

## Vitamin D Binding Protein-Macrophage Activating Factor Inhibits HCC in SCID Mice

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**Background.** A high incidence of recurrence after treatment is the most serious problem in hepatocellular carcinoma (HCC). Therefore, a new strategy for the treatment of the disease is needed. The aim of the present study was to investigate whether vitamin D binding protein-macrophage activating factor (DBP-*maf*) is able to inhibit the growth of HCC.

**Methods.** The effects of DBP-*maf* on endothelial cells and macrophage were evaluated by WST-1 assay and phagocytosis assay, respectively. Human HCC cells (HepG2) were implanted into the dorsum of severe combined immunodeficiency (SCID) mice. These mice were divided into control and DBP-*maf* treatment groups ( $n = 10/\text{group}$ ). The mice in the treatment group received 40 ng/kg/d of DBP-*maf* for 21 d.

**Results.** DBP-*maf* showed anti-proliferative activity against endothelial cells and also activated phagocytosis by macrophages. DBP-*maf* inhibited the growth of HCC cells (treatment group:  $126 \pm 18\text{mm}^3$ , untreated group:  $1691.5 \pm 546.9\text{mm}^3$ ,  $P = 0.0077$ ). Histologic examinations of the tumors revealed the microvessel density was reduced and more macrophage infiltration was demonstrated in the tumor of mice in the treatment group.

**Conclusion.** DBP-*maf* has at least two novel functions, namely, an anti-angiogenic activity and tumor killing activity through the activation of macrophages. DBP-*maf* may therefore represent a new strategy for the treatment of HCC. © 2010 Elsevier Inc. All rights reserved.

**Key Words:** DBP-*maf*; HCC; anti-angiogenesis; macrophage; SCID mouse.

### INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide [1]. The therapeutic options for HCC are limited and frequent recurrences develop even after curative treatments, although surgical resection and liver transplantation (for some cases) are the gold standards for the treatment of HCC [2–4]. Recent advances in the various kinds of treatment, including local ablation, systemic chemotherapy, radiotherapy, and hormone therapy, have led to an improved survival rate for HCC patients. The current treatments, however, are still not satisfactory in terms of reducing recurrence, and novel treatment strategies against HCC are therefore urgently needed [5, 6].

Solid tumors, including HCC, must establish an adequate vascular network to acquire nutrition [7]. Tumor angiogenesis is one of the fundamental requirements for tumor growth and proliferation. Folkman [8] advocated that tumor growth was angiogenesis-dependent, and that the absence of angiogenesis prevented tumor growth beyond 2–3 mm<sup>3</sup>, invasion, and/or metastasis. HCC is generally a vascular-rich tumor, and neovascularization plays an important role in its growth and progression [7–9]. We have previously reported that macrophage activating factor derived from vitamin D binding protein (DBP) is a novel angiogenesis inhibitor found in the medium of the human

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pancreatic cancer cell line, BxPC-3 [10]. DBP is a 55 kDa multi-domain serum protein secreted by the liver and belonging to the albumin superfamily. DBP binds vitamin D metabolites, especially 25-hydroxy-vitamin D, at its amino-terminal domain and actin at its carboxy-terminal domain [11, 12]. Selective deglycosylation of the protein has been shown to occur naturally as part of the inflammatory response [11]. Membrane-bound  $\beta$ -galactosidase and sialidase of activated B- and T-lymphocytes can hydrolyze the terminal galactose and sialic acid to yield a potent macrophage activating molecule- DBP-derived macrophage activating factor, called DBP-*maf* [11].

We herein describe the effects of DBP-*maf* on tumor growth in a severe combined immunodeficiency (SCID) mouse xenograft model human HCC.

## MATERIALS AND METHODS

### Preparation of Human DBP

DBP was purified by affinity chromatography with 25-hydroxy vitamin D3 [25(OH)D<sub>3</sub>]-sepharose from a 60% ammonium sulphate precipitation of the serum samples according to the method reported by Link *et al.* [13]. In brief, the precipitated proteins were diluted 1:5 with column buffer (50 mM Tris-HCl, pH 7.4) and applied to the 25(OH)D<sub>3</sub>-sepharose column. The column was washed with 100 mL of column buffer. The protein remaining on the matrix was eluted with 6 M guanidine-HCl. The peak fractions were pooled and dialyzed in 10 mM sodium phosphate buffer (pH 7.0) and then applied to a hydroxyapatite column (Econo-Pac HTP Cartridge 1; Bio-Rad, Hercules, CA) equilibrated in 10 mM sodium phosphate buffer (pH 6.0). A linear gradient elution was done from 10 to 200 mM sodium phosphate buffer (pH 6.0). The fractions of the protein peak were collected and buffer-exchanged into 10 mM sodium phosphate buffer (pH 7.0), and then concentrated by Centricon or Microcon filtration units (Millipore, Billerica, MA). The protein concentrations were measured using a BCA assay kit (Pierce Chemical Co., Rockford, IL) using BSA as a standard.

