Hydrogen peroxide-mediated nuclear factor κB activation in both liver and tumor cells during initial stages of hepatic metastasis

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Various factors involved in tumor metastasis are regulated by the transcription factor nuclear factor κB (NF-κB). Because NF-κB activation may contribute to establishment of hepatic metastasis, its activation in liver cells and tumor cells was separately evaluated in a mouse model of hepatic metastasis. pNF-κB-Luc, a firefly luciferase-expressing plasmid DNA depending on the NF-κB activity, was injected into the tail vein of mice by the hydrodynamics-based procedure, a well-established method for gene transfer to BALB/c male mouse liver. The luciferase activity in the liver was significantly increased by an intraportal inoculation of murine adenocarcinoma colon26 cells, but not of peritoneal macrophages, suggesting that the NF-κB in liver cells is activated when tumor cells enter the hepatic circulation. Then, colon26 cells stably transfected with pNF-κB-Luc were inoculated. The firefly luciferase activity, an indicator of NF-κB activity in tumor cells, was significantly increased when colon26/NFκB-Luc cells were inoculated into the portal vein of BALB/c male mice. The NF-κB activation in both liver and tumor cells was significantly inhibited by injection of catalase derivatives, which have been reported to inhibit hepatic metastasis of tumor cells. These findings indicate for the first time that NF-κB, a key agent regulating the expression of various molecules involved in tumor metastasis, is activated in both liver and tumor cells during the initial stages of tumor metastasis through a hydrogen peroxide mediated pathway. Thus, the removal of hydrogen peroxide will be a promising approach to treating hepatic metastasis. (*Cancer Sci* 2008; 99: 1546–1552)

Nuclear factor-κB (NF-κB) is a sequence-specific transcription factor that regulates the transcription of various genes, the products of which are involved in various biological processes, including inflammation, immune response, and the initiation and progression of cancer.[1–5] For example, tumor progression and metastasis are regulated by numerous NF-κB-regulated gene products, including matrix metalloproteinases (MMP), adhesion molecules, angiogenic factors and anti-apoptotic factors. Thus, the inhibition of NF-κB activation is a good target for inhibiting tumor growth and metastasis.[6] So far, several compounds, such as BAY11-7082, have been developed to inhibit the NF-κB activity by inhibiting proteasomal degradation of IκBα, the endogenous inhibitor of NF-κB.[7]

The activity of NF-κB and other transcription factors has been evaluated by an electrophoretic mobility shift assay (EMSA), which requires isolation of nuclear protein (radio)-labeled probes, and antibodies for its super-shift assay. In marked contrast, plasmid vectors expressing reporter genes under the control of the activity of a transcription factor, such as NF-κB, can be used to measure the activity of transcription factors. Using luciferase-expressing vectors is a simple, reliable and highly sensitive method of measurement. Cells stably transfected with vectors expressing the luciferase gene in response to the activation of any transcription factor have been developed and used to evaluate the activity of transcription factors in those cells.[8] Transgenic mice that express a luciferase, the transcription of which is dependent on NF-κB activity, have been developed and the NF-κB activation in whole animals has been examined by bioluminescence imaging.[9,10] We have developed a novel experimental system involving the injection of plasmid vectors into the tail vein of mice by the hydrodynamics-based procedure,[11–13] a well-established gene transfer method.[14,15] NF-κB activity in mouse liver was easily and quantitatively measured in this system, and we demonstrated that NF-κB in liver cells is activated when the liver suffers from thioacetamide-induced injury.[16]

Luciferase-based evaluation of NF-κB activity has advantages over conventional EMSA. First, the activity can be measured in live animals, so that the changes of the activity can be traced in individual animals. Next, the luciferase data are much more sensitive and quantitative than those obtained by gel-shift assays like EMSA. More importantly, NF-κB activity of a specific type of cell can be evaluated without any effect on the activity of other cells present in the system. The last characteristic is very important when the NF-κB activity is measured in a complex system, such as the hepatic metastasis of tumor cells. When plasmid vectors are selectively introduced to a specific type of cell, it is possible to examine the changes in NF-κB activity in those cells. Thus, the elucidation of NF-κB activity in liver cells and tumor cells during hepatic metastasis can help identify new targets for inhibition of tumor metastasis.

In the present study, the mouse model developed in our previous study[16] was used to evaluate the changes in NF-κB activity in liver cells when tumor cells enter the hepatic circulation. Murine adenocarcinoma colon26 cells were selected as tumor cells, which were inoculated into the portal vein of synergetic BALB/c mice. In addition, the NF-κB activity in colon26 cells was also evaluated by establishing colon26 cells stably transfected with the plasmid. The responsiveness of luciferase activity in colon26/NFκB-Luc cells was checked under culture conditions, and the transfectants were inoculated into the portal vein of mice. In both cases, catalase derivatives, which had been proved to inhibit reactive oxygen species (ROS)-dependent hepatic injuries and hepatic metastasis of tumor cells, were injected into the tail vein, and their effects on the NF-κB activity in both liver and tumor cells were quantitatively evaluated.

Materials and Methods

Animals. BALB/c and CDF_1 (6 weeks old, male) mice were purchased from the Shizuoka Agricultural Cooperative Association

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for Laboratory Animals (Shizuoka, Japan). Animals were maintained under conventional housing conditions. All animal experiments were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocols for animal experiments were approved by the Animal Experimentation Committee of Graduate School of Pharmaceutical Sciences of Kyoto University.

Chemicals. Bovine liver catalase (40 000 units/mg) and gadolinium chloride ($GdCl_3$) were purchased from Sigma Chemical. Two types of catalase derivatives, galactosylated (Gal-catalase) and succinylated (Suc-catalase) were synthesized as reported previously.[17] The enzymatic activity of catalase derivatives was measured by monitoring their ability to degrade hydrogen peroxide. D-luciferin was purchased from Promega.

