

**Fig. 4.** Induction of IGFBP-5 by gankyrin. (A) U-2 OS cells (lanes 1 and 2) and Huh-7 cells (lanes 3 and 4) transiently transfected with plasmids expressing gankyrin or vector alone were analyzed for expression of IGFBP-5 by western blotting using the indicated antibodies. Representative results from more than 3 experiments are shown. (B) Huh-7 cells, mock transfected or transfected with siRNA for gankyrin or control RNA as indicated, were analyzed as in (A). (C) Suppression of IGFBP-5 expression by siRNA. Huh-7 cells were transfected with control RNA or IGFBP-5-specific siRNA. IGFBP-5 transcript levels were determined by real-time RT-PCR and normalized with  $\beta$ -actin levels. Results from 3 repeats were averaged and expressed relative to control. Error bars refer to standard deviation of the average quantitated results. (D) Effect of IGFBP-5 down-regulation on cell growth. U-2 OS and Huh-7 cells were transfected with IGFBP-5 siRNA or control RNA, and 72 hours later viable cell numbers were determined. Values are mean  $\pm$  standard deviation ( $n = 3$ ) and expressed relative to controls. \*\* and \*,  $P < 0.01$  and  $P < 0.05$ , respectively.

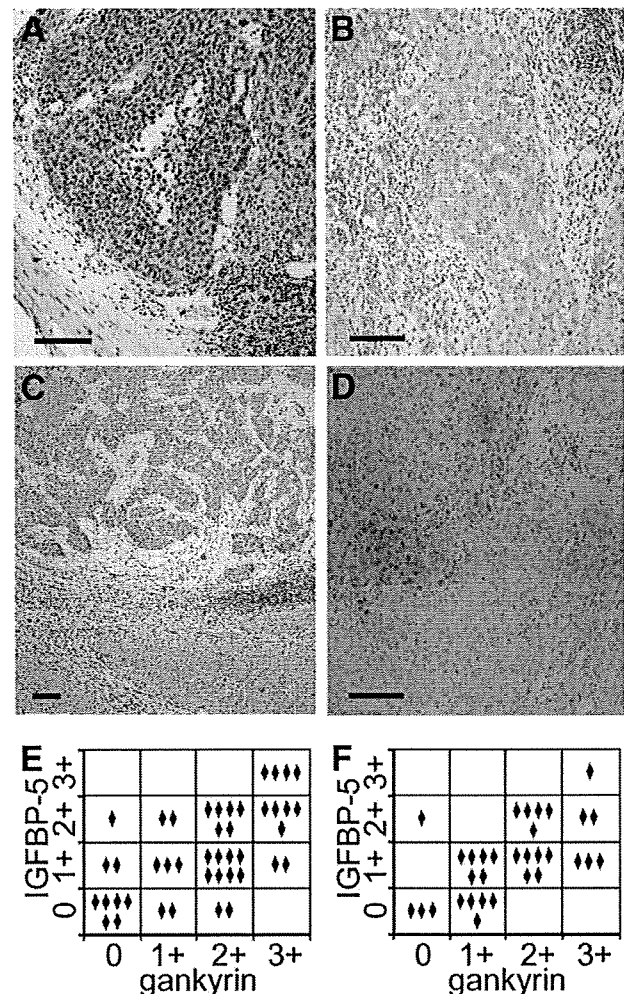
gankyrin (Table 3), and the levels of expression covaried both in HCCs ( $\rho = 0.629, P < 0.001$ ) (Fig. 5E) and non-cancerous hepatocytes ( $\rho = 0.606, P < 0.001$ ) (Fig. 5F).

**Expression of Gankyrin in HCC and Patient Prognosis.** When we examined the relationship between gankyrin expression in HCC cells and the survival of patients after surgical resection, a significant difference was observed between the patients with gankyrin-positive HCCs and those with gankyrin-negative HCCs (Fig. 6). We found no significant difference in the survival rates between the patients whose HCCs stained positively and negatively for p53, MDM2, or IGFBP-5.

**Discussion**

Gankyrin is as an oncogene, mRNA of which is over-expressed in almost all human HCCs.<sup>9,19</sup> Although less frequent, gankyrin has been found by RNA dot blot anal-

ysis to be overexpressed in additional tumors including those of the breast, colon, rectum, stomach, small intestine, pancreas, ovary, lung, and thyroid (A. Umemura and J. Fujita, unpublished data). In the current study, we immunohistochemically examined the gankyrin protein expression in HCCs using the monoclonal anti-gankyrin antibody and found that the protein was highly expressed in the cytoplasm of 63% of HCCs. Tan et al.<sup>20</sup> has simi-



**Fig. 5.** Immunohistochemical detection of IGFBP-5 in hepatocellular carcinoma (HCC). HCC sections were stained with anti-IGFBP-5 antibody and counterstained with hematoxylin. Positive immunostaining appears brown. (A) Positive staining for IGFBP-5 in the cytoplasm of HCC cells, especially at the invasive boundaries. (B) Presence of IGFBP-5 in non-cancerous cirrhotic hepatocytes. (C) Stronger staining for IGFBP-5 in HCC cells (upper) than the neighboring cirrhotic hepatocytes (lower). (D) Positive staining for IGFBP-5 in HCC cells (upper left), but negative in cirrhotic cells (lower right). Bar, 100  $\mu$ m. (E) Correlation of expression levels of gankyrin and IGFBP-5 in HCCs. The immunostaining levels were expressed as 0 (negative), 1+ (weakly positive), 2+ (moderately positive), or 3+ (strongly positive). Each diamond represents 1 case. The Spearman's  $\rho = 0.629, P < 0.001$ . (F) Correlation of expression levels of gankyrin and IGFBP-5 in noncancerous hepatocytes determined as in (E). The Spearman's  $\rho = 0.606, P < 0.001$ .

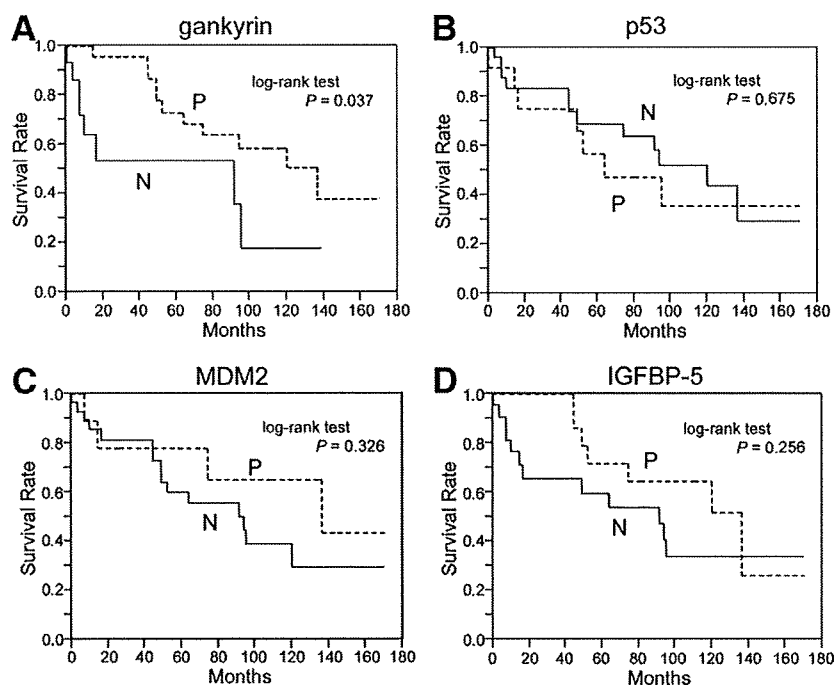


Fig. 6. Survival of patients and expression of molecular markers. The Kaplan-Meier method was used to determine the patient survival and log-rank test to compare survival between patients with HCC grouped according to (A) gankyrin positivity, (B) p53 positivity, (C) MDM2 positivity, and (D) IGFBP-5 positivity. P, positive. N, negative.

larly found overexpression of gankyrin protein in 60% of HCCs using a polyclonal antibody. The reason why the protein is not overexpressed in one-third of HCCs despite overexpression of its mRNA is unknown. The posttranscriptional, translational, and posttranslational regulations of gankyrin expression remain to be elucidated.

According to the 15th follow-up survey by the Liver Cancer Study group of Japan, the cumulative survival rates after surgical removal of HCC are 52.3% and 27.3% at 5 and 10 years, respectively, and better survival rates are associated with fewer numbers of tumors, lack of portal venous invasion, and early clinical stages.<sup>4-6</sup> Consistent with these observations, gankyrin positivity of HCC was associated with low TNM stage, lack of capsular invasion, portal venous invasion, and intrahepatic metastasis, and better prognosis of the patients. Patients with hyperdiploid acute lymphoblastic leukemia with more than 50 chromosomes, one of the 6 subtypes of pediatric acute lymphoblastic leukemia, have an excellent prognosis compared with other subtypes, and interestingly, overexpression of gankyrin is 1 of the diagnostic and subclassification markers for it.<sup>21</sup> Expression of gankyrin protein may be used as a marker for better prognosis of the patients with HCC as well.

The gankyrin oncoprotein plays a key role in regulation of cell cycle and apoptosis, at least in cultured cells, by inhibiting Rb and p53.<sup>10</sup> In a rodent hepatocarcinogenesis model, hypermethylation of the p16INK4A gene and p53 mutation appear at a late stage, whereas gankyrin is overexpressed from early after carcinogen treatment, pre-

ceding the loss of Rb protein and adenoma formation.<sup>22</sup> Clinically, p53 mutation is not so frequent in HCCs (15%-30%), especially in low-grade or low-stage HCCs.<sup>23,24</sup> Tan et al.<sup>20</sup> have immunohistochemically detected gankyrin overexpression in 82%, 63%, and 22% of Edmondson's grade I to II, III, and IV HCCs, respectively. We observed gankyrin positivity in 81% and 35% of low and high TNM stage HCCs, respectively. These results suggest that gankyrin plays an important role(s) at early stages of hepatocarcinogenesis by suppressing Rb, p53 and possibly other tumor suppressors. In advanced HCCs, by contrast, oncogenic mutations probably have accumulated in many genes including p53, and overexpression of gankyrin may not be so crucial as in early stage HCCs. This could explain the present association of gankyrin-negative HCCs with poorer prognosis and the finding that both cases of gankyrin-negative HCCs with gankyrin-positive noncancerous hepatocytes belonged to high TNM stages. This is, however, one of several possible explanations, and further work is necessary to clarify the exact reasons for the observed association.

By immunohistochemical staining, p53 has been detected in 20% to 30% of HCCs.<sup>25, 26</sup> Although strong immunohistochemical reactivity for p53 may not be an indicator of the presence of p53 gene mutations as initially suggested,<sup>26</sup> it has been associated in some studies with higher proliferative activity, lower differentiation of HCC cells, or poorer survival of patients. Endo et al.<sup>27</sup> immunohistochemically detected MDM2 in 28 of 107 (26%) HCCs, and the positive expression correlated with

the presence of p53 mutation and poorer prognosis, although it also correlated with smaller HCC size and the absence of vascular invasion. We immunohistochemically detected the expression of p53 and MDM2 in 30% and 23%, respectively, of HCCs, which is in accord with other studies, but no correlation was seen between expression and survival of the patients. Gankyrin accelerates degradation of Rb, p53, and MDM2 in cultured cells.<sup>9,16</sup> Although some correlation between expression of gankyrin and Rb has been suggested in HCC tissues,<sup>20</sup> we did not observe significant relationship between the gankyrin positivity and negative staining for p53 nor MDM2. The analysis of individual cells for protein expression, for example by double 2-color immunostaining, may have revealed the presence of some relationship. But most probably, our finding reflects complex interrelated mechanisms regulating the levels of these proteins and also suggests that the relevance of the effects of gankyrin on p53, MDM2, and Rb demonstrated in cultured cells to human hepatocarcinogenic process remains to be firmly established.