### Purification of DBP-*maf*

One microliter of DBP (5–10  $\mu$ g protein/ $\mu$ L) was diluted 1:50 with 10 mM sodium phosphate buffer (pH 6.0) and treated with 1  $\mu$ L of sialidase (10 U/mL) and 1  $\mu$ L of  $\beta$ -galactosidase from jack beans (10 U/mL) or 5  $\mu$ L of  $\beta$ -galactosidase from bovine testes (1 U/mL) at 37°C overnight. Next, the reaction was stopped on ice and then was immediately subjected to a biological assay.

### Phagocytosis Assay

Resident murine peritoneal macrophages were collected and centrifuged at 1000 rpm for 10 min at 4°C according to the method described by Yamamoto *et al.* [14]. The collected macrophages ( $5 \times 10^5$  cells/well) were suspended in RPMI-1640 medium and laid on a cover slip in 24-well plates. They were incubated at 37°C in a humidified 5% CO<sub>2</sub> incubator for 1 h. After washing the cells with the medium to remove non-adherent cells, the macrophages were treated with 100 pg/mL or 1 ng/mL of DBP-*maf*, and 1  $\mu$ g/mL of lipopolysaccharide (LPS) for 3 h, and then were used for the study. The macrophages in the control group were not exposed to any treatment, whereas those in the LPS group were treated with LPS alone as a positive control.

Sheep red blood cells (RBC) opsonized with rabbit hemolytic serum in RPMI-1640 medium were overlaid on each macrophage-coated cover slip and were incubated at 37°C in a humidified 5% CO<sub>2</sub> incubator for 90 min. Non-internalized erythrocytes were lysed by immersing a cover slip in a hypotonic solution (1/5 PBS) for 3 s. The macrophages were fixed with methanol, air-dried and stained with Giemza. The number of phagocytized erythrocytes per cell was determined microscopically, with 400 to 1000 macrophages being counted for each data point. The ingestion index was calculated from the data, and it was defined as the percentage of macrophages with phagocytized erythrocytes multiplied by the average number of erythrocytes phagocytized per ingested macrophage.

### Endothelial Cell Proliferation Assay

ECV304 endothelial cells and transformed rat lung endothelial cells (TRLECs) were both used to assess the effects of DBP-*maf* in this study. ECV304 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and cultured in DMEM (Invitrogen Co., Carlsbad, CA) containing 10% fetal bovine serum (FBS; Dainippon Pharmaceutical Co., Tokyo, Japan), 10 mg/mL penicillin, and 10,000 U/mL streptomycin (Invitrogen Co). ECV304 cells were originally considered to be human transformed endothelial cells, but were later discovered to be bladder cancer cells. ECV cells have been used for the studies of endothelial cells since they have perfect endothelial cell phenotype [15]. TRLEC cells were kindly supplied by Dr. Tsurufuji (Institute of Cytosignal Research, Tokyo, Japan) [16], and were maintained in DMEM supplemented with 10% FBS and penicillin-streptomycin under the same conditions. Since ECV304 cells and TRLECs were transformed, no growth factors, such as basic fibroblast growth factor (bFGF) or vascular endothelial cell growth factor (VEGF), were required for the cell proliferation.

Cell proliferation was evaluated by use of a WST-1 proliferation assay according to the previously described method [17]. Briefly,  $2 \times 10^4$  ECV304 or TRLEC cells in 100  $\mu$ L of DMEM containing 10% FBS were plated into each well of a 96-well plate and were cultured with or without DBP-*maf* (1  $\mu$ g/mL) in a 10% CO<sub>2</sub> incubator at 37°C. After 24 and 48 h incubation, 10  $\mu$ L of WST-1 solution (Dojinsha WAKO, Osaka, Japan) was added to each well and incubated for 2 h in 10% CO<sub>2</sub> at 37°C, and then the absorbance at 415 nm was determined with a microplate reader. All experiments were performed in triplicate.