Monitoring of NF-κB activity in mice. pNF-κB-Luc encoding firefly luciferase cDNA driven by a basic promoter element plus five tandem copies of NF-κB binding elements ([TGGGGACTTT-CCGC]5) was purchased from Stratagene (La Jolla, CA, USA). pRL-SV40 encoding the renilla luciferase cDNA under the control of SV40 promoter, which has no NF-κB binding sequences, was purchased from Promega. Plasmid DNA was injected into the tail vein of mice by the hydrodynamics-based procedure,[11–15] which is an established method for *in vivo* gene transfer to mouse liver. According to previous reports, plasmid DNA dissolved in 1.5 mL saline was injected into the tail vein within 5 s. Based on the sensitivity of the assays, the doses of plasmid DNA were set at 5 μg pNF-κB-Luc/mouse for *in vivo* imaging, and 1 μg each pNF-κB-Luc and pRL-SV40/mouse for quantitative analysis. Mouse tumor necrosis factor (TNF)-α was injected into the portal vein at a dose of 8 μg/kg to activate NF-κB in the liver.

Tumor cells. Murine colon adenocarcinoma colon26 cells were obtained from the Cancer Chemotherapy Center of the Japanese Foundation for Cancer Research (Tokyo, Japan). Cells were grown in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.15% $NaHCO_3$, 100 units/mL penicillin and 100 μg/mL streptomycin at 37°C in humidified air containing 5% CO_2. Colon26 cells were transfected with pNF-κB-Luc using Lipofectamine 2000 (Gibco–Invitrogen) to obtain colon26/NFκB-Luc cells, according to the method of Terouanne *et al*.[18] Then, the cells were treated with medium containing 1 mg/mL G418 (Geneticin; Sigma, St. Louis, MO, USA) and single colonies of G418-resistant cells were identified and examined for their luciferase activity as described below. TNF-α (1 ng/mL) was added to colonies, and a clone that showed a marked response to the stimulus in terms of the expression of the luciferase was selected as colon26/NFκB-Luc. To examine the response to TNF-α, colon26/NF-κB-Luc cells were added with a varying concentration of TNF-α (0–10 nM) and incubated for 12 h. Then, cells were lysed using a lysis buffer and the luciferase activity in cell lysates was measured as described below.

Experimental hepatic metastasis and treatment with catalase derivatives. Mice were anesthetized by an i.p. injection of pentobarbital sodium (50 mg/kg). A midline abdominal incision was made to expose the portal vein then colon26 or colon26/NFκB-Luc cells (1×10^5 cells/0.1 mL HBSS) were injected into this vein using a syringe with a 29 G-needle. Then, the incision was sutured and mice were allowed to recover. Catalase derivatives were injected into the tail vein at a dose of 200 000 units/kg (~5 mg/kg for unmodified catalase).

Measurement of luciferase activity. Mice were transfected with pNF-κB-Luc and pRL-SV40 as described above, and injected with TNF-α (8 μg/kg), colon26 cells or peritoneal macrophages (1×10^5 cells/mouse) into the portal vein at a 24 h-interval. Eight hours after injection, the liver was excised, homogenized and centrifuged as previously reported.[19] Then, 10 μL of the supernatant was assayed for luciferase activity using a luminometer (Lumat LB 9507, EG&G Berthold, Bad Wildbad, Germany), Picagene (Toyo Ink, Tokyo, Japan) or the Dual Luciferase Reporter assay system (Promega). Following subtraction of the background activity for the liver homogenate without injection, the ratio of the activity of the firefly luciferase to the renilla luciferase (F/R) was calculated to correct for differences in transfection efficiency among mice.

Luciferase imaging. Mice received injections of TNF-α or colon26 cells into the portal vein 24 h after transfection with pNF-κB-Luc. Then, 4, 8 and 12 h after injection, mice were injected i.p. with 2 mg D-luciferin, anesthetized with pentobarbital in phosphate-buffered saline, and then placed in a NightOwl LB 981 Molecular Light Imager (Berthold Technologies, Bad Wildbad, Germany). Imaging was then performed in a two-step process using WinLight32 software (Berthold Technologies, Bad Wildbad, Germany). First, a black and white photographic image was acquired using a 1-s exposure. Next, the luminescent image was acquired using a 5-min photon integration period with background subtraction. The luminescent image was processed by the software to color the luminescence intensity, and then overlaid onto the photographic image. The parameters in the WinLight32 software used to obtain luminescent images were the color threshold and indicated color scheme.

Results

TNF-α-induced increase in the luciferase activity in mouse liver. Mice transfected with pNF-κB-Luc received an injection of TNF-α, an activator of NF-κB signaling, into the portal vein 24 h after gene transfer. Fig. 1(a) shows the luminescent images of mice receiving pNF-κB-Luc. Sham-operated, control mice showed some luminescent signals in the liver, indicating that some firefly luciferase was produced in the liver under these conditions. An injection of TNF-α greatly increased the luminescence in the liver. A dual luciferase assay was applied to quantitatively evaluate the effects of TNF-α on firefly luciferase activity in the liver (Fig. 1b). Compared with the sham-operated, control group, the TNF-α-treated group showed an approximately sixfold greater F/R ratio. When TNF-α was injected to mice at different doses from 0.08 to 8 μg/kg, the luciferase activity in the liver increased in a dose-dependent manner (Fig. 1a). These results indicate that mice receiving a hydrodynamic delivery of plasmid DNA expressing luciferase in an NF-κB activity-dependent manner can be used to monitor the activation of NF-κB in mouse liver.