The 6 members of IGFBP family (IGFBP-1 through IGFBP-6) are important components of the insulin-like growth factor (IGF) axis, and regulate the activity of both IGF-I and IGF-II polypeptide growth factors.<sup>28</sup> IGF-I, IGF-II, and their receptors are expressed in a wide variety of cells, and the liver is the main source of circulating IGF-I. IGFBPs are also secreted by many cell types, and their expression is regulated in a cell-dependent and tissue-type-dependent manner. In the current study, we found up-regulation of IGFBP-5 mRNA and protein levels by overexpression of gankyrin in human osteosarcoma and HCC cell lines and consistently detected a significant association between the protein levels of gankyrin and IGFBP-5 in HCC specimens. In the proximal promoter region of the IGFBP-5 gene, there are several putative transcription-factor-binding sites including those for AP-2, c-Myb, C/EBP, and NF-1, and responsive elements to prostaglandin E<sub>2</sub>, cyclic adenosine monophosphate, progesterone/retinoic acid, and Akt.<sup>28</sup> Whether the effect of gankyrin on IGFBP-5 expression is mediated by these factors is unknown.

The IGFBPs bind IGFs with high affinity, and they are able to enhance or inhibit the activity of IGFs in a cell-specific and tissue-type-specific manner.<sup>28</sup> In addition, IGFBPs have IGF-independent effects. There are several reports on the relationship between the IGF axis and HCC.<sup>29-31</sup> IGFBP-3 is the most abundant IGFBP present in noncancerous liver tissue and could serve as a negative regulator of cell proliferation in human HCCs.<sup>32</sup> Although the presence of IGFBP-5 in numerous tumors and cell lines has been demonstrated, its expression and signif-

icance in human HCC have not been documented. We found positive staining for IGFBP-5 in 42% of HCCs, and the positivity correlated with absence of portal venous invasion, low TNM stage, and small tumor size. Although not statistically significant, patients with IGFBP-5-positive HCCs tended to survive longer than those with IGFBP-5-negative HCCs. These findings are essentially similar to those observed for gankyrin. Regarding the effect of IGFBP-5 on cell proliferation, there are contradictory findings.<sup>28</sup> In breast cancer cells, many studies have reported inhibition of growth, but there are some indicating a stimulatory effect.<sup>33</sup> IGFBP-5 is up-regulated in involuting prostate but is also implicated in growth stimulation of prostate tumor cells.<sup>34</sup> We found that down-regulation of IGFBP-5 suppresses growth of Huh-7 HCC cells. Thus, these findings are consistent with a notion that high expression of IGFBP-5 and gankyrin play oncogenic roles in HCCs of early clinical stages. Clarification of the exact roles played by them will shed more light on the molecular mechanisms of human hepatocarcinogenesis and lead to development of new therapeutic and preventive strategies.

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# Low-intensity ultrasound and microbubbles enhance the antitumor effect of cisplatin

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Cell permeabilization using microbubbles (MB) and low-intensity ultrasound (US) have the potential for delivering molecules into the cytoplasm. The collapsing MB and cavitation bubbles created by this collapse generate impulsive pressures that cause transient membrane permeability, allowing exogenous molecules to enter the cells. To evaluate this methodology *in vitro* and *in vivo*, we investigated the effects of low-intensity 1-MHz pulsed US and MB combined with *cis*-diamminedichloroplatinum (II) (CDDP) on two cell lines (Colon 26 murine colon carcinoma and EMT6 murine mammary carcinoma) *in vitro* and *in vivo* on severe combined immunodeficient mice inoculated with HT29-luc human colon carcinoma. To investigate *in vitro* the efficiency of molecular delivery by the US and MB method, calcein molecules with a molecular weight in the same range as that of CDDP were used as fluorescent markers. Fluorescence measurement revealed that approximately  $10^6$ – $10^7$  calcein molecules per cell were internalized. US–MB-mediated delivery of CDDP in Colon 26 and EMT6 cells increased cytotoxicity in a dose-dependent manner and induced apoptosis (nuclear condensation and fragmentation, and increase in caspase-3 activity). *In vivo* experiments with xenografts (HT29-luc) revealed a very significant reduction in tumor volume in mice treated with CDDP + US + MB compared with those in the US + CDDP groups for two different concentrations of CDDP. This finding suggests that the US–MB method combined with chemotherapy has clinical potential in cancer therapy. (*Cancer Sci* 2008; 99: 2525–2531)

Microbubbles (MB) have been developed as ultrasound (US) contrast agents with a diameter of less than 10  $\mu\text{m}$ . The components of their shell membrane vary (albumin, lipid, or polymer), and gases such as air or perfluorocarbons are internalized in them.<sup>(1–3)</sup> These bubbles oscillate non-linearly in an US field and emit harmonic and subharmonic acoustic signals, thereby enabling differentiation between acoustic scattering and vascular signatures. In addition, because these bubbles behave similar to red blood cells, they have been used to evaluate the blood pool and blood flow at the microvascular level.<sup>(4)</sup>

Microbubbles collapse in the presence of low-intensity US. There is evidence that impulsive pressures generated by either collapsing MB<sup>(5,6)</sup> or the cavitation bubbles created by this collapse may permeabilize the plasma membrane of neighboring cells.<sup>(7,8)</sup> This process results in the diffusion of nearby exogenous molecules into the cytoplasm and a subsequent biological response.<sup>(8–11)</sup> Because these impulsive pressures can be induced by high-intensity US,<sup>(12,13)</sup> substantial thermal and mechanical side effects can be reduced using the US–MB method.

The US–MB method is non-toxic and non-immunogenic, and allows local or systemic administration. This method can be used to deliver exogenous molecules into dividing and non-dividing cells and has been investigated as an approach for *in vivo* gene transfer and molecular delivery.<sup>(14–16)</sup> In any case,

the efficiency of molecular delivery depends on the size of the molecules to be delivered.<sup>(17,18)</sup> The amount of molecules increases with decreasing molecular weight. Thus, it is expected that this methodology will be useful for therapeutic strategies involving drugs with small molecular sizes. *Cis*-diamminedichloroplatinum (II) (cisplatin; CDDP) is one of the most effective and commonly used chemotherapeutics, possessing a molecular weight of 300. CDDP has been used for the treatment of many solid tumors, including those of the ovaries, testicles, bladder, lung, and head and neck.<sup>(19)</sup> Increasing CDDP penetration into the tumor cells could further improve its therapeutic efficacy. In the present study, we estimated the number of CDDP molecules internalized by the US–MB method using calcein molecules with molecular weights in the same range as that of CDDP as fluorescent markers. Subsequently, we assessed the therapeutic potential of the combination of CDDP and US with MB *in vitro* and *in vivo* and demonstrated that this combination induces apoptotic effects, and increases the therapeutic efficacy.

## Materials and Methods

*In vitro* and *in vivo* studies were carried out in accordance with the ethical guidelines approved by Tohoku University.

**Cell preparation.** Human embryonic kidney (293T) cells were a generous gift from Dr Ono of Tohoku University. Murine mammary carcinoma (EMT6) cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Murine colon carcinoma (Colon 26) cells were obtained from the Cell Resource Center for Biomedical Research of the Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). Human colon carcinoma (HT-29-luc) cells stably transfected with a plasmid carrying the firefly luciferase gene driven by a cytomegalovirus promoter were obtained from Xenogen (Alameda, CA, USA). Colon 26 and HT-29-luc cells were cultured under standard conditions in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 1% L-glutamine–penicillin–streptomycin (Sigma-Aldrich, St Louis, MO, USA), whereas 293T and EMT6 cells were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich) containing the same supplements as those added to the RPMI-1640 medium. HT29-luc cells were selected in 1 mg/mL geneticin (G418) (Sigma-Aldrich). Cells cultured in a 10-cm culture dish were maintained in a humidified incubator at 37°C under an atmosphere containing 5% CO<sub>2</sub> and

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95% air. The total cell counts and viability were counted in a hemocytometer by the trypan blue dye exclusion method<sup>(20)</sup> prior to US exposure. Only cells in their exponential growth phase with a viability  $\geq 99\%$  were used for the study.

**Microbubbles.** MB were created in an aqueous dispersion of 2 mg/mL 1,2-distearoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids, Alabaster, AL, USA) and 1 mg/mL polyethylene glycol 40 stearate (Sigma-Aldrich) using a 20-kHz sonicator (Vibra Cell; Sonics and Materials, Danbury, CT, USA) in the presence of  $C_3F_8$  gas.<sup>(21)</sup> The lipid molecules that formed components of the MB surface were confirmed by staining the molecules with 3  $\mu\text{mol/L}$  FM1-43 (excitation 479 nm, emission 598 nm; Molecular Probes, Eugene, OR, USA) and observing them under an inverted microscope (IX81; Olympus, Tokyo, Japan). The peak diameter and the zeta potential of the MB were determined to be  $1272 \pm 163$  nm ( $n = 7$ ) and  $-4.1 \pm 0.85$  mV ( $n = 3$ ), respectively, by using a laser diffraction particle size analyzer (particle range 0.6 nm–7  $\mu\text{m}$ ; ELSZ-2; Otsuka Electronics, Osaka, Japan).

**Ultrasound exposure.** Three 1-MHz submersible US probes were used. A 12-mm (Fuji Ceramics, Fujinomiya, Japan) and a 30-mm diameter probe (BFC Applications, Fujisawa, Japan) were used for the *in vitro* experiments, whereas 38-mm diameter probes (Fuji Ceramics) were used for the *in vivo* experiments. Each probe was placed in the test chamber (380 mm  $\times$  250 mm  $\times$  130 mm) that was previously filled with tap water. Signals of 1 MHz were generated by a multifunction synthesizer (WF1946A; NF Co., Yokohama, Japan) and amplified with a high-speed bipolar amplifier (HSA4101; NF Co.). The pressure values were measured using a polyvinylidene fluoride (PVDF) needle hydrophone (PVDF-Z44-1000; Specialty Engineering Associates, Soquel, CA, USA) at a stand-off distance of 1 mm from the transducer surface by using a stage control system (Mark-204-MS; Sigma Koki, Tokyo, Japan). The signals from both the amplifier and the hydrophone were recorded onto a digital phosphor oscilloscope (Wave Surfer 454, 500 MHz, 1 M $\Omega$  [16 pF]; LeCroy Co., Chestnut, NY, USA) in top water degassed with transducer (SPN-620) generated by ultrasonic generator  $\alpha 2$  (GP-622D) (Tiyoda Electric Co., Chikuma, Japan). The positive and negative peak values of the pressures were the same. Two intensities, 0.5 and 1.0 W/cm<sup>2</sup>, were used in the *in vitro* experiments. The duty cycle was 50%, the number of pulses was 2000, the pulse repetition frequency was 250 Hz, and the exposure time was 10 s. For the *in vivo* experiments, the intensity was 3.0 W/cm<sup>2</sup>, the duty cycle was 20%, the number of pulses was 200; the pulse repetition frequency was 1000 Hz, and the exposure time was 60 s. The intensity was defined as the average rate of flow of energy through a unit area placed normal to the direction of propagation.

***In vitro* quantization of calcein uptake.** The 293T cells ( $5 \times 10^4$  cells/well) were seeded in complete medium onto 48-well plates and incubated at 37°C in a 5% CO<sub>2</sub> incubator. On the next day, the medium was replaced with fresh medium containing 200  $\mu\text{mol/L}$  calcein (molecular weight 622) (excitation 494 nm, emission 517 nm; Sigma-Aldrich) with and without MB (10% v/v). After US exposure for 10 s, the cells were washed with phosphate-buffered saline (PBS), trypsinized, and collected in a 15-mL conical tube. Thereafter, the cells were washed three times and transferred to a 1.5-mL conical tube in which they were pelleted. The pellets were lysed in 200  $\mu\text{L}$  reporter lysis buffer (Promega, Madison, WI, USA) and subsequently frozen at  $-80^\circ\text{C}$  for 15 min. The cells were thawed on ice. Each cell lysate was centrifuged at 12 000g for 2 min to pellet the cell debris. Twenty microliters of the supernatant was examined for the uptake of fluorescent molecules using Mx3000P software (Stratagene, La Jolla, CA, USA). The fluorescence of these molecules was excited using a quartz tungsten halogen lamp (350–750 nm), and the emission was collected with a 492–516-nm bandpass filter. The fluorescence

data were analyzed with MxPro QPCR Software (Stratagene). The total protein content from an aliquot of each sample of supernatant was calculated by establishing albumin standard curves (BCA protein assay kit; Pierce, Rockford, IL, USA). In addition, two other standard curves were utilized: one to determine the total protein content of the cells, and the other to determine the concentration and intensity of the fluorescence. The experiment was carried out with samples and standards in duplicate, and the absorption of the protein was measured at 562 nm using a plate reader (Sunrise; Tecan Austria, Salzburg, Austria) with the data analysis software LS-Plate manager RD 2001 (Win) (Sunrise). The number of equivalent fluorescent molecules per cell was determined from the calibration curves.