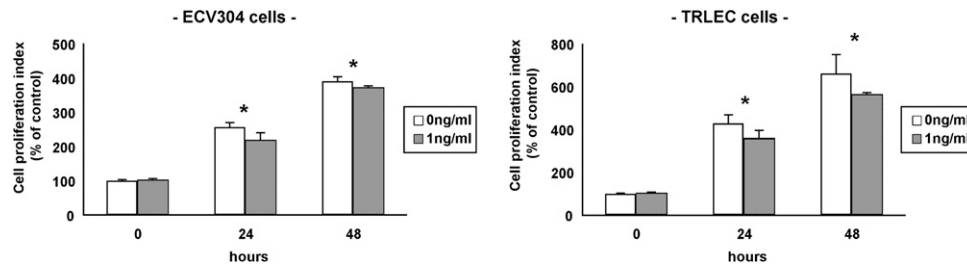
### Cell Culture

The human HCC cell line, HepG2, was obtained from the ATCC. HepG2 cells were incubated in DMEM (Invitrogen Co.) and supplemented with 10% fetal bovine serum (Equitech-Bio, Kerrville, TX), 50 unit/mL penicillin G, and 50  $\mu$ g/mL streptomycin (Invitrogen Co). Cells were maintained in a 10 cm tissue culture dish and grown at 37°C in a humidified 5% CO<sub>2</sub> environment.

### In Vivo Treatment with DBP-*maf*

Animal care and experimental procedures described in this study were done in accordance with the Guidelines for Animal Experiments of Nagasaki University with the approval of the Institutional Animal Care and Use Committee (no.0609190529). Male 6-wk-old immunocompromised mice (SCID mice; Charles River Japan, Tokyo, Japan) were used in this experiment.

A suspension of HepG2 cells ( $2.5 \times 10^6$  cells in 0.2 mL saline) was injected into the dorsal subcutaneous space of mice in the proximal midline. The mice were weighed, and the tumors were measured with a dial-caliper, and the volumes were determined using the formula: volume = width<sup>2</sup>  $\times$  length  $\times$  0.52. When the tumor volumes were approximately 100 mm<sup>3</sup>, the mice were divided into two groups (10 mice/group). The treatment group received 40 ng/kg of DBP-*maf* in 0.2 mL saline subcutaneously. The nontreatment group received comparable injections of only 0.2 mL saline. DBP-*maf* was previously



**FIG. 1.** Effect of DBP-*maf* on the proliferation of endothelial cells. In the WST-1 cell proliferation assay, the proliferation of ECV304 cells and TRLEC cells was significantly suppressed by a 24 and 48 h treatment with 1 ng/mL of DBP-*maf* (A) and (B), respectively). TRLEC = transformed rat lung endothelial cell.

administered at 4 ng/kg for the treatment of pancreatic cancer in the SCID mice [10, 18]. Because HepG2 cells form more hypervascular tumors, we chose a 10-fold dose of this agent for the present study.

### Histologic Examinations

The tumor tissues were harvested and fixed in 10% neutral buffered formalin at 4°C for 12 h. All the tissues were embedded in paraffin. Sections (4 μm thick) were first stained with hematoxylin and eosin (H and E) to evaluate tissue viability and quality. The microvessel density (MVD) was determined by immunocytochemical staining using a CSA II biotin-free Tyramide Signal Amplification System (DAKO Japan, Kyoto, Japan) with the anti-CD34 antibody (monoclonal Ab, dilution: 1:100, eBioscience, San Diego, CA) according to the manufacturers' instructions. MVD was analyzed as described by Weidner *et al.* [19]. In brief, the microvessels were highlighted by staining with anti-CD34 antibody, and then regions of high vessel density found at low magnifications (from × 40 to × 100) were scanned. The vessels highlighted by stained endothelial cells were counted at 200× magnification (0.738 mm<sup>2</sup> field) by two observers who did not know the treatment schedule. The microvessels in six fields were counted in a representative tumor section and the mean number of microvessels was determined.

Macrophages were immunostained with rat anti-mouse F4/80 antigen (1:500; Morphosys, Oxford, UK). F4/80 antigen is a glycoprotein expressed on the cell surface membrane which is an antigen specific for murine macrophages [20]. After incubation with the same secondary antibody as above, a CSA II biotin-free Tyramide Signal Amplification System (DAKO Japan, Kyoto, Japan) was used for detection.