NF-κB activity in liver cells during the initial stages of hepatic metastasis of tumor cells. Fig. 2(a) shows the luminescence images of mice receiving an i.v. injection of pNF-κB-Luc by the hydrodynamics-based procedure. Inoculation of colon26 cells into the portal vein transiently increased the intensity of luciferase activity in the liver with a peak at 8 h after tumor inoculation. A quantitative luciferase assay showed that inoculation of 1×10^5 colon26 cells into the portal vein resulted in an approximately ninefold increase in the expression of firefly luciferase (Fig. 2b), indicating that NF-κB in liver cells is activated when tumor cells enter the hepatic circulation. The increase was dependent on the number of colon26 cells injected (data not shown). In addition, it was almost completely blocked by pretreatment of mice with dexamethasone (90 mg/kg), a well-known suppressor of NF-κB (YuKi Kobayashi, unpublished data, 2007). No significant increase in firefly luciferase of liver cells was observed when mouse peritoneal macrophage (1×10^5 cells) were inoculated instead of colon26 cells in the same manner (Fig. 2c). Injection of catalase derivatives greatly inhibited the colon26 cell-induced increase in the firefly luciferase activity of liver cells (Fig. 2b,d). Gal-catalase, a catalase derivative targeting hepatocytes through the recognition by asialoglycoprotein

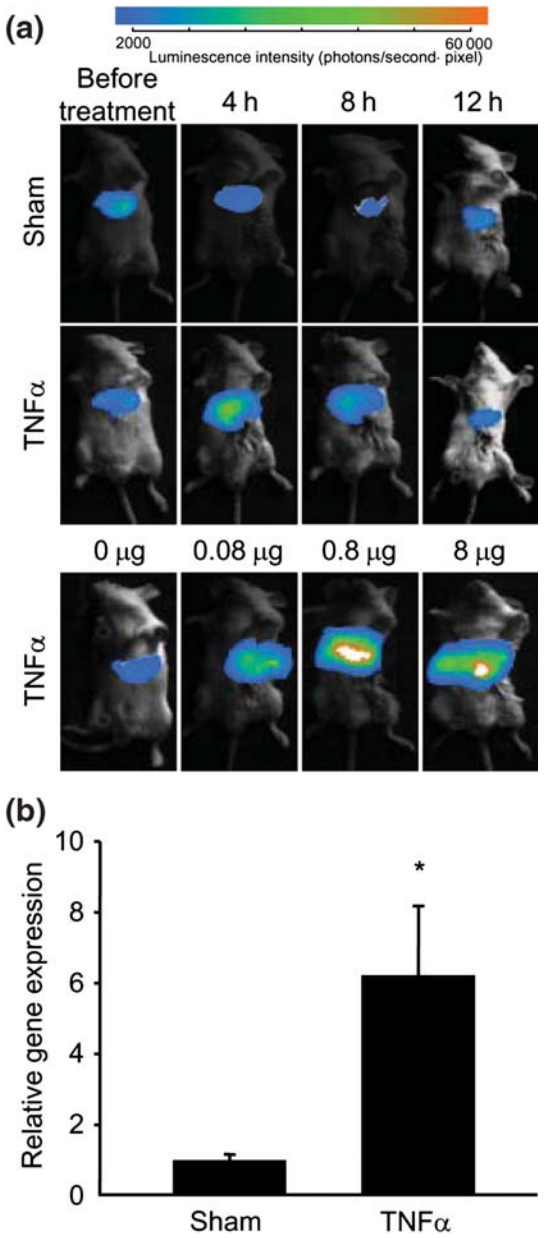

Fig. 1. Tumor necrosis factor (TNF)-α-induced changes in luciferase expression in mouse liver transfected with pNF-κB-Luc. Mice transfected with pNF-κB-Luc were injected with saline (sham operated) or TNF-α into the portal vein at 24 h after gene transfer. (a) At 4, 8 and 12 h after injection of saline or TNF-α (8 μg/kg), mice were injected with D-luciferin and the luminescent image was obtained (upper panels). At 4 h after injection of TNF-α at a dose of 0.08, 0.8 or 8 μg/kg, mice were injected with D-luciferin and the luminescent image was obtained (lower panels). (b) Mice were killed at 8 h after injection of saline or TNF-α (8 μg/kg), and the luciferase activity in the liver was measured. Results are expressed as mean ± standard deviation of at least three mice. *$P < 0.01$, statistically significant difference compared with the sham-operated group.

receptors, and Suc-catalase, one targeting liver non-parenchymal cells through the recognition by scavenger receptors, were more effective than unmodified catalase. Injection of any catalase derivative hardly affected the luciferase activity in the liver of tumor-free mice receiving pNFκB-Luc (Fig. 2e). In a separate experiment, injection of catalase, Gal-catalase or Suc-catalase hardly influenced the expression from pCMV-Luc (data not shown). These results clearly demonstrate that NF-κB in liver cells is activated when tumor cells, not macrophages, enter the hepatic circulation, and this activation is blocked by catalase derivatives targeting liver-constituting cells.

Reduction in colon26 inoculation-induced NF-κB activation by depletion of Kupffer cells. To evaluate the role of Kupffer cells on the NF-κB activation in liver cells, gadolinium chloride ($GdCl_3$, 45 mg/kg), which induces a selective and complete destruction of Kupffer cells,[20] was injected into the tail vein of mice 12 h before gene transfer. Depletion of Kupffer cells by $GdCl_3$ markedly prevented the NF-κB activation in liver cells induced by inoculation of colon26 cells into the portal vein (Fig. 3a,b). No significant differences were observed in the luciferase activity between the saline- and GdCl3-treated mice receiving pCMV-Luc, suggesting that GdCl3 hardly interfere with the detection method. Taken together, these results suggest an important role for Kupffer cells in the NF-κB activation in liver cells during initial stages of hepatic metastasis of tumor cells.

Characterization of colon26/NFκB-Luc cells. To evaluate NF-κB activity in tumor cells, we established cell lines stably transfected with pNF-κB-Luc. Several colonies of colon26/NFκB-Luc cells were obtained with different levels of luciferase activity. A colony expressing a high luciferase activity upon addition of TNF-α was selected as colon26/NFκB-Luc. Fig. 4 shows the firefly luciferase activity in colon26/NFκB-Luc cells. The baseline level of expression was 0.387 ± 0.015 relative light units (RLU)/sec/cells, and this level significantly increased on addition of TNF-α. The luciferase activity of cells increased with an increasing concentration of TNF-α (Fig. 4) or hydrogen peroxide (data not shown). These data indicate that the luciferase activity of cells is an indicator of the NF-κB activity of colon26/NFκB-Luc cells.

