Hepatoma-derived growth factor induces tumorigenesis *in vivo* through both direct angiogenic activity and induction of vascular endothelial growth factor

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Hepatoma-derived growth factor (HDGF) is highly expressed in tumor cells, and stimulates their proliferation. In the present study, we investigated the role of HDGF in tumorigenesis and elucidated the mechanism of action. Stable transfectants of NIH3T3 cells overexpressing HDGF did not show significant anchorage-independent growth in soft agar assay. However, these stable transfectants overexpressing HDGF generated sarcomatous tumors in nude mice. These tumors were red-colored macroscopically, and histologically showed a rich vascularity. Immunohistochemical analysis using CD31 antibody showed new vessel formation. Recombinant HDGF stimulated proliferation of human umbilical vein endothelial cells in a dose-dependent manner, and stimulated tubule formation. Furthermore, vascular endothelial growth factor (VEGF) was detected immunohistochemically in the tumor tissues. Transient expression of HDGF induced both *VEGF* gene and protein expression as demonstrated by a reporter assay using *VEGF* gene promoter. The administration of anti-VEGF neutralizing antibody significantly suppressed, but did not block, the tumor growth of HDGF-overexpressing cells in nude mice. Thus, these findings suggested that HDGF-induced tumor formation *in vivo* involves induction of VEGF as well as direct angiogenic activity. (Cancer Sci 2003; 94: 1034–1041)

Oncogenesis is induced by genetic alterations of various proteins involved in the cell cycle and cellular proliferation, especially by up-regulation of oncogenic genes and/or down-regulation of tumor suppressor genes. Overexpression of growth factors, receptors for growth factors, proteins related to signal transduction from the respective receptors and positive transcriptional regulatory factors, and suppressed expression of growth-inhibitory genes, including negative signal transducers and transcriptional regulatory factors, have all been associated with tumor formation in nude mice.[1–9)]

Hepatoma-derived growth factor (HDGF) is a heparin-binding protein purified from the conditioned media of Huh-7 hepatoma cells, which proliferate autonomously in a serum-free chemically defined medium.[10, 11)] HDGF contains a well-conserved N-terminal amino acid sequence, which is called the *hath* (*h*omologous to *a*mino *t*erminus of *H*DGF) region.[11, 12)] HDGF is translocated to the nucleus via nuclear localization signals and its nuclear translocation is essential for induction of cell growth activity.[13, 14)] It is highly expressed in fetal tissues and may be involved in development of organs, including the cardiovascular system, kidney and liver.[15–18)] HDGF has mitogenic activity for some hepatocellular carcinoma (HCC) cells, in addition to fibroblasts, endothelial cells, vascular smooth muscle cells and fetal hepatocytes.[10, 11, 13, 15, 16, 18, 19)] It is abundantly expressed in tumor cell lines, and HDGF antisense oligonucleotides suppress the proliferation of hepatoma cells expressing HDGF endogenously.[19)] In human and murine models of HCC, HDGF expression increases at an early stage, even before tumor formation. Furthermore, HDGF is more abundantly expressed in HCC than in adjacent non-tumor liver tissues.[20)] HDGF is a unique nuclear/growth factor, possibly playing an important role in the development and progression of cancer cells.

Increasing evidence suggests that HDGF might be implicated in tumorigenesis. However, whether HDGF really induces transformation of cells and tumor formation *in vivo* is not known. Therefore, we established stable cell lines overexpressing HDGF from NIH3T3 fibroblasts by transfection, and investigated their oncogenic potential. In this report, we demonstrate that mouse fibroblasts overexpressing HDGF induce sarcomatous tumors after injection into nude mice, and that tumor formation *in vivo* by overexpression of HDGF is induced mainly by angiogenesis due to induction of the vascular endothelial growth factor (VEGF), in addition to direct angiogenic activity.