**Imaging of confocal fluorescence microscopy.** The 293T cells ( $5 \times 10^4$  cells/well) were seeded in complete medium onto alternate 48-well plates to prevent US exposure of neighboring cells.<sup>(8)</sup> On the next day, the medium was replaced with fresh medium (110  $\mu\text{L}$ ) containing calcein (200  $\mu\text{mol/L}$ ) with and without MB (10% v/v). After US exposure of 10 s, the plates were incubated for 24 h. Thereafter, the cells were washed three times with PBS and trypsinized. Finally, the cell pellet was resuspended in 60  $\mu\text{L}$  propidium iodide (PI) (excitation 536 nm, emission 617 nm; Molecular Probes) (0.7  $\mu\text{g/mL}$ ) and incubated at room temperature for 10–15 min. The calcein and PI fluorescence intensities were determined with a confocal microscope (FV1000; Olympus). A  $\times 60$  oil-immersion objective lens with a numerical aperture of 1.25 was used. Calcein and PI fluorescence were excited with the 488-nm line of an argon laser. The laser excitation beam was directed to the specimen through a 488-nm dichroic beam splitter. The emitted fluorescence was collected through a 510–550-nm bandpass emission filter in the green channel and a 580-nm longpass filter in the red channel. Computer-generated images of 1- $\mu\text{m}$  optical sections were obtained at the approximate geometric center of the cell as determined by repeated optical sectioning.

***In vitro* delivery of CDDP.** CDDP (molecular weight 300) was donated by Nihon Kayaku (Tokyo, Japan). Colon 26 and EMT6 cells were seeded in complete medium onto 10-cm culture dishes. Both cells were trypsinized, counted, and transferred into 15-mL round tubes at concentrations of  $5 \times 10^5$  and  $3 \times 10^5$  cells/mL, respectively. For control samples, 1 mL complete medium was used as the sample solution; for treated samples, the sample solution was mixed with 800  $\mu\text{L}$  complete medium, 100  $\mu\text{L}$  CDDP solution (0.5–1.0 mmol/L), and 100  $\mu\text{L}$  MB solution (7% v/v). Each tube was positioned above a 30-mm diameter US probe that was immersed in tap water and exposed to US (10 s; 0.5 W/cm<sup>2</sup>). After exposure, 4 mL PBS was added to each tube and centrifuged for 5 min at 4°C (350g). The cells were washed twice with PBS and subsequently seeded in 1 mL complete medium onto 24-well plates. The plates were incubated for 24 h at 37°C in a 5% CO<sub>2</sub> incubator. The cell viability was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously.<sup>(22)</sup> Each experiment was carried out with five samples. For each experiment, the mean percentage of treated samples was divided by the mean percentage of control samples to obtain the survival fraction. The mean of five survival fractions was calculated for each condition. The survival fraction of each cell line was measured at the CDDP concentration at which the highest statistical significance was obtained.

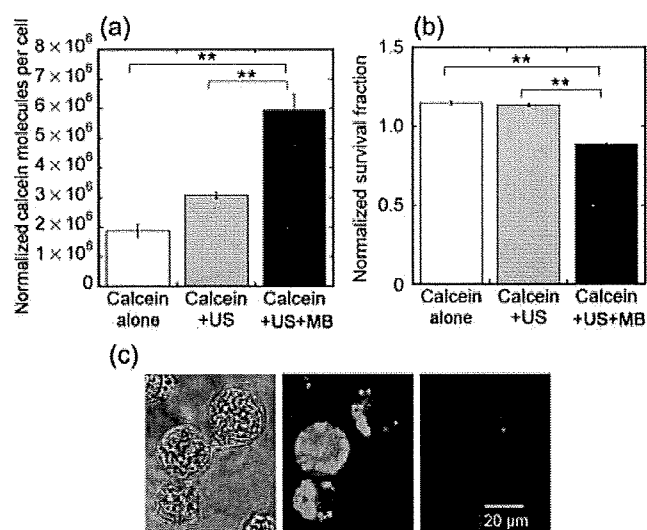
***In vitro* analysis of apoptosis.** Colon 26 ( $5 \times 10^4$  cells/well) cells were seeded onto alternate 48 wells to prevent the US exposure of neighboring cells.<sup>(8)</sup> The medium was replaced with fresh medium (110  $\mu\text{L}$ ) containing CDDP (1.5 mg) with and without MB (7% v/v). After US exposure, the plates were incubated for 1 h in a 5% CO<sub>2</sub> incubator, supplemented with 390  $\mu\text{L}$  complete medium; subsequently, the plates were incubated for an additional 24 h at 37°C in the same incubator. The final

concentration of CDDP was 10  $\mu\text{mol/L}$ . The cell viability was determined by an MTT assay as described previously.<sup>(22)</sup> Staining with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) was carried out for observing nuclear condensation and fragmentation. Twenty-four hours after the addition of CDDP, the cells were washed with PBS, stained with 100  $\mu\text{L}$  DAPI (100 ng/mL) solution, and observed under an inverted microscope (IX 81). DAPI fluorescence of the cell nuclei was visualized by excitation at 330–385 nm with a 420-nm barrier filter. For determining the induction of apoptotic mediator proteins, caspase-3 activity was measured using a colorimetric assay kit (Medical and Biological Laboratories, Woburn, MA, USA) 24 h after the treatment. In brief, the treated cells were collected from 12 wells of the 48-well plates and suspended in cell lysis buffer. Aliquots of protein were incubated in a reaction buffer containing 10 mmol/L dithiothreitol (DTT) at 37°C for 1 h. A p-nitroaniline-conjugated synthetic peptide was used as the substrate. The caspase activity was calculated by measuring the optic absorbance at 400 nm using a plate reader with the data analysis software LS-Plate manager RD 2001 (Win).

**In vivo therapeutic effects.** To evaluate the antitumor effects of MB, the antitumor effects of CDDP + US and CDDP + US + MB were compared using xenografts of HT29-luc cells. Two CDDP concentrations (0.5 and 1.25  $\mu\text{g/g}$  bodyweight) were used. HT29-luc cells ( $1 \times 10^6$  cells) in 100  $\mu\text{L}$  saline were injected subcutaneously into the right and left flanks of 16 male severe combined immunodeficient mice aged 6–9 weeks (mouse bodyweight was set to 20 g). The mice were assigned randomly into two groups. On days 3, 7, and 10, all mice were injected intratumorally with the following assigned treatments. (i) Four mice received 20  $\mu\text{L}$  CDDP (0.5  $\mu\text{g}/\mu\text{L}$  bodyweight) with 80  $\mu\text{L}$  saline per site following US exposure (CDDP + US), and four others received 20  $\mu\text{L}$  CDDP (0.5  $\mu\text{g}/\mu\text{L}$  bodyweight) with 30  $\mu\text{L}$  saline and 50  $\mu\text{L}$  MB per site following US exposure (CDDP + US + MB). (ii) Four mice received 50  $\mu\text{L}$  CDDP (1.25  $\mu\text{g}/\mu\text{L}$  bodyweight) with 50  $\mu\text{L}$  saline per site following US exposure (CDDP + US), and four others received 50  $\mu\text{L}$  CDDP (1.25  $\mu\text{g}/\mu\text{L}$  bodyweight) with 50  $\mu\text{L}$  MB per site following US exposure (CDDP + US + MB). The tumors were immersed in tap water with a temperature of 37°C, positioned just above the 38-mm diameter US probe, and exposed to US (3.0 W/cm<sup>2</sup>, 60 s). Bioluminescence induced by CDDP + US + MB was normalized with that of CDDP + US at each concentration (0.5 and 1.25  $\mu\text{g/g}$  bodyweight) on days 4, 7, 9, and 11 to provide the antitumor effects of MB.

**Bioluminescence imaging.** On days 4, 7, 9, and 11, the mice were anesthetized with isoflurane. Subsequently, they were injected intraperitoneally with luciferin (150  $\mu\text{g/g}$  bodyweight) and placed on the *in vivo* imaging system (IVIS100; Xenogen). The bioluminescence signals were monitored at 10-s time intervals after 10 min luciferin administration. The signal intensity was quantified as the sum of all detected photon counts within the region of interest after subtraction of the measured background luminescence. The light intensity closely correlated with the tumor volume (EMT6-luc) up to 100 mm<sup>3</sup>, at which point the tumor volume was calculated according to the formula  $(\pi/6) \times (\text{width})^2 \times (\text{length})$ .<sup>(21)</sup> In the present experiment, all tumors (HT29-luc) with a volume less than 100 mm<sup>3</sup> were subjected to treatment.

**Statistical analysis.** All measurements are expressed as mean  $\pm$  SEM. An overall difference between the groups was determined by one-way analysis of variance (one-way ANOVA). When the one-way ANOVA results were significant for three samples, the differences between each group were estimated using the Tukey–Kramer test. Simple comparisons of the mean and SEM of the data were carried out using Student's *t*-test. The differences were considered to be significant at  $P < 0.05$ .



**Fig. 1.** Uptake of fluorescent molecules by 293T cells. (a) Mean fluorescence uptake under various conditions (calcein alone, calcein + ultrasound [US], and calcein + US + microbubbles [MB]). The calcein + US + MB condition results in a significant increase in the uptake of fluorescent molecules ( $6.0 \pm 0.5 \times 10^6$  molecules per cell) compared to calcein alone and calcein + US. Calcein alone ( $n = 4$ ), calcein + US ( $n = 4$ ), calcein + US + MB ( $n = 4$ ). (b) Cell viability measured by the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The survival fraction is decreased slightly by the effect of US + MB. Calcein alone ( $n = 4$ ), calcein + US ( $n = 4$ ), calcein + US + MB ( $n = 4$ ). (c) Confocal microscopy showing differential interference contrast (left) and fluorescence images (middle) or representative viable 293T cells (right) exposed to US in the presence of MB. Propidium iodide (PI) staining was carried out with fluorescence staining in some cases to confirm that the cells that acquired calcein were viable and excluded PI. Scale bars = 20  $\mu\text{m}$ . Ultrasound intensity 1.0 W/cm<sup>2</sup>; duty cycle 50%; number of pulses 2000; pulse repetition frequency 250 Hz; and exposure time 10 s. \*\* $P < 0.01$ .

## Results

**Uptake of fluorescent molecules.** Calcein, which has a molecular weight of 622 (calculated Stokes radius of 0.68),<sup>(23)</sup> was used as a fluorescent marker to evaluate small-molecule entry in cancer cells upon US–MB stimulation. As the molecular weight of CDDP is 300 (calculated Stokes radius is 0.48 nm), calcein can be considered to represent a realistic marker of CDDP entry into tumor cells.

The exposure of cells to US in the presence of MB resulted in the delivery of  $10^6$ – $10^7$  calcein molecules per cell (Fig. 1a). This represents a significant increase in the uptake of fluorescent molecules compared to calcein alone and calcein + US. Figure 1b shows that this effect was achieved with a very limited loss of cell viability that was measured by MTT assay, where the survival fraction rate due to MB alone was not investigated as it was found that MB alone did not contribute to cell viability.<sup>(24)</sup> To confirm that the calcein molecules actually entered the cytoplasm, confocal fluorescence microscopic analysis was carried out. Figure 1c shows the differential interference contrast and fluorescence images or the representative viable 293T cells exposed to US in the presence of MB. PI staining was also carried out with some instances of fluorescence staining to confirm that the cells that acquired calcein were viable and excluded PI. Some cells treated with US in the presence of MB demonstrated intense fluorescence distributed uniformly throughout the entire cell, whereas other cells demonstrated localized intense fluorescence (Fig. 1c).