NF-κB activation in colon26/NFkB-Luc cells. Fig. 5 shows the firefly luciferase activity in the liver after inoculation of colon26/NF-κB-Luc cells into the portal vein. The activity was measured at 8 h after inoculation of tumor cells, when more than 80% of cells inoculated remain within the liver organ, determined using colon26/Luc cells (YuKi Kobayashi, unpublished data, 2007). Inoculation of colon26/NF-κB-Luc cells into the portal vein significantly increased the firefly luciferase activity of cells compared with the value under *in vitro* culture conditions. These results suggest that NF-κB in colon26 cells is also activated during the initial stages of hepatic metastasis. To examine the effect of catalase derivatives, mice inoculated with colon26/NF-κB-Luc cells were injected with catalase, Gal-catalase or Suc-catalase. Suc-catalase, but not catalase or Gal-catalase, significantly inhibited the increase in the luciferase activity of colon26/NF-κB-Luc cells.

Discussion

Tumor metastasis is the primary cause of death among cancer patients. It involves many steps, such as tumor cell survival and arrest in the bloodstream, and progressive outgrowth at distant sites. Tumor metastasis is regulated by numerous factors, such as MMP, adhesion molecules, angiogenic factors and anti-apoptotic factors. Activation of transcription factors, such as NF-κB, is involved in the expression of these factors. NF-κB could be constitutively activated in tumor cells, but it may be activated by the interaction of tumor cells with other cells. Several reports have shown that NF-κB inhibitors suppress tumor promotion and metastasis.[7,21] The importance of NF-κB activation on tumor growth and metastasis was suggested in a report showing that the activation suppresses the apoptotic potential of chemotherapeutic agents and contributes to drug resistance.[22,23]

One of the most popular methods for analyzing NF-κB activity is an EMSA. A standard EMSA typically uses ^{32}P-labeled DNA to detect the presence of any proteins binding to the DNA. Although isotopic labeling provides high detection sensitivity, the use of radioisotopes is subject to regulatory procedures, disposal

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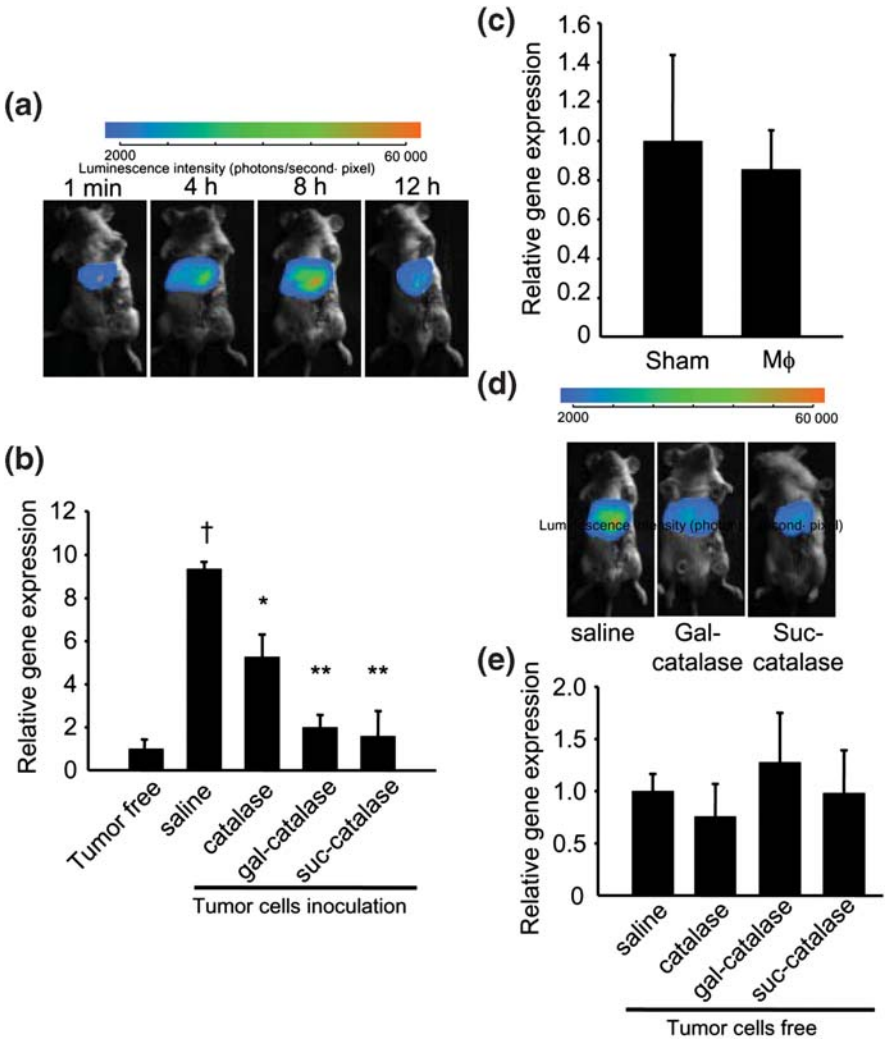