### Statistical Analysis

The mean and standard deviation were calculated for each of the parameters. In order to assess the statistical significance of inter-group differences, a Student's *t*-test was used. A *P* value < 0.05 was considered to be statistically significant. The statistical analysis was performed using the StatView for Windows software program ver. 5.0 (SAS Institute Inc., Cary, NC).

## RESULTS

### Effect of DBP-*maf* on the Proliferation of Endothelial Cells

In the WST-1 cell proliferation assay, 1 ng/mL of DBP-*maf* significantly suppressed the proliferation of ECV304 and TRLEC cells following incubation with the compound for 24 and 48 h (Fig. 1). The suppressive activity of DBP-*maf* was similar in both endothelial cell lines.

### Effect of DBP-*maf* on Macrophage Phagocytic Activity

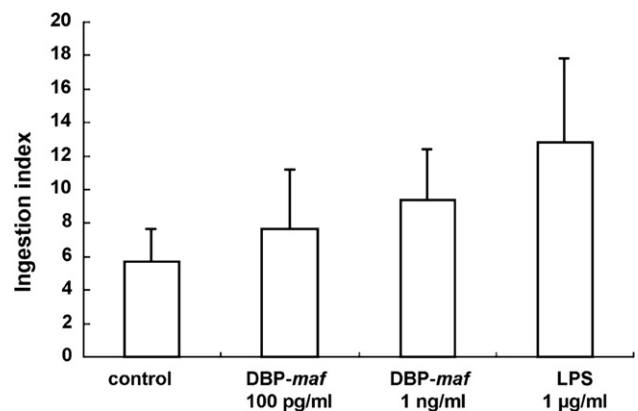
We evaluated the *in vitro* phagocytic activities of DBP-*maf* in resident murine peritoneal macrophages using the ingestion index of sheep RBC. Macrophages treated with 100 pg/mL or 1 ng/mL of DBP-*maf* showed higher phagocytic activities than control macrophages (control:  $5.7 \pm 2.0$ , 100 pg/mL DBP-*maf*:  $7.7 \pm 3.4$ , 1 ng/mL DBP-*maf*:  $9.4 \pm 3.0$ , LPS:  $12.8 \pm 5.0$ ) (Fig. 2).

### Treatment of HepG2 Tumors with DBP-*maf*

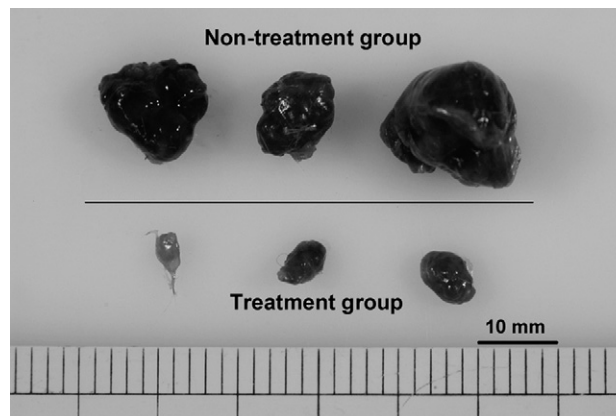
Twenty-one days after the course of animal study, the growth of HepG2 tumors was significantly inhibited in the mice treated with DBP-*maf* ( $126 \pm 18$  mm<sup>3</sup>, *P* < 0.0077), whereas the size of the tumors in the untreated mice increased with time ( $1691.5 \pm 546.9$  mm<sup>3</sup>) (Figs. 3 and 4). No significant decrease was observed in the body weight in the animals that received DBP-*maf* (Fig. 5), and this dose of DBP-*maf* did not lead to the observation of any adverse effects in the animals during their course of treatment.

### Histologic Findings

In the analysis with H and E stain, the untreated tumors showed features compatible with human HCC



**FIG. 2.** Phagocytosis assay after a 3 h treatment of murine macrophages. All the data are shown as the means ± standard deviation (*n* = 5).



**FIG. 3.** Representative tumors removed 21 d after administration of DBP-*maf* (40 ng/kg/d).

(Fig. 6A), whereas necrotic tissues with infiltration of inflammatory cells were observed in the tumors of treated animals (Fig. 6B).