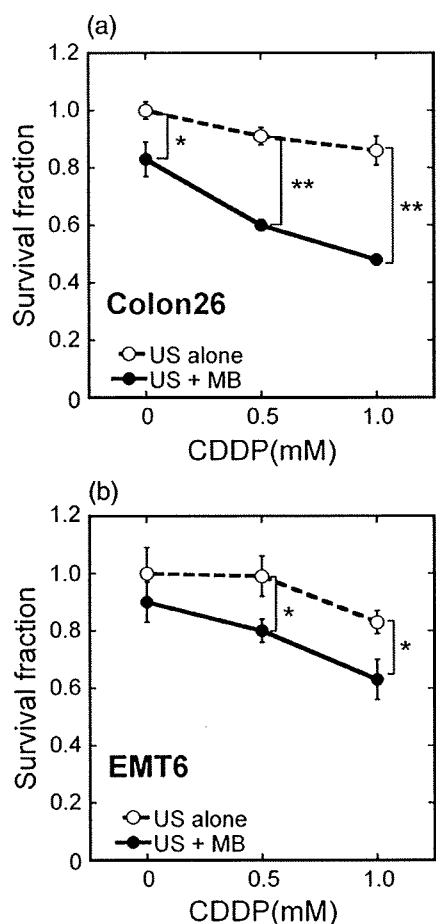


Fig. 2. Potentiation of *in vitro* cis-diamminedichloroplatinum (II) (CDDP) cytotoxicity in Colon 26 and EMT6 cells. (a) Colon 26: (s) ultrasound (US) alone (n = 5) and (d) US + microbubbles (MB) (n = 5). (b) EMT6: (s) US alone (n = 5) and (d) US + MB (n = 5). Cell survival was measured by a 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay 24 h after US exposure. US intensity 0.5 W/cm<sup>2</sup>; duty cycle 50%; number of pulses 2000; pulse repetition frequency 250 Hz; and exposure time 10 s. The bars represent mean  $\pm$  SEM. \*P < 0.05, \*\*P < 0.01.

**Cytotoxicity *in vitro*.** The cytotoxicity of various doses of CDDP in the presence of US with and without MB was tested on Colon 26 and EMT6 cells (Fig. 2). A marked increase in CDDP toxicity was observed under the US–MB conditions, whereas US alone did not significantly affect cell survival with various CDDP concentrations. The CDDP toxicity depended slightly on the cell type.

**Apoptosis assay.** CDDP is known to induce apoptosis.<sup>(25)</sup> We confirmed the involvement of apoptosis in mediating cytotoxicity in response to CDDP. Cells undergoing apoptosis demonstrate characteristic nuclear morphological changes with DAPI staining. Figure 3 shows phase contrast and DAPI images of Colon 26 cells. Untreated control cells (Fig. 3a,b) and cells treated with US + MB (Fig. 3c,d), CDDP alone (Fig. 3e,f), and CDDP + US (Fig. 3g,h) showed extremely little condensed or fragmented chromatin. The majority of cells treated with CDDP + US + MB (Fig. 3i,j) displayed apoptotic features, including condensed nuclei and nuclear fragmentation.

Induction of caspase-3 has been suggested as a marker of apoptosis.<sup>(26)</sup> Figure 4 shows that treatment with US + MB activates caspase-3 as compared to treatment with CDDP alone or with CDDP + US. The caspase activity increases with time. Taken together, the data presented in Figures 3 and 4 demonstrate that the CDDP + US + MB combination decreases cell viability and that this reduction in cell survival is associated with increased induction of apoptosis. In the present study, we did not consider the activation of caspase-3 by US because US alone did not contribute to the survival fraction (Fig. 2) and subsequent apoptotic induction (Fig. 3).

***In vivo* therapeutic effects of MB.** From the above *in vitro* experiments, we found that MB associated with US are able to trigger the uptake of small molecules (Fig. 1), thereby inducing antitumor effects (Fig. 2) and apoptosis (Figs 3,4) in conjunction with CDDP. Thereafter, we investigated the *in vivo* antitumor effects of using MB, in cases in which the xenografts of HT29-luc cells were used. We investigated the antitumor effects of CDDP + US + MB on the xenografts with two different CDDP concentrations (0.5 and 1.25  $\mu$ g/g bodyweight) on days 4, 7, 9, and 11. The luciferase activity for each concentration of CDDP + US + MB was normalized with each concentration of CDDP + US that was administered previously (Fig. 5a) in order to determine the antitumor effects of MB alone. The activities of the MB were recognized after day 7 (second treatment) in both groups. On day 11, a significant reduction was observed in the CDDP + US + MB group compared to the control and US + MB

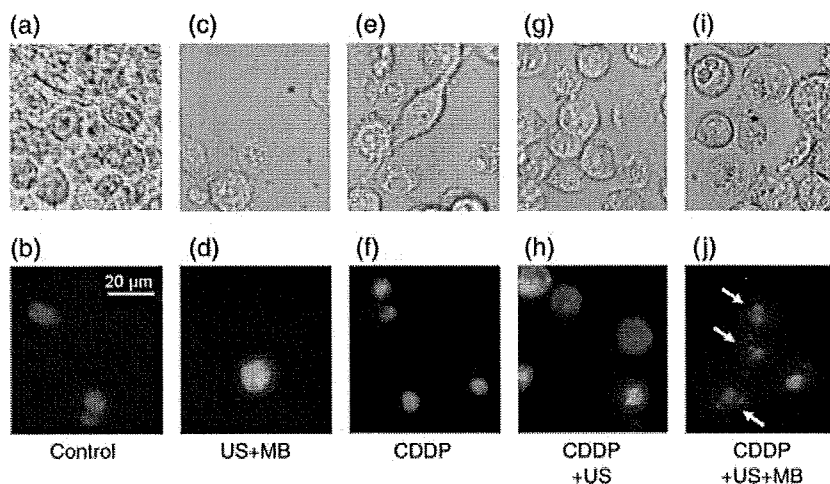
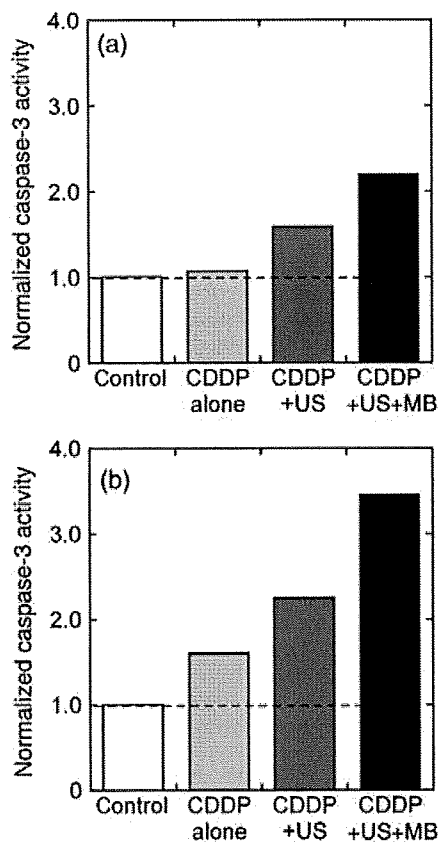


Fig. 3. Nuclear condensation and fragmentation. (a,c,e,g,i) Differential interference contrast and (b,d,f,h,j) 4',6-diamidino-2-phenylindole (DAPI) fluorescence images of representative viable Colon 26 cells 24 h after treatment. 10  $\mu$ mol/L cis-diamminedichloroplatinum (II) (CDDP): (a,b) control, (c,d) ultrasound (US) + microbubbles (MB), (e,f) CDDP, (g,h) CDDP + US, and (i,j) CDDP + US + MB. Round or shrunken nuclei of DAPI-stained cells (white arrows) are hallmarks of apoptosis in (j). Experiments were repeated three times with similar results. Scale bar = 20  $\mu$ m. Ultrasound intensity 1.0 W/cm<sup>2</sup>; duty cycle 50%; number of pulses 2000; pulse repetition frequency 250 Hz; and exposure time 10 s.



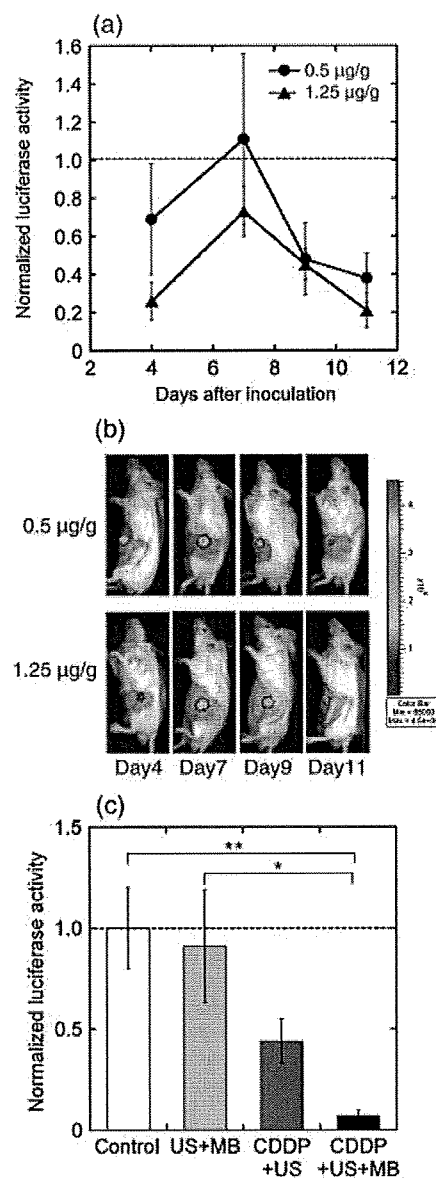


**Fig. 4.** Upregulation of the proapoptotic gene caspase-3. Colon 26 cells were treated with cis-diamminedichloroplatinum (II) (CDDP) (10  $\mu\text{mol/L}$ ) in the presence of ultrasound (US) with and without microbubbles (MB). Caspase-3 activity was measured at 24 h after treatment. Twelve wells from 48-well plates were analyzed for each condition. Results are expressed as the number of molecules of p-nitroaniline (pNA) (nmol) released by 1 mg of protein in (a) 1 and (b) 2 h. Ultrasound intensity 1.0 W/cm<sup>2</sup>; duty cycle 50%; number of pulses 2000; pulse repetition frequency 250 Hz; and exposure time 10 s.

groups. These effects were recognized by bioluminescence images (Fig. 5b). Figure 5c shows antitumor effects for different conditions (US + MB, CDDP + US, CDDP + US + MB) at day 11, where the CDDP concentration was 1.25  $\mu\text{g/g}$  bodyweight, and the bioluminescence of each condition was normalized with that of the control at day 11. There was no significant difference between control and US + MB. CDDP alone was recognized as the difference between US + MB and CDDP + US, where CDDP alone decreased US + MB by 48.4%. MB further reduced CDDP + US by 84.1%.

## Discussion

The US-MB method permeabilizes the cell membrane directly, thereby allowing the delivery of exogenous molecules into the cells. Electroporation is also a method that is used to permeabilize the cell membrane by direct application of an external electric field. Cemazar *et al.* delivered CDDP into both murine sarcoma cisplatin-sensitive TBL.C12 cells and their resistant subclones, namely, TBL.C12.Pt cells.<sup>(27)</sup> These cells were treated *in vivo* by electroporation, and their platinum content was measured by atomic absorption. Based on their findings, the authors suggested that  $10^6$  platinum molecules were delivered into TBL.C12 and TBL.C12.Pt cells at 0.05 and



**Fig. 5.** Antitumor effects of cis-diamminedichloroplatinum (II) (CDDP) + ultrasound (US) + microbubbles (MB) on HT29-luc xenografts with two different CDDP concentrations (0.5 and 1.25  $\mu\text{g/g}$  bodyweight) on days 4, 7, 9, and 11. Ultrasound intensity 3.0 W/cm<sup>2</sup>; duty cycle 20%; number of pulses 200; pulse repetition frequency 1000 Hz; and exposure time 60 s. Luciferase activity after (a) treatment and (b) bioluminescence imaging. The luciferase activity under each concentration of CDDP + US + MB was normalized with each concentration of CDDP + US. CDDP (0.5  $\mu\text{g/g}$  bodyweight) + US ( $n = 4$ ), CDDP (0.5  $\mu\text{g/g}$  bodyweight) + US + MB ( $n = 4$ ), CDDP (1.25  $\mu\text{g/g}$  bodyweight) + US ( $n = 4$ ), CDDP (1.25  $\mu\text{g/g}$  bodyweight) + US + MB ( $n = 4$ ). (c) The luciferase activity normalized with that of control at day 11, where the concentration of CDDP was 1.25  $\mu\text{g/g}$  bodyweight. Control ( $n = 5$ ), US + MB ( $n = 4$ ). The bars represent mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ .

0.46  $\mu\text{g/mL}$ , respectively, where these concentrations correspond to the 50% inhibitory concentration values of platinum for these cells. Our finding that  $10^6$ – $10^7$  calcein molecules were internalized per cell at 200  $\mu\text{mol/L}$ , as shown in Figure 1a, is in agreement with the finding of Cemazar *et al.* and demonstrates that US-MB permeabilization is as efficient as electroporation in the permeabilization of cell membranes.

There are several anticancer drugs with a similar molecular weight to CDDP. For example, the molecular weight of vincristine is 923, and that of taxol is 854. There is not even one order difference between these molecules and cisplatin, which has a molecular weight of 300. Therefore, we think that the same number of molecules will be delivered into cells by this method. However, it is noted that the number of molecules internalized is not directly correlated with subsequent antitumor effects.

Following the application of MB and US, the cell membrane surface becomes rough and is characterized by depressions that are reversible within 24 h after US exposure.<sup>(28,29)</sup> Collapsed MB or cavitation bubbles generated by collapsed MB induce impulsive pressures such as liquid jets and shock waves; these pressures affect the neighboring cells. The shock wave propagation distance from the center of a cavitation bubble that has the potential to damage the cell membrane is considerably larger than the maximum radius of the cavitation bubble.<sup>(6)</sup> Molecular dynamic simulation has revealed that the cell membrane affected by the shock wave is deformed, thereby allowing the entry of exogenous molecules into the cells.<sup>(30)</sup> Although the membrane permeabilization time has not been measured accurately, it has been reported that the membrane reseals within 80 s when it is permeabilized by shock waves.<sup>(31)</sup> When 1176 water molecules are delivered into a lipid bilayer comprising 128 dipalmitoylphosphatidylcholine molecules, a water pore of 1.9-nm diameter is formed in the lipid bilayer;<sup>(30)</sup> this water pore is larger than the CDDP with a diameter of 0.48 nm.

As shown in Figure 1, the US-MB method enhances cell permeability, thereby allowing the delivery of a large number of CDDP molecules into the cells. The combination of high-intensity ultrasound (shock waves) and generated cavitation bubbles – the same concept as that used in the US-MB method – can deliver CDDP molecules into the cells.<sup>(32–35)</sup> However, the cytotoxicity of CDDP depends on the cell type (Fig. 2); therefore, resistance to CDDP is not completely overcome simply by the application of US and MB (or cavitation bubbles).

The delivery of CDDP into cells induces apoptosis.<sup>(36)</sup> Apoptotic pathways involved in mediating CDDP-induced cellular effects have been investigated thoroughly.<sup>(37)</sup> The apoptotic induction resulting from CDDP delivery was confirmed by DAPI staining (Fig. 3) and measurement of caspase-3 activity (Fig. 4). The upregulation of caspase-3 coincides with the observations of previous reports.<sup>(10,11,38,39)</sup> Caspase-3 is a key effector of apoptosis that is responsible for the proteolytic cleavage of cytoskeletal proteins, kinases, and DNA repair enzymes.<sup>(40)</sup> The signaling pathway that mediates US-induced apoptosis has been investigated previously.<sup>(11,41)</sup>

In the *in vivo* experiments, CDDP and MB were injected intratumorally on days 3, 7, and 10 after the injection with tumor cells (HT29-luc), and the tumor was exposed to US. The normalized luciferase activity increased by day 7 and decreased afterwards. This indicates that the antitumor effects resulted from the activity of MB becoming dominant against the tumorigenesis. Tumor growth was suppressed effectively, indicating that the US-MB method provides a synergistic effect with antitumor drugs.<sup>(42,43)</sup>

We used a local administration system in the *in vivo* experiments (direct injection of CDDP + MB and local US exposure).

This local exposure to US has more advantages than the currently available local therapies such as surgery and radiotherapy. However, the usefulness of local therapy is expected to be limited to a particular tumor or a particular tumor stage. For example, hepatocellular carcinomas and brain gliomas seldom metastasize to other organs. Instead, hepatocellular carcinomas and gliomas grow within the liver and brain, respectively, and eventually cause death without metastasizing to other organs. Because these tumors are fed by a tumor-feeding artery, the arterial injection of any anticancer agent (ACA) into the tumor-feeding artery is the most direct way of delivering it into the tumors, mainly because of the first-path effect. With regard to superficial bladder cancer recurrences, half of all such cases recur, and 10–30% progress to a higher grade or stage and form local invasive cancer. Intravesical administration of ACA is known to prolong the duration of progression-free survival. For a clinically randomized phase III trial conducted in patients with stage III ovarian cancer, local intraperitoneal injection of ACA can prolong the duration of overall survival, compared to systemic intravenous injection.<sup>(44)</sup>

According to the above mentioned evidence, we believe that it is possible to establish a local ACA delivery system in experimental animal models in anticipation of future clinical trials. The US-MB method has the advantages of tissue specificity and non-invasiveness. In addition, this method can be applied repeatedly to patients without immunogenicity.<sup>(21)</sup> However, the efficiency of molecular delivery into cells is low, and the subsequent bioeffects are not adequate to be investigated by clinical trials. Recently, many types of MB with characteristics of tissue specificity and drug incorporation have been developed<sup>(45)</sup> and the US exposure conditions have been investigated.<sup>(46,47)</sup> Combination of the US-MB method with other physical methods such as hyperthermia has also been investigated.<sup>(48)</sup> In our laboratory, we have investigated the relationship between the physicochemical properties (zeta potential, size, and lipid components) of MB and the transfection efficiency (data not shown).

In conclusion, in the present study we have demonstrated that the US-MB method combined with the well-known chemotherapeutic agent CDDP has great therapeutic potential in cancer therapy. By reducing the dose of CDDP required to induce cell death through the abovementioned method it may be possible to increase the therapeutic action of the drug and to limit the toxicity of the treatment.

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# Prostaglandins, Leukotrienes and Essential Fatty Acids

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## Fatty acid-binding protein regulates LPS-induced TNF- $\alpha$ production in mast cells

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### ABSTRACT

There has been increasing evidence for the involvement of fatty acid-binding proteins (FABPs) in the cytokine production of macrophages and dendritic cells probably through the control of cellular lipid metabolism and signal transduction. Since mast cells (MCs) are recently shown to be involved in immune response through modification of cytokine production, it is possible that some FABPs could also be involved in the immune response of MCs. In this study, we found that epidermal-type FABP (E-FABP) was expressed in murine bone marrow-derived MCs (BMMCs). Using BMMCs from genetically E-FABP-null mutated mice, we demonstrated that E-FABP in BMMCs plays a key role in the production of TNF- $\alpha$  following lipopolysaccharide (LPS) stimulation. In the *in vivo* septic peritonitis model (cecal ligation and puncture model), E-FABP-null mice showed a significantly increased mortality compared to wild-type mice. However, no significant difference in antigen-induced cytokine production was observed between wild-type and E-FABP-null BMMCs, and systemic anaphylaxis was equally induced *in vivo* in both wild-type and E-FABP-null mice. These results suggest that E-FABP is specifically involved in the LPS-induced cytokine production of MCs, and could play a role in the host-defense against bacterial infection, possibly through regulation of TNF- $\alpha$  production.

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### 1. Introduction

Mast cells (MCs) induce profound allergic responses through release of a variety of inflammatory mediators, including histamine and a number of immuno-regulatory cytokines, by cross-linking high-affinity receptors for IgE (Fc $\epsilon$ RI) on their plasma membrane and appropriate antigens [1,2]. In contrast to the harmful role of MCs, recent studies have revealed that MCs that reside in high numbers at the host–environment interface play a protective role in the host defense against bacteria [3,4]. When MCs are exposed to lipopolysaccharide (LPS), a bacterial cell wall component from gram-negative bacteria, they produce proinflammatory cytokines such as TNF- $\alpha$  and IL-6 through activation of toll-like receptors (TLRs) on their cell surface [5,6].

This cytokine response of MCs to various pathogens is strictly regulated by the cellular chemical environments including eicosanoids such as leukotrienes (LTs) and prostaglandins (PGs) [7–10], derivatives from long-chain fatty acids (LCFA). Thus, LCFAs are supposed to directly modulate the inflammatory response through modulation of intracellular signal transduction pathways of MCs [11]. However, the molecular mechanism underlying such regulation by LCFAs is still poorly understood.

Due to the hydrophobic property of LCFAs, intracellular transport or storage of LCFAs requires an interaction with carrier proteins such as fatty acid-binding proteins (FABPs) [12]. FABPs constitute a multigene family of structurally homologous cytosolic proteins capable of binding LCFAs and various eicosanoids, and could work as vehicles of water-insoluble ligands. Recent evidence suggests that the FABP family of proteins could play a role in (i) prompting cellular flux of poorly water-soluble ligands and their subsequent metabolic utilization or transformation, (ii) sequestration of ligands that limits their association with alternative-binding sites in the cell, and (iii) transport of ligands in

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a manner that promotes their association with alternative-binding sites in the cell [13]. Among members of the FABP family, epidermal-type FABP (E-FABP), also called mal1, KLBP, fabp5 or C-FABP, is expressed not only in keratinocytes [14,15] but also in macrophages and dendritic cells (DCs), which are known to be involved in inflammatory response [16–18]. The present authors have revealed that E-FABP-null splenic DCs show an increased production of IL-12 in response to LPS, suggesting a role of E-FABP as a modulator of cytokine production in the immune cells [19,20]. These findings suggest the possibility that FABP may also be involved in inflammatory response mediated by MCs through regulation of MC cytokine production. Here, we show that E-FABP, which is specifically expressed in MCs, modulates MC response to bacteria through regulation of TNF- $\alpha$  production.

## 2. Materials and methods

### 2.1. Animals

The generation of E-FABP gene knockout mice was described previously [21]. Mice used in this study were backcrossed from a mixed 129SVJ  $\times$  C57BL/6 background onto the C57BL/6 for at least eight generations and were intercrossed to produce wild-type and E-FABP-null mice. All experimental protocols were reviewed by the committee on the ethics of animal experiments of Yamaguchi University.

### 2.2. Preparations of BMMCs

Bone marrow-derived MCs (BMMCs) were isolated from the bone marrow of wild-type and E-FABP-null mutant mice on postnatal 12 week as previously described [22]. Briefly, mice were sacrificed, and intact femurs and tibias were removed. Sterile endotoxin-free medium was repeatedly flushed through the bone shaft using needles and syringes, and the bone marrow cells were passed through a nylon mesh to remove the bone fragments. The cell suspension was centrifuged at  $320 \times g$  for 20 min at 4 °C and cultured at a concentration of  $0.5\text{--}1 \times 10^6$  nucleated cells/ml in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 1 mM sodium pyruvate,  $1 \times$  non-essential amino acid, 50  $\mu$ M 2-mercaptoethanol, and 5 ng/ml murine IL-3 (R&D Systems, St. Louis, MO, USA). The medium was replaced three times a week with the fresh complete medium.

BMMCs were monitored for purity after 4 weeks by flow cytometry (FACS Caliber, Becton Dickinson, San Jose, CA, USA). The cells were incubated with fluorescently labeled antibodies in 5% FCS in PBS and were analyzed with CELLQuest Software. BMMCs were identified as double-positive cells for FITC-conjugated anti-IgE and PE-conjugated anti-c-Kit (BD Bioscience Pharmingen, San Diego, CA, USA). After 4 weeks of culture of BMMCs, more than 95% of the cells were identified as BMMCs (data not shown), and no significant differences were observed between wild-type and E-FABP-null BMMCs in the expression of these two markers (data not shown). Furthermore, no significant differences were detected in the increase of BMMC population during the time course of culture between wild-type and E-FABP-null mice (data not shown).