Fig. 2. Tumor cell-induced changes in luciferase expression in mouse liver transfected with pNF-κB-Luc. Mice were transfected with pNF-κB-Luc, and injected with colon26 cells (1×10^5 cells) into the portal vein over a 24-h interval. (a) At 1 min, 4, 8 and 12 h after injection of saline or tumor cells, mice were injected with D-luciferin and a luminescent image was obtained. (b,c) Mice were killed at 8 h after injection of saline, colon26 cells (b) or mouse peritoneal macrophages (c, 1×10^5 cells), and the luciferase activity in the liver was measured. Each catalase derivative was injected into the tail vein at a dose of 200 000 units/kg immediately after the injection of cells. The ratio of firefly and renilla luciferase activities was calculated as an indicator of the nuclear factor κB activity in liver cells. The ratio of each treatment was normalized to that in the saline-treated group. Results are expressed as mean ± standard deviation of at least four mice. $^{\dagger}P < 0.01$ statistically significant difference compared with the tumor-free, saline-treated group. $^{*}P < 0.05$, $^{**}P < 0.01$, statistically significant difference compared with the tumor, saline-treated group. (d) Mice were given an injection of each catalase derivative into the tail vein at a dose of 200 000 units/kg immediately after the injection of colon26 cells. The luminescent images were obtained 8 h after the inoculation of colon26 cells. (e) Mice were transfected with pNF-κB-Luc, and each catalase derivative was injected into the tail vein at a dose of 200 000 units/kg over a 24-h interval. Mice were killed at 8 h after injection of each catalase, and the luciferase activity in the liver was measured. Gal-catalase, galactosylated catalase; Suc-catalase, succinylated catalase.

limitations and the short half-life of the label, and requires a long development period. In marked contrast, the luminescence-based technology developed in our previous study[16] provides a simple, sensitive and quantitative method for detecting the close to real-time transcriptional activity of NF-κB in live animals.

In the present study, reporter genes were introduced into mouse liver by an i.v. injection of plasmid DNA by the hydrodynamics-based procedure. This method produces a very high transgene expression, although a transient increase in liver enzyme levels in plasma within the first day of gene transfer has been reported.[13] Because tumor cells were inoculated with a 24-h interval from the hydrodynamic injection, it can be considered that the injection-induced liver damages could hardly affect the metastatic profile of tumor cells. We previously showed that mice treated by the procedure can be used to examine the NF-κB activity in liver cells of mice with fulminant hepatitis and indicated that the luciferase activity in mouse liver receiving pNF-κB-Luc is correlated well with the NF-κB activity determined by an EMSA assay.[16] The luciferase activity, which was monitored by an *in vivo* imaging system or quantitatively measured by a standard assay, corresponded to the changes in the NF-κB activity in the liver measured by an EMSA. Because gene transfer only occurs in liver cells, such as hepatocytes, sinusoidal endothelial cells and Kupffer cells, the luciferase activity detected indicate the NF-κB activity in liver cells even when tumor cells are present in the organ. This is in marked contrast to EMSA analysis, which requires separation of cells for measuring the NF-κB activity in a specific type of cell. NF-κB signaling is well known to be oscillating depending on a well-orchestrated negative-feedback system.[24,25] The analytical system in the present study is a system where a transient increase in the NF-κB activity can be easily and sensitively detected without considering the changes in the NF-κB activity. In addition, the half-life of firefly luciferase in cells is reported to be approximately 3 h. These conditions strongly suggest that this analytical method is quite useful for examining the effect of catalase derivatives on tumor metastasis.

Biological labeling of cells enables researchers to detect the cells of interest with a high sensitivity and specificity. Tumor cell lines stably transfected with a reporter gene have been widely used for the evaluation of tumor growth and metastasis *in vivo*.[26–28] We reported that melanoma B16 cells and colon26 cells stably labeled with firefly luciferase have been successfully used for the evaluation of tumor metastasis in various experimental settings.[19,29–32] Replacement of a constitutively reporter gene expressing vector with one expressing in response to NF-κB activation can be a reliable method of evaluating its activity in tumor cells. Transgenic mice, with luciferase expressed depending on NF-κB activity, have been developed[9] but, to our knowledge, no tumor cells expressing luciferase depending on NF-κB activity have been reported. In the present study, we established several clones of colon26/NFκB-Luc cells and selected one that was sensitive to the stimulation of NF-κB activation. It was reported that rat colon cancer cells inoculated into the portal vein localize

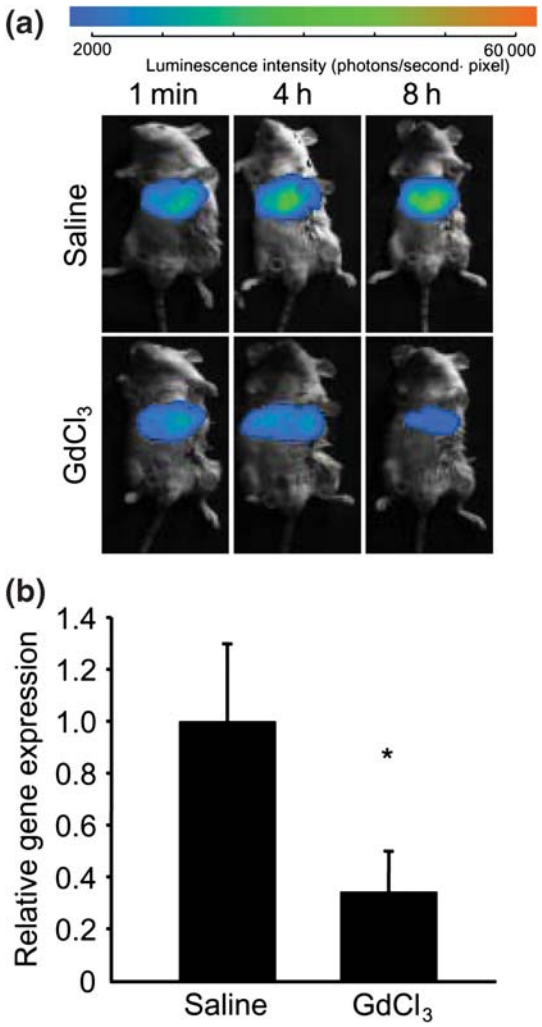

Fig. 3. Tumor cell-induced changes in luciferase expression in gadolinium chloride ($GdCl_3$)-treated mouse liver transfected with pNF-κB-Luc. Mice received an i.v. injection of saline or $GdCl_3$ (45 mg/kg) saline solution 12 h prior to gene transfer with pNF-κB-Luc. At 24 h after transfection, mice were given an injection of colon26 cells (1×10^5 cells) into the portal vein. (a) At 0, 4 and 8 h after injection of the colon26 cells, mice were injected with D-luciferin and a luminescent image was obtained. (b) Mice were killed at 8 h after injection of colon26 cells, and the luciferase activity in the liver was measured. Results are expressed as mean ± standard deviation of at least four mice. $*P < 0.01$, statistically significant difference compared with the saline-treated group.