Immunohistochemical staining directed against the macrophage-specific marker F4/80 revealed that the tumors in the treatment group had been infiltrated by more macrophages (Fig. 7A) than the tumors of the control mice (Fig. 7B).

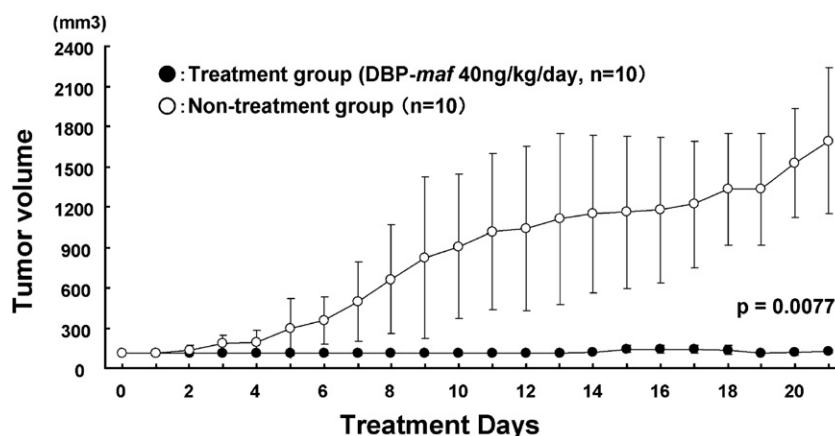
MVD was determined by the number of the intratumoral microvessels highlighted by immunohistochemical staining for CD34. The tumors in the treatment group had fewer microvessels than those in the untreated control group (Fig. 7C and D). In the untreated group, the number of intratumoral microvessels per high power field was  $97.0 \pm 28.2$ , whereas the number of vessels was  $37.1 \pm 14.1$  in the treated mice ( $P < 0.001$ , Fig. 8). These results indicate that DBP-*maf* inhibits tumor angiogenesis.

## DISCUSSION

The present *in vivo* study demonstrated that DBP-*maf* can reduce tumor progression of xenografted

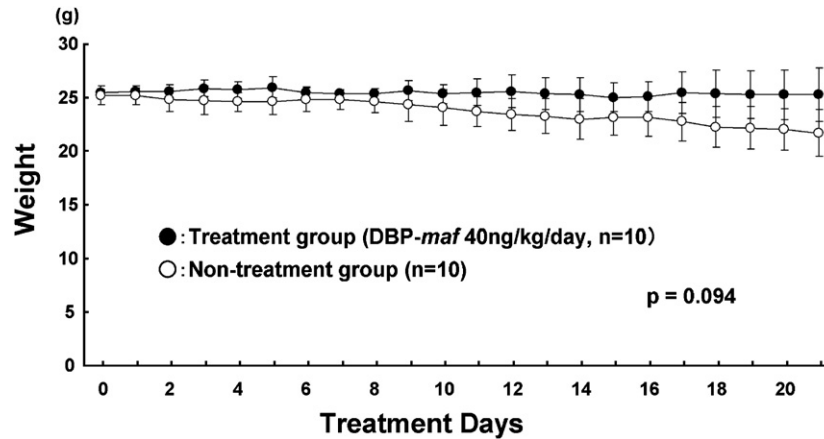
HCC cells in SCID mice. The *in vitro* study also showed that DBP-*maf* has at least two biologic functions; anti-angiogenic activity and enhancement of macrophage activation.

Anti-angiogenesis is considered to be one of the most effective methods for inducing tumor regression or dormancy [18]. Our previous study showed that DBP-*maf* suppressed the tumor growth of the pancreatic cancer cell line BxPC-3 [10]. Kanda et al. [21] demonstrated that DBP-*maf* could inhibit endothelial cell proliferation, chemotaxis, and tube formation, even under stimulation by fibroblast growth factor-2 (FGF-2), VEGF, or angiopoietin 2. Kalkunte et al. [22] also showed the anti-endothelial activity of DBP-*maf* by inhibiting the signaling cascade of VEGFR-2 and ERK1/2 in human endothelial cells. These reports support our results, in which DBP-*maf* acts as an anti-angiogenic agent and inhibits the growth of HCC in mice. Hepatocellular carcinoma has been reported to show increased MVD, as well as increased VEGF levels in the serum or tumor tissue [23, 24]. Bevacizumab (Avastin; a humanized murine anti-VEGF monoclonal antibody) is clinically used to treat colorectal cancer [25]. Several studies



**FIG. 4.** Changes in the tumor volume ( $n = 10$ ) after 21 d of treatment. The tumors grew time-dependently in the untreated group open circles: (○—○), whereas tumor growth in the treatment group was significantly inhibited filled circles: (●—●).