### 2.3. Immunocytochemistry

Immunocytochemistry was performed as described previously with slight modification [19]. Briefly, BMMCs were attached onto glass slides using a centrifugal cell collector (TOMY SEIKO, Tokyo, Japan). The cells on glass slides were incubated with a rabbit

anti-rat antibody against E-FABP [21] at a concentration of 0.5  $\mu$ g/ml for 12 h at 4 °C. After incubation with the primary antibody, the cells were incubated with an anti-rabbit antibody labeled by Alexa488 (Invitrogen, Carlsbad, CA, USA). After nuclear-counter staining with DAPI (4',6'-diamidino-2-phenylindole), the cells were covered with Gel/Mount (Biomed, Foster City, CA, USA), and observed by confocal laser microscope (LSM5 Pascal; Carl Zeiss, Oberkochen, Germany).

### 2.4. Immunoblotting

Cells were lysed in a lysis buffer solution that consisted of 12.5 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerin, 0.01% bromophenol blue, 1 mM PMSF, 1 mM  $\text{Na}_3\text{VO}_4$ , and protease inhibitor cocktail tablet (Roche, Basel, Switzerland). Lysates were cleared by centrifugation at 15,000 rpm for 5 min and boiled in a sample buffer solution. Cell lysates (10  $\mu$ g of total protein from BMMCs or tissues) were loaded onto 10% SDS-PAGE, fractionated and transferred to PVDF membranes (Osmonic Inc., Livmore, CA, USA). After blocking with 5% normal goat serum, the membrane was incubated with an appropriate primary antibody. Primary antibodies used in the present study were anti-rat E-FABP antibody [21], anti-mouse B (brain type)-FABP antibody [23], anti-mouse L (liver type)-FABP antibody [24], anti-mouse H (heart type)-FABP antibody [25] and anti-mouse A (adipocyte type)-FABP antibody [26]. After incubation with the primary antibody, the membranes were subsequently incubated with the anti-rabbit secondary antibody conjugated with HRP (Sigma, St. Louis, MO, USA). The immunopositive bands were visualized with the enhanced chemiluminescence immunoblotting detection system (Amersham Bioscience, Buckinghamshire, UK), and the images were digitally captured by ChemiDoc XRS (Bio-Rad Laboratories, Hercules, CA, USA).

### 2.5. Activation of BMMCs

BMMCs were seeded at  $1 \times 10^6$  cells per well in 96-well plates. For antigen stimulation, BMMCs were sensitized overnight with 1  $\mu$ g/ml trinitrophenyl (TNP) hapten-specific IgE (TNP-IgE) and 1 ng/ml TNP-conjugated ovalbumin (TNP-OVA) (fraction VII; Sigma). For LPS stimulation, BMMCs were stimulated by LPS (*E. coli* 055:B5, Sigma) at a dose of 0.1  $\mu$ g/ml. For application of fatty acids together with LPS, a given fatty acid was mixed with RPMI medium for 30 min to allow binding of the fatty acid with fatty acid free-albumin (Sigma) prior to addition to BMMCs at indicated concentrations.

### 2.6. Determination of cytokine production by ELISA

Cell supernatants after 24 h of LPS or IgE+OVA stimulation were collected, and used for cytokine measurement by ELISA. Commercially available ELISA kits (BD OptEIA Set; BD Biosciences Pharmingen, San Diego, CA, USA) were used for measuring the concentration of TNF- $\alpha$  and IL-6 in supernatant fraction samples of the cell culture according to the manufacture's manual.

### 2.7. RT-PCR

Total RNAs were isolated from BMMCs at 30 min after LPS stimulation using TRIzol solution (GIBCO-BRL, Burlington, ON, Canada). The detail of semi-quantitative RT-PCR was described elsewhere [20]. First-strand cDNA was synthesized using a reverse transcription system (Promega, Madison, WI, USA). PCR was carried out with following primers:  $\beta$ -actin: 5'-CAGGAGAT-GGCCACTGCCGCA and 5'-CTCCTTCTGCATCTGTCTAGCA; TNF- $\alpha$ :

5'-CTGGGACAGTGACCTGGACT and 5'-GCACCTCAGGGAAGAGTCTG;  
IL-6: 5'-GAAATGAGAAAAGAGTTGTGC and 5'-ATTGGAAATTGGGG-  
TAGGAAG.

### 2.8. Cecal ligation and puncture (CLP)

CLP were performed as previously described by others with slight modification [3,27]. In brief, mice were anesthetized by i.p. injection of 50 mg/kg sodium pentobarbital. A 1-cm midline incision on the anterior abdominal wall was made. The cecum was exposed and filled with feces by squeezing stool gently back from the ascending colon. The cecum was 50% ligated below the ileocecal valve and then punctured using a 21 G needle followed by gentle squeezing of the cecum. Mice were observed for mortality at least five times daily over a period of 10 days. Before CLP was performed, the mice were coded so that the CLP was done without identifying individual groups.

### 2.9. Passive anaphylactic response

For IgE-mediated systemic anaphylaxis, 10 weeks-old male mice were used ( $n = 6$  from each genotype). Mice were intravenously injected with TNP-IgE (125  $\mu$ g per mouse dissolved with 200  $\mu$ l of saline). After 24 h, TNP-OVA (1 mg per mouse dissolved with 200  $\mu$ l of saline) was intravenously injected, and the body temperature were monitored with a rectal thermometer (Shibaura Electronics Co. Ltd., Saitama, Japan) for mice without general anesthesia at 0, 10, 20, 30, 40, 50, 60 and 80 min after the injection.

### 2.10. Histological quantification of MCs

Tissues including tongue, ear skin and stomach were extirpated from wild-type and E-FABP-null mice, fixed in 10% neutral buffered formalin, and embedded in paraffin. Sections with 4  $\mu$ m thickness were cut using a sledge microtome (Leica, Wetzlar, Germany). Peritoneal lavages were obtained by washing repeatedly the peritoneal cavity with ice-cold PBS, and the smear sections were prepared. After Giemsa staining, the population of MCs in tissues and peritoneal lavages was counted under light microscope. The data were expressed as the number of MCs/mm<sup>2</sup> of tissues.

### 2.11. Statistical analysis

Data were presented as average  $\pm$  S.E.M. The survival curve was calculated with the aid of the Kaplan-Meier life-table method and log-rank test using a GraphPad Prism version 4 (GraphPad Software Inc., San Diego, CA, USA). Comparable data were performed using the Student's *t*-test. Differences were considered statistically at  $P < 0.05$ .

## 3. Results

### 3.1. Identification of E-FABP in the MCs

First, we examined the expression of FABP family members, including A-, B-, E-, H- and L-FABP in the murine BMMCs. As shown in Fig. 1A, E-FABP was expressed in BMMCs and no significant expression of B-, H- and L-FABP was observed in BMMCs. In immunocytochemical analysis, immunoreactivity for E-FABP was detected in the cytosol as well as nucleus of BMMCs (Fig. 1B). No positive immunoreactivity was detected when

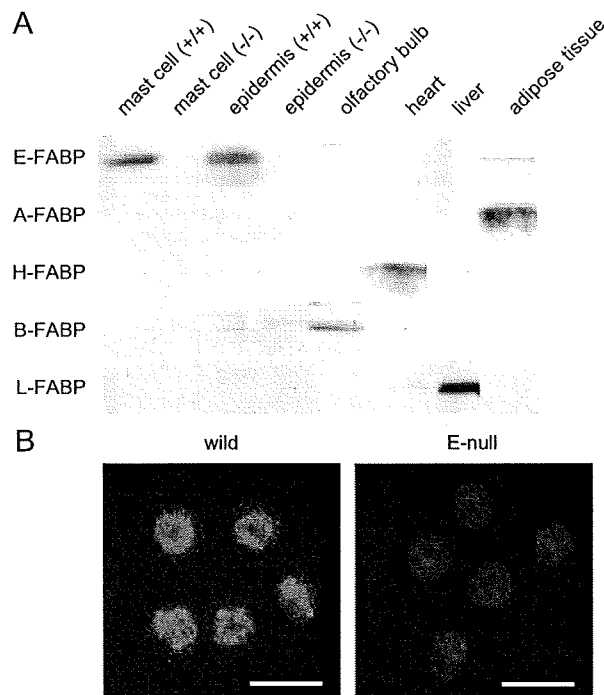


Fig. 1. E-FABP is expressed in mouse BMMCs. (A) Immunoblot analysis of five species of FABPs in BMMCs. Immunopositive single band for E-FABP is detected in BMMCs, while no positive bands for A-, B-, H- or L-FABP are observed. (B) Light micrographs of wild-type and E-FABP-null BMMCs stained with E-FABP antibody (green) and DAPI (blue). Note that E-FABP is localized in the cytoplasmic compartment as well as the nucleus. The bands shown in (A) are obtained from different gels. Bars = 10  $\mu$ m.

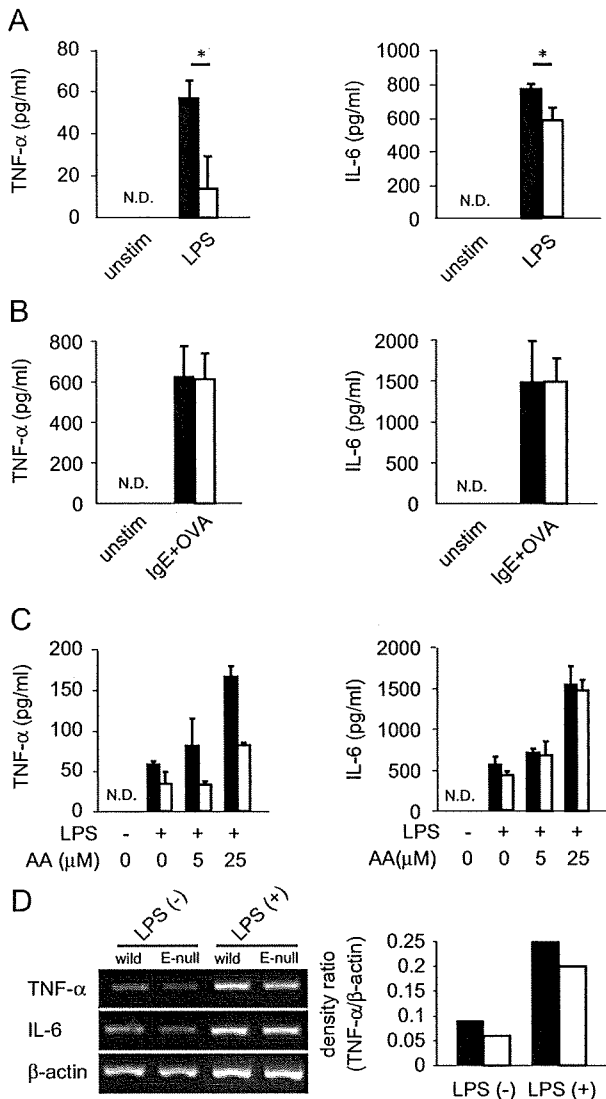
E-FABP antiserum was applied to sections of BMMCs from E-FABP-null mutant mice.

### 3.2. E-FABP deficiency in BMMCs results in decreased TNF- $\alpha$ and IL-6 production after LPS stimulus

To examine the functional significance of E-FABP in BMMCs, cytokine production in response to LPS- or antigen-stimulation, which is known to be mediated by TLR4 or Fc $\epsilon$ IR, respectively, was measured in E-FABP-null BMMCs. As shown in Fig. 2, a marked decrease in amount of TNF- $\alpha$  and IL-6 was detected in E-FABP-null BMMCs 24 h after LPS stimulus (Fig. 2A). However, no significant difference in TNF- $\alpha$  and IL-6 was detected between wild-type and E-FABP-null BMMCs when cells were stimulated with a combination of IgE and OVA (Fig. 2B), which is known to be mediated by Fc $\epsilon$ IR. Gene expression of TNF- $\alpha$  and IL-6 was decreased in E-FABP-null BMMCs 6 h after LPS stimulation when examined by semi-quantitative RT-PCR (Fig. 2D).