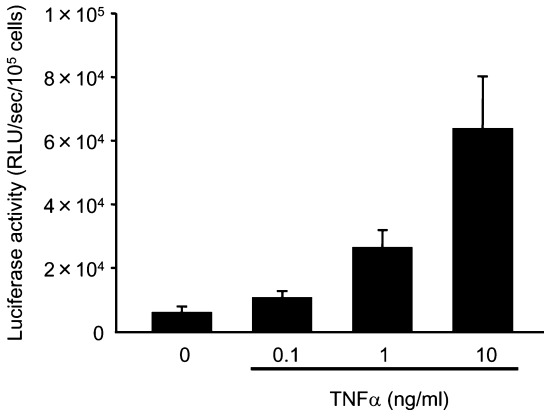

Fig. 4. Tumor necrosis factor (TNF)-α induced changes in luciferase expression in colon26/NF-κB-Luc cells. Colon 26/NF-κB-Luc cells were incubated with a range of concentrations of TNF-α (0–10 nM) for 12 h. Then, cells were lysed using a lysis buffer and the luciferase activity in the cell lysates was measured. RLU, relative light units.

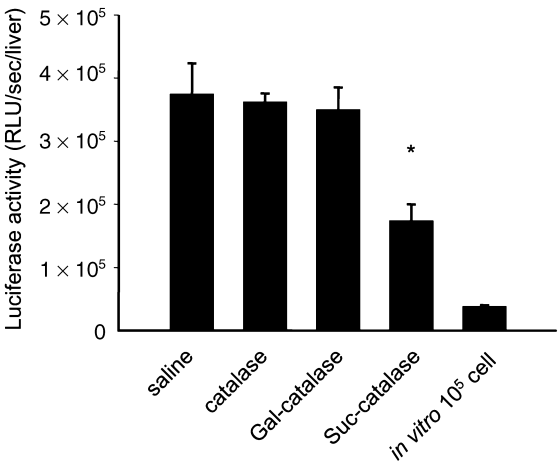

Fig. 5. Luciferase expression in colon26/NF-κB-Luc cells inoculated into the portal vein of mice. Mice were given an injection of colon26/NF-κB-Luc (1×10^5 cells) into the portal vein, then each catalase derivative (5 mg/kg) was injected into the tail vein after a minimal interval. At 8 h after tumor inoculation, mice were killed and the luciferase activity in the liver was measured. The luciferase activity of 1×10^5 colon26/NF-κB-Luc cells under culture conditions was also shown for comparison. $*P < 0.05$, statistically significant difference compared with the saline-treated group. Gal-catalase, galactosylated catalase; Suc-catalase, succinylated catalase.

to sinusoids of the liver at the early stage of metastasis.[33] In our previous studies,[34] colon26 cells and colon26/Luc cells formed metastatic colonies in the liver after inoculation into the portal vein. In the present study, mouse liver inoculated with colon26/NFκB-Luc cells clearly showed that the luciferase protein, which should be derived from colon26/NFκB-Luc cells in these mice, exists in the organ. Imaging of untreated mice or ones receiving colon26 cells showed no significant chemiluminescence under the same conditions (data not shown). Therefore, it is reasonable to speculate that tumor cells localize sinusoids of the liver at the early stage of metastasis under the conditions used in the present study.

Tumor necrosis factor-α is reported to be produced as a part of the host pro-inflammatory response to tumor cells during the initial stages of hepatic metastasis. Although TNF-α is cytotoxic, it also facilitates the formation of hepatic metastasis by increasing the expression of adhesion molecules, by promoting tumor cell migration, survival and invasion, and by inducing an angiogenic response.[35,36] It is well known that TNF-α activates NF-κB in many types of cells through binding to TNF receptors.[37] Thus, we first examined whether luciferase activity in mouse liver and tumor cells was responsive to TNF-α. In both cases, the luciferase activity was significantly increased by administration or addition of TNF-α. On the other hand, TNF-α had no significant effects on the luciferase activity in mouse liver when pTK-Luc, which has no binding sequences for transcription factors including NF-κB, was injected in place of pNF-κB-Luc (data not shown). These results strongly support the hypothesis that luciferase activity can be used as an indicator of NF-κB activity in cells in which the plasmid vector pNF-κB-Luc was introduced.

When tumor cells were injected into the portal vein, luciferase activity was significantly increased irrespective of the type of cells expressing the gene; that is, liver cells or colon26 cells.

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Injection of peritoneal macrophages did not increase the luciferase activity in liver cells, suggesting that the activation of NF-κB is not mediated by nonspecific events relating to the injection of cells into the portal vein. On the other hand, the luciferase activity in colon26/NFκB-Luc cells was significantly higher when the cells were inoculated into the portal vein compared with the activity of cells under culture conditions. The NF-κB activation induced by tumor cell inoculation into the portal vein would be followed by various events resulting from the activation. It has been reported that NF-κB activation increases the expression of anti-apoptotic factors in tumor cells. The activation also increases the expression of adhesion molecules, pro-inflammatory cytokines and MMP in host cells.[38] These changes would produce pro-tumor effects and therefore the inhibition of NF-κB activation can be used as a cancer treatment.