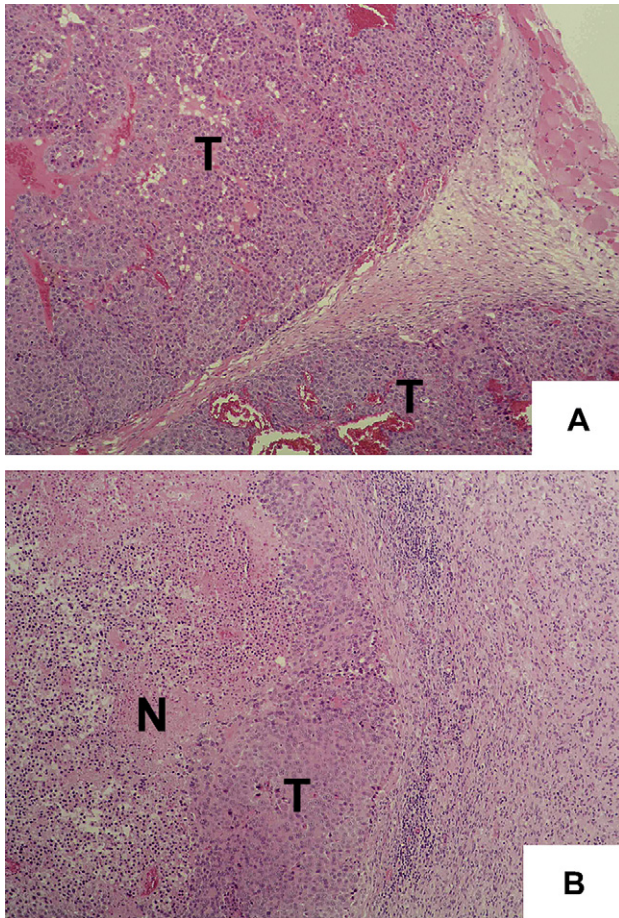


**FIG. 5.** Mouse body weight. The mice in the treatment group did not lose any weight filled circles: (●—●), while the body weight of the mice in the untreated group decreased time-dependently open circles: (○—○).

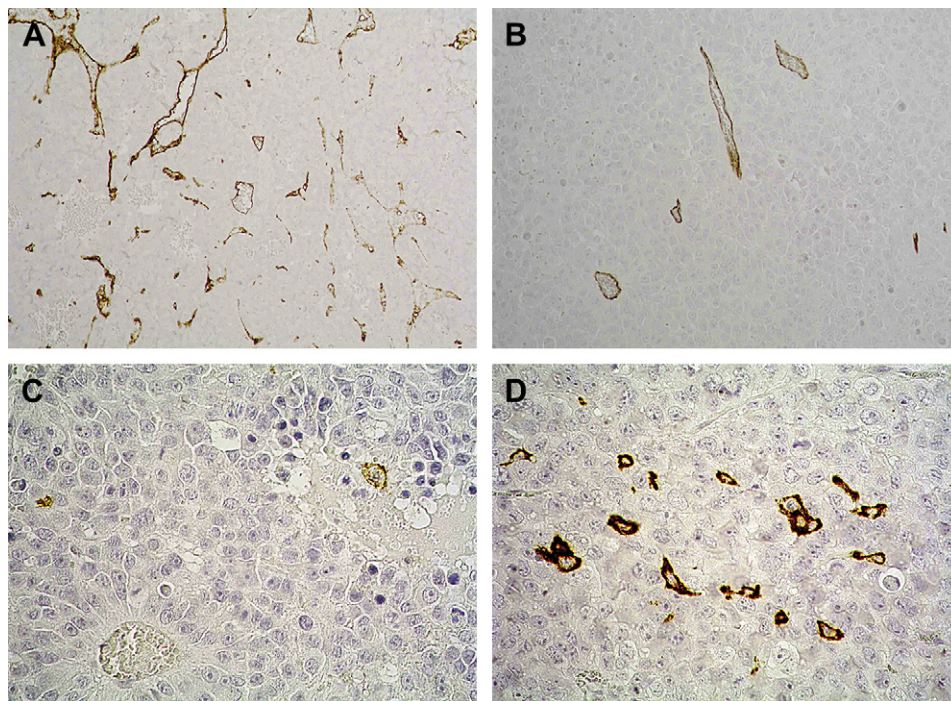
have explored the use of bevacizumab as a single agent or in combination with cytotoxic or molecularly-targeted agents in patients with advanced HCC