### 3.3. AA enhances cytokine production in BMMCs

E-FABP expression in MCs could mean that LCFAs, which are E-FABP ligands, modulate the immune response in the same way as other immune cells [28]. Thus, we next examined the impact of exogenously applied LCFA on cytokine production of BMMCs after LPS stimuli. As shown in Fig. 2C, AA enhanced production of TNF- $\alpha$  and IL-6 from wild-type and E-FABP-null BMMCs after LPS stimulation in a dose-dependent manner, whereas a combination of IgE and OVA stimulation with each dose of AA examined has no effects (data not shown). Furthermore, no significant changes in production of TNF- $\alpha$  and IL-6 were observed when other fatty



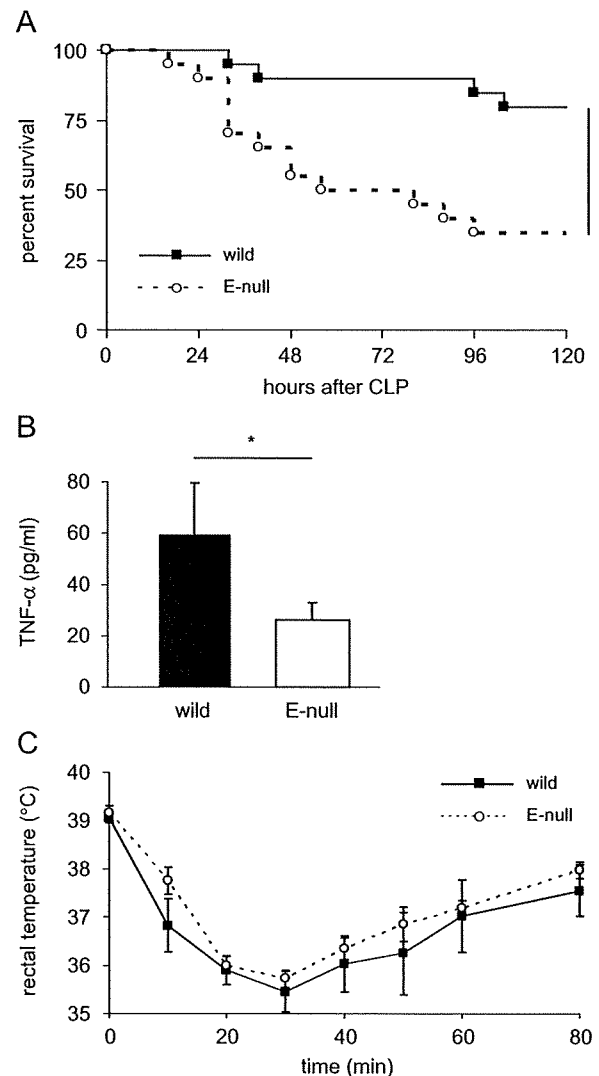
**Fig. 2.** Decreased production of TNF- $\alpha$  and IL-6 in E-FABP-null BMMCs after LPS stimulation and effect of AA on the production of LPS-induced TNF- $\alpha$  and IL-6. (A) Production of two cytokines after LPS (0.1  $\mu$ g/ml) stimulation in wild-type and E-FABP-null BMMCs by ELISA. Note a marked decrease of TNF- $\alpha$  and IL-6 in E-FABP-null BMMCs (open column) compared with wild-type (filled column) in the media containing BMMCs 24 h after LPS stimulation. \* $P$ <0.05. (B) In contrast to (A), note no significant difference in the amount of TNF- $\alpha$  and IL-6 after antigen-stimulation (IgE+OVA) between the two groups. Data are shown as means  $\pm$  S.E.M. and representative of two independent experiments. (C) LPS (0.1  $\mu$ g/ml) combined with each dose of AA (0, 5, 25  $\mu$ M) were added to the culture media containing wild-type (filled column) and E-FABP-null BMMCs (open column). Note that AA enhances LPS-induced production of TNF- $\alpha$  and IL-6 in a dose-dependent manner in BMMCs, but such enhancement of TNF- $\alpha$  by AA was markedly reduced in the E-FABP-null BMMCs especially at a dose of 5  $\mu$ M AA. Data are shown as means  $\pm$  S.E.M. and representative of two independent experiments. (D) Expression of mRNA for TNF- $\alpha$  and IL-6 was examined by RT-PCR. A significant decrease of TNF- $\alpha$  mRNA expression is detected in the E-FABP-null BMMCs at 30 min after LPS stimulation. Data are representative of two independent experiments.

acids including palmitic acid, oleic acid, linoleic acid, linolenic acid, stearic acid and docosahexaenoic acid were applied (data not shown). When we examined whether E-FABP deficiency in BMMCs modifies such enhancement of LPS-induced TNF- $\alpha$  or IL-6 production by AA, the induction rate of TNF- $\alpha$  production by AA was significantly decreased in E-FABP-null BMMCs in 5  $\mu$ M AA application (TNF- $\alpha$  with 5  $\mu$ M AA/TNF- $\alpha$  without AA = 1.41 in wild versus 0.96 in E-FABP-null BMMCs,  $P$ <0.05, see Fig. 2C left).

However, no impact of E-FABP deficiency was detected in the enhancement of IL-6 production by AA (Fig. 2C right). In addition, we did not observe significant differences between wild-type and E-FABP-null BMMCs in the enhancement of TNF- $\alpha$  production after antigen (IgE-OVA)-stimulation with a combination of various doses of AA incubation (data not shown).

#### 3.4. Lack of E-FABP in MCs leads to increased mortality in a model of acute septic peritonitis

The CLP model was used to evaluate whether decreased TNF- $\alpha$  production after LPS stimulation due to E-FABP deficiency in MCs could affect the MC-mediated host defense against bacteria *in vivo*. As shown in Fig. 3A, E-FABP-null mice showed a significantly increased mortality compared to wild-type mice following CLP.



**Fig. 3.** E-FABP is required for protection of mice from CLP-induced acute septic peritonitis. Wild-type mice and E-FABP-null mice ( $n = 25$  per each genotype) were subjected to CLP (cecal ligation and puncture), and the survival rate was monitored. (A) Note a significant increase in the mortality rate of E-FABP-null mice as compared with wild-type mice. (B) Note a decrease in the amount of TNF- $\alpha$  in peritoneal lavage in E-FABP-null mice (open column) as compared with wild-type (filled column) 6 h after CLP. (C) No significant differences in the antigen-induced passive anaphylactic response between wild-type and E-FABP-null mice ( $n = 10$  per each genotype) as indicated by rectal temperature after antigen injection. \* $P$ <0.05. Data are representative of two independent experiments.

**Table 1**  
Populations of tissue-resident MCs are normal in E-FABP-null mice

	Peritoneal cavity ( $\times 10^4$ MCs/ml)	Tongue (MCs/mm <sup>2</sup> )	Ear skin (MCs/mm <sup>2</sup> )	Stomach (MCs/mm <sup>2</sup> )
Wild (n = 5)	2.6 $\pm$ 2.1	48.1 $\pm$ 14.6	136.8 $\pm$ 17.6	19.5 $\pm$ 9.1
E-null (n = 5)	3.2 $\pm$ 1.7	44.3 $\pm$ 7.7	139.4 $\pm$ 17.9	17.4 $\pm$ 4.2

The number of MCs in wild-type and E-FABP-null were assessed as described in Section 2. Data are shown as means $\pm$ S.E.M.

Furthermore, the amount of TNF- $\alpha$  in peritoneal lavage 6 h after CLP was significantly decreased in E-FABP-null mice compared with wild-type (Fig. 3B). In order to exclude the possibility that the decreased amount of TNF- $\alpha$  is due to a decreased population density of tissue resident MCs, the population of MCs in various tissues was examined by histological examination, and no significant differences were observed (Table 1). Also, no significant differences were observed between wild-type and E-FABP-null mice in the antigen-induced passive anaphylactic response represented by changes in body temperature after OVA injection (Fig. 3C).

#### 4. Discussion

In the present study, we revealed that E-FABP was solely expressed in mouse BMMCs, and E-FABP gene ablation in BMMCs resulted in a marked decrease of proinflammatory cytokine production after LPS stimulation. The modulatory role of E-FABP in cytokine production in MCs could be specific to LPS because no such decrease of cytokine production was found after antigen (IgE+OVA) stimulation. Furthermore, mice genetically deficient in E-FABP showed a higher mortality rate compared to wild-type mice in the CLP-induced acute septic peritonitis model, which has been shown to reflect LPS-mediated MC function in the innate immunity [3]. Collectively, the present findings suggest that deteriorated protection against acute septic peritonitis in E-FABP-null mice is at least in part due to a decreased production of proinflammatory cytokines including TNF- $\alpha$  in MCs.

Several reports have so far demonstrated the expression and/or possible functions of different FABPs in various immune cells: A-, B-, L- and E-FABPs in monocytes/macrophages [17,29–31], A-FABP in lymphocytes [26] or A- and E-FABPs in DCs [19,20,32]. However, this is the first study about the functional significance of FABP in MCs. LCFAs, cellular ligands of FABP, have been shown to modulate cytokine production after antigen-stimulation in a rat mast cell line [11]. In agreement with these previous results, the present study revealed that exogenously applied AAs enhanced LPS-induced production of TNF- $\alpha$  from BMMCs in a dose-dependent manner. Considering the preferable binding of E-FABP with LCFAs including AA [33], and a possible role of E-FABP as a cellular shuttle of such ligands to various cell organelles such as the nucleus and plasma membrane, it is possible that impaired intracellular transport of AA and/or its bioactive derivatives onto nuclear receptors could occur in E-FABP-null BMMCs. Indeed, in the present study, we detected the elimination of enhancement of TNF- $\alpha$  production by AA in the E-FABP-null BMMCs. However, the further studies are necessary to explain why such elimination of the impact by AA in E-FABP-null BMMCs was observed only in TNF- $\alpha$  but not in IL-6 production.

How the present E-FABP ablation in BMMCs specifically reduced LPS-induced proinflammatory cytokine production remains unclear. It has been shown that the production of inflammatory cytokines by BMMCs is regulated by LPS/TLR4 signaling [5] and/or IgE/Fc $\epsilon$ RI signaling [34]. The activation of

these receptors on MCs is considered to be followed by activation of the NF- $\kappa$ B signaling pathway [5,34]. Recent evidence has suggested that FABPs are involved in the control of intracellular signaling pathways activated by peroxisome proliferators-activated receptors (PPARs) and NF- $\kappa$ B, possibly through regulation of the availability of lipid ligands for these signaling molecules [24,35,36]. In the present study, E-FABP ablation in MCs did not alter the response to antigen-stimulation, suggesting that E-FABP is specifically involved in LPS/TLR4 signaling, but not in IgE/Fc $\epsilon$ RI signaling. According to previous findings by others, ligand-activated TLR4 recruits cytosolic adaptor molecules that include myeloid differentiation protein 88 (MyD88), MyD88-adaptor like/TIR associated protein (MAL/TIRAP), toll-receptor associated molecule (TRAM). The recruitment of Myd88 by TLR4 results in sequential activation of protein kinase interleukin-1 receptor-associated kinases (IRAKs) and transforming growth factor- $\beta$ -activated kinase (TAK1). TAK1, in turn, regulates activation of mitogen-activated (MAP) kinases and the transcription factor, NF- $\kappa$ B, with ensuing production of inflammatory cytokines [37]. Thus, it is likely that the alteration of fatty acid metabolism in MCs due to E-FABP ablation may negatively modify the LPS/TLR4-activated signaling pathway at a site upstream of NF- $\kappa$ B, resulting in decreased production of TNF- $\alpha$  and IL-6.

We have recently reported that E-FABP gene ablation in DCs results in enhanced production of IL-12 in response to LPS compared with wild-type DCs. Such an enhancement of IL-12 production in E-FABP-null DCs seems to be contradictory to the present finding that E-FABP-null BMMCs showed a decreased production of TNF- $\alpha$  and IL-6. In this regard, it should be noted that the effect of AA on the production of cytokines in these cells is also opposite: under LPS stimulation, AA enhances TNF- $\alpha$  and IL-6 production in MCs as shown in Fig. 2C but eliminates IL-12 production in DCs [20]. Furthermore, the bioactive AA metabolites such as PGD<sub>2</sub>, PGE<sub>2</sub> and LXA<sub>4</sub> are shown to modulate the LPS-activated cytokine production positively in MCs and negatively in DCs, respectively [38,39]. Considering that AA and its bioactive metabolites are cellular ligands of E-FABP, individual E-FABP/ligand complexes may interact with and/or activate discrete molecules and thereby modify the cytokine production differently between MCs and DCs, although the mechanism underlying these different responses to LPS is to be elucidated in the further study.