Reactive oxygen species have been proposed to be involved in tumor metastasis. It has been reported that ROS generated from activated phagocytes through nicotinamide adenine dinucleotide phosphate oxidase facilitated tumor metastasis.[39] In the early stages of hepatic metastasis, tumor cells entering the liver through the portal vein interact with Kupffer cells. As a consequence of this interaction, 92% of tumor cells are phagocytosed, 6% were untouched and only 2% of tumor cells remained and were metastasized to the liver.[40] It has previously been reported that the host inflammatory response promotes hepatic metastasis of tumor cells by increasing the arrest and extravasation of tumor cells. It has also been demonstrated that hydrogen peroxide is involved in the TNF-α-induced NF-κB activation.[41] In a series of investigations, we have demonstrated that chemically-modified catalase derivatives effectively inhibit tumor metastasis to various organs, including the liver, lung and peritoneal cavity.[19,29–31] Administration of catalase derivatives was found to be effective in inhibiting the NF-κB activation in both liver cells and tumor cells. Targeting delivery of catalase leads to inhibit increase the expression of anti-apoptotic factors in tumor cells and increase the expression of adhesion molecules, pro-inflammatory cytokines and MMP in host cells by suppression of NF-κB activation. Gal-catalase, a derivative targeting hepatocytes, was highly effective in inhibiting the NF-κB activation in liver cells, but not so effective for tumor cells. These results can be explained by the fact that more than 90% of Gal-catalase is delivered to hepatocytes[17] whereas tumor cells are localized within the sinusoids. On the other hand, Suc-catalase was effective in inhibiting the activation in both types of cells. Suc-catalase can be targeted not only to hepatocytes, but also to Kupffer cells and sinusoidal endothelial cells. The difference in activity between Gal- and Suc-catalase on the inhibition of the activation in tumor cells strongly suggests that the delivery of catalase to liver non-parenchymal cells, such as Kupffer and endothelial cells, is important for inhibiting the NF-κB activation in tumor cells. Localization of tumor cells to the sinusoids in the early stages of metastasis would explain the different effects of these catalase derivatives. In Kupffer cell-depleted mice, NF-κB activation in liver-constituting cells was almost abolished. Soluble factors from Kupffer cells, such as pro-inflammatory cytokines and ROS, will be a major cause of NF-κB activation during the initial stages of hepatic metastasis of tumor cells. Therefore, targeted delivery of catalase or any other compounds to inhibit the activation to Kupffer cells will be an effective treatment in hepatic metastasis.

In conclusion, an experimental system has been successfully developed in which the NF-κB activity during the initial stages of hepatic metastasis can be quantitatively evaluated both in liver cells and metastasizing tumor cells. Using this system, we have clearly demonstrated that NF-κB is activated both in liver cells and tumor cells as a consequence of the interaction of tumor cells with liver cells, through a hydrogen peroxide-mediated pathway. Because this activation will lead to a facilitated hepatic metastasis of tumor cells, removal of hydrogen peroxide can be theoretically considered to be an effective approach to inhibiting hepatic metastasis of tumor cells, as we have reported in previous papers.[34,41–43]

References

1 Hayden MS, Ghosh S. Signal to NF-kappaB. *Genes Dev* 2004; **18**: 2195–224.
2 Inoue J, Gohda J, Akiyama T, Semba K. NF-kappaB activation in development and progression of cancer. *Cancer Sci* 2007; **98**: 268–74.
3 Luo JL, Kamata H, Karin M. IKK/NF-kappaB signaling: balancing life and death – a new approach to cancer therapy. *J Clin Invest* 2005; **115**: 2625–32.
4 Karin M. Nuclear factor-kappaB in cancer development and progression. *Nature* 2006; **441**: 431–6.
5 Park BK, Zhang H, Zeng Q *et al*. NF-kappaB in breast cancer cells promotes osteolytic bone metastasis by inducing osteoclastogenesis via GM-CSF. *Nat Med* 2007; **13**: 62–9.
6 Bassères DS, Baldwin AS. Nuclear factor-kappaB and inhibitor of kappaB kinase pathways in oncogenic initiation and progression. *Oncogene* 2006; **25**: 6817–30.
7 Scaife CL, Kuang J, Wills JC *et al*. Nuclear factor kappaB inhibitors induce adhesion-dependent colon cancer apoptosis: implications for metastasis. *Cancer Res* 2002; **62**: 6870–8.
8 Liu YX, Cao WS, Irani K, Li XJ. Establishment of a T-Ag and NF-kappaB binding elements gene co-transfected stable HUVECs cell line. *J Cell Biochem* 2003; **89**: 1285–90.
9 Carlsen H, Moskaug JO, Fromm SH, Blomahoff R. *In vivo* imaging of NF-kappa B activity. *J Immunol* 2002; **168**: 1441–6.
10 Maggi A, Ciana P. Reporter mice and drug discovery and development. *Nat Rev Drug Discov* 2005; **4**: 249–55.
11 Kobayashi N, Kuramoto T, Yamaoka K, Hashida M, Takakura Y. Hepatic uptake and gene expression mechanisms following intravenous administration of plasmid DNA by conventional and hydrodynamics-based procedures. *J Pharmacol Exp Ther* 2001; **297**: 853–60.
12 Kobayashi N, Kuramoto T, Chen S, Watanabe Y, Takakura Y. Therapeutic effect of intravenous interferon gene delivery with naked plasmid DNA in murine metastasis models. *Mol Ther* 2002; **6**: 737–44.
13 Kobayashi N, Nishikawa M, Hirata K, Takakura Y. Hydrodynamics-based procedure involves transient hyperpermeability in the hepatic cellular membrane: implication of a nonspecific process in efficient intracellular gene delivery. *J Gene Med* 2004; **6**: 584–92.
14 Liu F, Song Y, Liu D. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther* 1999; **6**: 1258–66.
15 Zhang G, Budker V, Wolff JA. High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. *Hum Gene Ther* 1999; **10**: 1735–7.
16 Hyoudou K, Nishikawa M, Kobayashi Y, Kuramoto Y, Yamashita F, Hashida M. Analysis of *in vivo* nuclear factor-kappaB activation during liver inflammation in mice: prevention by catalase delivery. *Mol Pharmacol* 2007; **71**: 446–53.
17 Yabe Y, Nishikawa M, Tamada A, Takakura Y, Hashida M. Targeted delivery and improved therapeutic potential of catalase by chemical modification: combination with superoxide dismutase derivatives. *J Pharmacol Exp Ther* 1999; **289**: 1176–84.
18 Térouanne B, Tahiri B, Georget V *et al*. A stable prostatic bioluminescent cell line to investigate androgen and antiandrogen effects. *Mol Cell Endocrinol* 2000; **160**: 39–49.
19 Hyoudou K, Nishikawa M, Umeyama Y, Kobayashi Y, Yamashita F, Hashida M. Inhibition of metastatic tumor growth in mouse lung by repeated administration of polyethylene glycol-conjugated catalase: quantitative analysis with firefly luciferase-expressing melanoma cells. *Clin Cancer Res* 2004; **10**: 7685–91.
20 Jahnke C, Mehrabi A, Golling M *et al*. Evaluation of microperfusion disturbances in the transplanted liver after Kupffer cell destruction using GdCl3: an experimental porcine study. *Transplant Proc* 2006; **38**: 1588–95.
21 Aggarwal BB, Shishodia S, Takada Y *et al*. Curcumin suppresses the paclitaxel-induced nuclear factor-kappaB pathway in breast cancer cells and inhibits lung metastasis of human breast cancer in nude mice. *Clin Cancer Res* 2005; **11**: 7490–8.
22 Baldwin AS. Control of oncogenesis and cancer therapy resistance by the transcription factor NF-kappaB. *J Clin Invest* 2001; **107**: 241–6.
23 Nakanishi C, Toi M. Nuclear factor-kappaB inhibitors as sensitizers to anticancer drugs. *Nat Rev Cancer* 2005; **5**: 297–309.
24 Hoffmann A, Levchenko A, Scott ML, Baltimore D. The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation. *Science* 2002; **298**: 1241–5.

