF-A with heparin-binding domains. On the other hand, NAC, which had less influence on lung metastasis formation than PTX, sup-

Fig. 5. Inhibition of lung metastasis formation. (A) Significant suppression in the PTX group and a tendency for inhibition of development were observed in the NAC group, but no suppressive effects were observed in the ASP group. (B) The size of metastatic nodules was also significantly reduced with PTX or NAC, but not ASP. * P<0.05, significantly different from the control value. Number of rats examined in the CON, PTX, NAC, and ASP groups were 4, 6, 5, and 3, respectively.

Fig. 6. Western blot analysis for IκB. (A) Western blotting were examined for proteins extracted from HCCs in animals treated with PTX, NAC, and ASP. (B) Relative IκB protein levels in HCCs were decreased by the treatments in the order of PTX, NAC, and ASP. * P<0.05, significantly different from the control value. Values are shown as the mean±SE of five HCCs. Numbers of rats examined in the CON, PTX, NAC, and ASP groups were 4, 5, 5, and 3, respectively.

Fig. 7. Results of quantitative RT-PCR for VCAM-1, ICAM-1, and E-selectin in HCCs. Y-axis, mean±SE of relative expression values (eight HCCs in each group). * P<0.05, significantly different from the control value. Values are shown as the mean±SE of five HCCs. Numbers of rats examined in the CON, PTX, NAC, and ASP groups were 4, 5, 5, and 3, respectively.
Fig. 8. Results of quantitative RT-PCR for VEGF-A188, VEGF-A144, and VEGF-A120 in HCCs. Y axis, mean±SE of relative expression values (eight HCCs in each group). * P<0.05, significantly different from the control value.

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