[26, 27]. DBP-*maf* has been shown to exert its anti-angiogenic effect through the inhibition of the VEGF signaling cascade [22], in a similar manner as bevacizumab. All of these results indicate that DBP-*maf* is a promising angiogenesis inhibitor, although further exploration of its mode of action is required.

DBP-*maf* has also been reported to have another interesting function as an inducer of macrophage activation. As a result of this activity, DBP-*maf* increases macrophage infiltration into the HCC tumor. While macrophages prevent carcinogenesis and decrease the spread of tumor cells [28, 29], they have also been shown to support tumor growth and dissemination [30]. These conflicting characteristics reflects the different functional programs that are activated in response to different microenvironment signals, as exemplified in the M1 (classic)-M2 (alternative or non-classic) paradigm of macrophage polarization [31]. Our present studies demonstrated that DBP-*maf* induced the massive infiltration of macrophages into the transplanted HCC cells also activated macrophages as antigen presentation cells against tumor antigens [10]. These macrophages thus not only acted as innate immune cells, but also played the same role as dendritic cells [29]. DBP-*maf* has also been demonstrated to enhance macrophage activity as measured by Fc receptor-mediated phagocytotic and superoxide-generating capacities, and to enhance the cytotoxic activity of macrophages themselves to the transplanted HCC cells [32]. In the present study, DBP-*maf* was active at a very low dose (40 ng/kg/d) in comparison to other anti-angiogenic agents [33, 34]. We observed no anti-proliferative effect on HCC cells by DBP-*maf* itself (data not shown), thus suggesting that DBP-*maf* synergistically functioned by inhibiting angiogenesis and killing tumor cells as a result of the activated macrophages.



**FIG. 6.** Representative HepG2 tumor (H and E stain, ×100). (A) HepG2 tumor from untreated mouse; (B) HepG2 tumor from a treated mouse. T = HepG2 tumor; N = necrotic tissue.



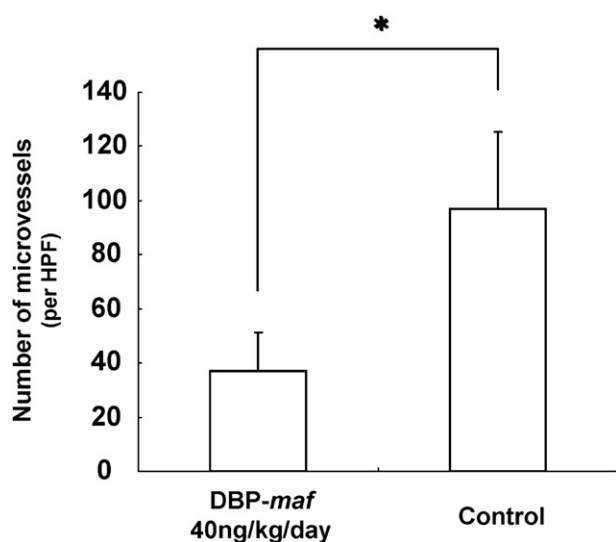
**FIG. 7.** Immunohistochemical analyses of the transplanted HepG2 tumors. Immunohistochemical staining for CD34 ( $\times 200$ ) showed that the untreated tumors had more microvessels (A) than the tumors in treated mice (B). Immunohistochemical staining for F4/80 ( $\times 400$ ) revealed few macrophages in the untreated tumors (C). In contrast, the tumors in the treatment group had considerable infiltration of macrophages (D).

In conclusion, we have herein shown for the first time that the treatment of SCID mice bearing subcutaneous HepG2 with DBP-*maf* resulted in tumor growth suppression. Although further studies are called for to elucidate its potential clinical applications for HCC, we

believe that we have nevertheless demonstrated that DBP-*maf* treatment may represent a promising new strategy for the treatment of HCC.

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**FIG. 8.** Number of microvessels in the transplanted HepG2 tumors. The number of microvessels in the tumors from treated mice was significantly fewer than that in the untreated tumors ( $*P < 0.001$ ).



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