25 Nelson DE, Ihekwaba AE, Elliott M *et al*. Oscillations in NF-kappaB signaling control the dynamics of gene expression. *Science* 2004; **306**: 704–8.
26 Zhang L, Hellstrom KE, Chen L. Luciferase activity as a marker of tumor burden and as an indicator of tumor response to antineoplastic therapy *in vivo*. *Clin Exp Metastasis* 1994; **12**: 87–92.
27 El Hilali N, Rubio N, Martinez-Villacampa M, Blanco J. Combined noninvasive imaging and luminometric quantification of luciferase-labeled human prostate tumors and metastases. *Lab Invest* 2002; **82**: 1563–71.
28 Bouvet M, Tsuji K, Yang M, Jiang P, Moossa AR, Hoffman RM. *In vivo* color-coded imaging of the interaction of colon cancer cells and splenocytes in the formation of liver metastases. *Cancer Res* 2006; **66**: 11 293–7.
29 Hyoudou K, Nishikawa M, Kobayashi Y, Umeyama Y, Yamashita F, Hashida M. Pegylated catalase prevents metastatic tumor growth aggravated by tumor removal. *Free Radic Biol Med* 2006; **41**: 1449–58.
30 Hyoudou K, Nishikawa M, Kobayashi Y, Kuramoto Y, Yamashita F, Hashida M. Inhibition of adhesion and proliferation of peritoneally disseminated tumor cells by pegylated catalase. *Clin Exp Metastasis* 2006; **23**: 269–78.
31 Hyoudou K, Nishikawa M, Kobayashi Y *et al*. Inhibition of peritoneal dissemination of tumor cells by cationized catalase in mice. *J Control Release* 2007; **119**: 121–7.
32 Kuramoto Y, Nishikawa M, Hyoudou K, Yamashita F, Hashida M. Inhibition of peritoneal dissemination of tumor cells by single dosing of phosphodiester CpG oligonucleotide/cationic liposome complex. *J Control Release* 2006; **115**: 226–33.
33 Mook OR, Van Marle J, Vreeling-Sindelárová H, Jonges R, Frederiks WM, Van Noorden CJ. Visualization of early events in tumor formation of eGFP-transfected rat colon cancer cells in liver. *Hepatology* 2003; **38**: 295–304.
34 Nishikawa M, Tamada A, Hyoudou K *et al*. Inhibition of experimental hepatic metastasis by targeted delivery of catalase in mice. *Clin Exp Metastasis* 2004; **21**: 213–21.
35 Khatib AM, Auguste P, Fallavollita L *et al*. Characterization of the host proinflammatory response to tumor cells during the initial stages of liver metastasis. *Am J Pathol* 2005; **167**: 749–59.
36 Auguste P, Fallavollita L, Wang N, Burnier J, Bikfalvi A, Brodt P. The host inflammatory response promotes liver metastasis by increasing tumor cell arrest and extravasation. *Am J Pathol* 2007; **170**: 1781–92.
37 Smith CA, Farrah T, Goodwin RG. The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. *Cell* 1994; **7**: 959–62.
38 Balkwill F, Coussens LM. Cancer: an inflammatory link. *Nature* 2004; **431**: 405–6.
39 Okada F, Kobayashi M, Tanaka H *et al*. The role of nicotinamide adenine dinucleotide phosphate oxidase-derived reactive oxygen species in the acquisition of metastatic ability of tumor cells. *Am J Pathol* 2006; **169**: 294–302.
40 Timmers M, Vekemans K, Vermijlen D *et al*. Interactions between rat colon carcinoma cells and Kupffer cells during the onset of hepatic metastasis. *Int J Cancer* 2004; **112**: 793–802.
41 de Oliveira-Marques V, Cyrne L, Marinho HS, Antunes F. A quantitative study of NF-kappaB activation by H2O2: relevance in inflammation and synergy with TNF-alpha. *J Immunol* 2007; **178**: 3893–902.
42 Nishikawa M, Tamada A, Kumai H, Yamashita F, Hashida M. Inhibition of experimental pulmonary metastasis by controlling biodistribution of catalase in mice. *Int J Cancer* 2002; **99**: 474–9.
43 Nishikawa M, Hyoudou K, Kobayashi Y, Umeyama Y, Takakura Y, Hashida M. Inhibition of metastatic tumor growth by targeted delivery of antioxidant enzymes. *J Control Release* 2005; **109**: 101–7.

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