It has recently been known that FABPs are functionally located at the interface of inflammatory and metabolic systems, and may serve as a subtle-modulator for immune cell responses [40]. We demonstrated in this study that E-FABP-null MCs showed a decreased production of proinflammatory cytokines upon LPS stimulation, leading to a decreased protection against the acute septic peritonitis induced by CLP in mice. This finding suggests that E-FABP in MCs positively regulates the innate immune responses against gram-negative bacteria. Considering various dietary effects of lipids on the immune function and onset of inflammatory diseases [28], and the preferable binding of E-FABP with common dietary LCFAs, the modulation of E-FABP expression in MCs could become a novel therapeutic target in the



regulatory response of MCs against gram-negative bacterial infections.

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## Original contribution

## Characterization of lymphangiogenesis in various stages of idiopathic diffuse alveolar damage

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**Summary** In pulmonary fibrosis, an abnormal healing process, is believed to be involved in the damage to lung tissue. This process has not been correlated with lymphangiogenesis, which has garnered current interest in relation to wound healing. The aim of the present study was to clarify the characteristics of lymphangiogenesis in pulmonary fibrosis associated with idiopathic diffuse alveolar damage. Formalin-fixed and paraffin-embedded lung tissues from 13 autopsy cases with idiopathic diffuse alveolar damage were used. Antibodies specific to CD34 and D2-40 were used to detect blood vessels and lymphatics, respectively, and immunohistochemical examinations and morphometry analyses were performed. The standardized density of capillaries was increased significantly in the exudative stage of diffuse alveolar damage, whereas that of the lymphatics remained unchanged. In the proliferative stage, new lymphatics emerged, primarily in the intra-alveolar fibrotic lesions where capillaries were absent. In the fibrotic stage, in which the lung was shrunken, as revealed by the elevated density of pulmonary arteries, the standardized density of capillaries was reduced significantly. The standardized area density of the interstitium was elevated in the proliferative stage and subsequently reduced in the fibrotic stage. Three-dimensional reconstruction of images revealed that some new lymphatics lacked connection to existing lymphatics. During the progression of diffuse alveolar damage, lymphangiogenesis occurs independent of capillary angiogenesis.

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## 1. Introduction

Diffuse alveolar damage (DAD) is a histologic manifestation of acute lung injury and is associated clinically with acute respiratory distress syndrome [1-4]. The condition also is manifested as an idiopathic disease, acute interstitial pneumonia [5-9]. These conditions generally are refractory to anti-inflammatory treatment and often cause lethal respiratory failure [1,5-9]. On the basis of histopathologic evidence, DAD is divided into 3 consecutive stages: exudative, defined by the formation of a hyaline membrane and edema of the alveolar wall; proliferative, defined by the presence of intra-alveolar fibrosis; and fibrotic, defined by the shrunken interstitium neighboring dilated air spaces (honeycomb formation) [1,4]. The mechanism by which DAD occasionally progresses to irreversible fibrosis of the lung is unclear.

Inflammatory fibrosis has been hypothesized as the explanation for the tissue destruction and fibrogenic remodeling associated with pulmonary fibrosis [10]. However, findings from experimental pulmonary fibrosis do not necessarily agree with this hypothesis. Mice deficient in the  $\beta 6$ -integrin chain ( $\beta 6^{-/-}$ ) develop exaggerated inflammation. However, they are protected from bleomycin-induced pulmonary fibrosis, although a similar concentration of transforming growth factor- $\beta$  was detectable in both  $\beta 6^{-/-}$  and  $\beta 6^{+/+}$  mice [11]. A fibrogenic response in the lung has been demonstrated in a mouse model of hyperoxic stress under blood-free conditions where the development of inflammation is limited [12]. Clinical outcomes in patients poorly compliant with anti-inflammatory therapy for DAD indicate that a pathogenic mechanism other than inflammatory fibrosis causes progression of the disease [10].

The lymphatic system plays various roles in fluid homeostasis and the activation of adaptive immunity by fluid drainage and cell transport [13]. Exploiting the ability to detect lymphatics by immunohistochemistry techniques, lymphangiogenesis has been discovered in various pathophysiological conditions [14-18], and its significance in

tumor metastasis and wound healing has been postulated [19-23]. It has been demonstrated that in wound healing, lymphangiogenesis occurs after angiogenesis by the sprouting of existing lymphatics, and the resultant lymphatic system exists as a transient structure [22]. Lymphatics were observed to be less developed in a prolonged healing condition such as chronic skin ulcers than in normal healing [22]. It is reasonable to hypothesize that newly formed lymphatics play important roles in the later processes of wound healing, such as fibrosis.

Pulmonary fibrosis is believed to be a pathological consequence of wound healing in the lung [10]. We have focused on lymphangiogenesis in pulmonary fibrosis. The principal aim of this study was to characterize lymphangiogenesis in a fibrogenic condition associated with idiopathic DAD.

## 2. Materials and methods

### 2.1. Materials

We examined autopsy lung specimens of 11 men and 2 women with idiopathic DAD that were obtained from the archives of the Department of Pathology at Saka General Hospital, Ishinomaki Red Cross Hospital, Sendai Medical Center, or Iwate Medical University. Patients with idiopathic DAD fulfilled the clinical and pathological criteria for acute interstitial pneumonia described by Katzenstein et al [5]. None had any evidence of systemic infection, iatrogenic causes of immunosuppression, toxic exposure, cancer for which they were undergoing cytotoxic chemotherapy, or cardiac failure. Every patient had previously been healthy without established interstitial lung disease. All patients were observed to have acute onset of symptoms, severe hypoxemia, and diffuse pulmonary infiltrates on chest radiography and computed tomography scan. The mean age of the patients was  $69.2 \pm 5.5$  years (range, 61-78 years). Every specimen was categorized into 1 of 3 consecutive stages of

**Table 1** Primary antibodies used

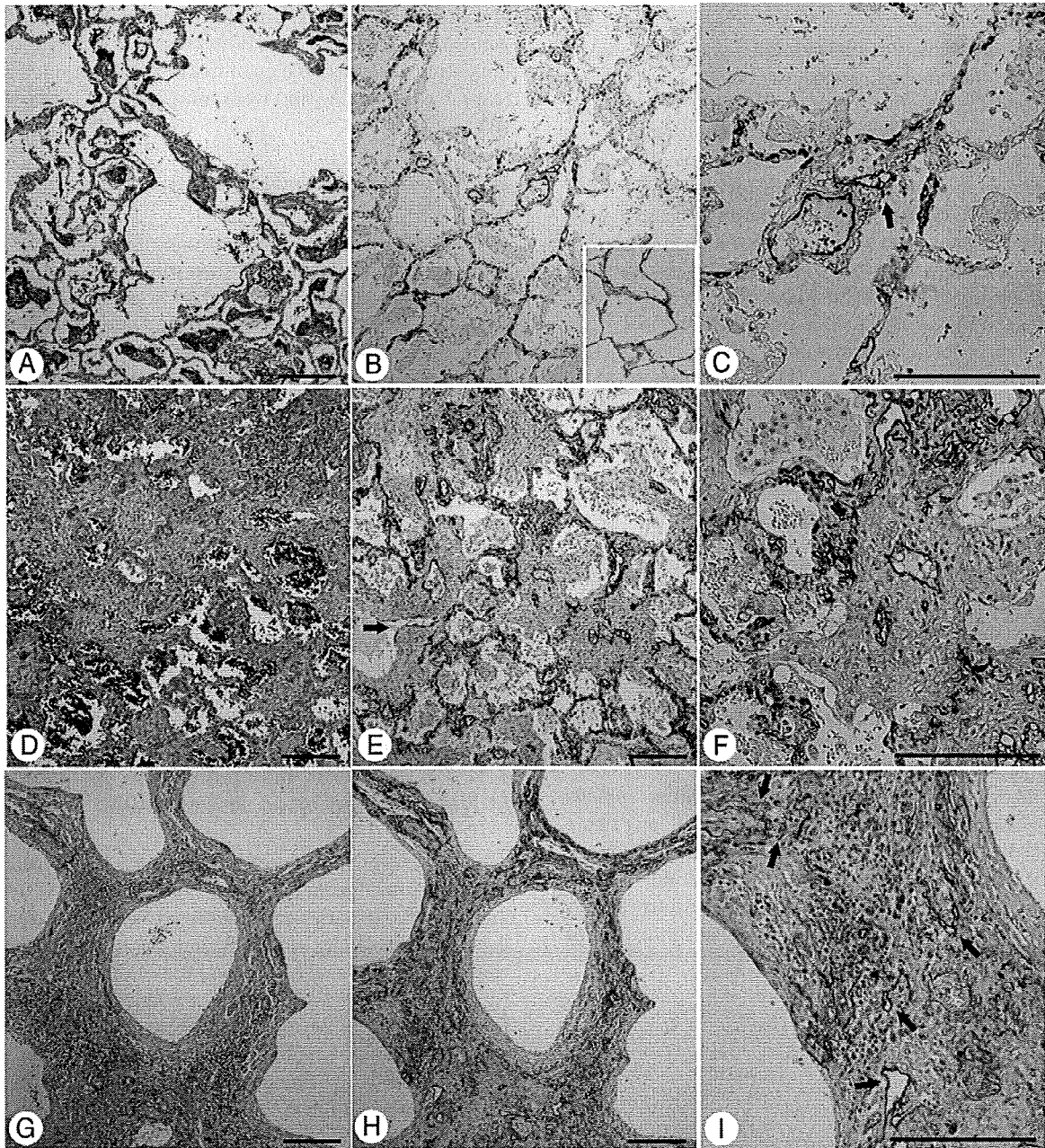
Primary antibody	Source	Isotype	Antigen retrieval method	Concentration
CD34, NU-4A1 (Nihirei, Tokyo, Japan)	Mouse	IgG1	None	4
D2-40 (Dako Japan, Tokyo, Japan)	Mouse	IgG1	MW	0.26
Podoplanin (Angiobio, Del Mar, CA)	Mouse	IgG1	None	0.3
Cytokeratin, AE1/AE3 (Dako Japan)	Rabbit	IgG1 kappa	None	0.53
Prox-1 (Angiobio)	Rabbit	Polyclonal	MW	2
von Willebrand factor (Dako Japan)	Rabbit	Polyclonal	None	2.9
Laminin (Dako Japan)	Rabbit	Polyclonal	Trypsin+ pepsin	2
Vascular endothelial growth factor receptor-3 (R&D Systems, Minneapolis, MN)	Goat	Polyclonal	MW	0.4

NOTE. Trypsin and pepsin were applied for 30 minutes. Abbreviation: MW, microwave irradiation for 10 minutes in citrate buffer, pH 6.0.

DAD on the basis of morphologic criteria previously summarized by Travis et al [1]. Two stages were present in 4 cases. Normal lung tissues were obtained from 4 autopsies of patients who died independently of pulmonary diseases.

All lungs were inflated by injecting 10% buffered formalin and embedded in paraffin wax.

The use of all autopsy samples was approved by The Ethical Committees of Saka General Hospital and Iwate



**Fig. 1** Histopathologic and immunohistochemical characteristics of the 3 stages of DAD. A, Exudative stage represents mild fibrosis in alveolar wall and hyaline membrane in alveolar ducts. B and C, Number of CD34<sup>+</sup> capillaries (red) in alveolar wall is more than that in normal lung (inset in panel B). Existing lymphatics are shown as D2-40<sup>+</sup> (brown) around vessels (arrow). D, Proliferative stage represents increased fibrous thickening of alveolar wall and the presence of intra-alveolar fibrotic lesions. E and F, D2-40<sup>+</sup> lymphatics (brown) but not CD34<sup>+</sup> capillaries (red) are apparent in intra-alveolar fibrotic lesions. Heterotopic expression of D2-40 on surface of the intra-alveolar fibrotic lesion (arrow). G, Fibrotic stage exhibits dense interstitium, including collapsed alveoli and enlarged airspaces. H and I, Capillaries (red) and lymphatics (brown, arrows) in dense interstitium. A, D, and G, Elastica-Goldner stain; B, C, E, F, and I, dual immunostaining with CD34 and D2-40 counterstained by resorcin-fuchsin and hematoxylin. Scale bar, 200  $\mu$ m.