Materials and Methods

Cell culture and establishment of stable transfectants. NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS at 37°C in a humidified atmosphere of 5% CO_2 in air. Two stable transfectants were established as described previously.[13)] In brief, for NIH3T3-HDGF, the parent NIH3T3 cells were co-transfected with 18 μg of pEF-BOS containing myc-tagged HDGF cDNA and 2 μg of pST vector with the neomycin-resistance gene, and were selected with neomycin in DMEM-10% FCS. For NIH3T3-Neo, NIH3T3 cells were co-transfected with 18 μg of pEF-BOS and 2 μg of pST vector. Two transfectants were maintained in DMEM supplemented with 10% FCS and 0.9 mg/ml geneticin (G418). To confirm the establishment of stable tranfectants, western blot analysis was performed. To detect myc-tagged HDGF proteins, mouse monoclonal anti-c-Myc antibody (Calbiochem, Cambridge, UK) and rabbit polyclonal anti-C terminus HDGF (aa 231–240) IgG[18, 19)] were used as a primary antibody.

Anchorage-independent growth in soft agar. To assess the anchorage dependency of growth, 1×10^5 cells were plated in 0.35% agar layered on top of 0.5% agar in 60-mm plates, and the colonies were counted after 3 to 4 weeks of incubation at 37°C and 5% CO_2 in air. Two transfectants were used in each

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group, and each assay was completed in triplicate. The number of colonies that consisted of more than 20 cells per colony in each well was scored.

Tumorigenicity in nude mice. Four-week-old BALB/c *nu/nu* mice were obtained from Japan Clea (Hamamatsu). Following trypsinization, NIH3T3-HDGF and NIH3T3-Neo cells were harvested (2×10^6), resuspended in 200 μl of PBS and inoculated subcutaneously into the flank of the mice. Six animals and two transfectants were used in each group. The tumor size was measured with a caliper once or twice per week, and the volumes were estimated according to the following formula: volume=length×width^2×0.52.

Transfection for transient expression. pEF-Bos-HDGF or pEF-BOS was transfected onto the NIH3T3 cells with Polyfect Transfection Reagent (QIAGEN, Tokyo), according to the manufacturer's protocol. The cells were incubated and harvested at 6, 12, 24, 48, and 72 h after transfection.

Western blot analysis. The transfectant cells were washed once with PBS, added to 300 μl of "Cellytic"-M Reagent (Sigma-Aldrich, Inc.), and incubated for 15 min on a shaker. The lysate was centrifuged at 15,000*g* for 15 min, and the supernatants were collected. The proteins were quantified by means of the BCA assay (Pierce). Protein samples (15 μg) were electrophoresed on 10% SDS-PAGE gel and transferred to an Immobilon membrane (Millipore Co.). The membrane was blocked with 5% skimmed milk, then incubated with a 1:2000 dilution of rabbit polyclonal anti-C terminal HDGF (aa 231–240) IgG,[18,19] or a 1:1000 dilution of rabbit anti-mouse VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and reacted with horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Pharmacia Biotech, Buckinghamshire, UK). For analysis of basic fibroblast growth factor (FGF) and cyclooxygenase (Cox)-2, rabbit anti-human FGF-2 antibody (Santa Cruz Biotechnology) and goat anti-human Cox-2 antibody (Santa Cruz Biotechnology) were used as the primary antibodies at 1:200 and 1:500 dilution, respectively. The blots were detected with the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech.).

Immunohistochemical staining. Tumor sections from the nude mice were fixed in 10% neutral buffered formalin, and embedded in paraffin. For preparing frozen sections, the tumor tissues were resected from the nude mice, fixed in 2% paraformaldehyde for 6 h, embedded in cryopreservative compound (OCT) after having been washed with 10–30% sucrose in PBS overnight at 4°C, and then frozen. Sections from the frozen specimens (6-μm thick) and from the paraffin blocks (5-μm thick) were stained with hematoxylin and eosin by means of standard methods. For the immunohistochemical analysis, the paraffin sections were deparaffinized, and microwaved in 1 m*M* EDTA solution (pH 8.0) for 5 min three times for antigen retrieval. Both types of sections were treated for 15 min with 0.3% H_2O_2 dissolved in methanol to inactivate endogenous peroxidase. For detection of the endothelial marker CD31, the frozen sections were blocked with normal rabbit serum and stained with a 1:50 dilution of rat anti-mouse CD31 (BD Pharmingen). For detection of HDGF and VEGF, the paraffin sections were blocked with normal goat serum, and stained with a 1:5000 dilution of rabbit polyclonal anti-C terminus HDGF IgG and a 1:100 dilution of rabbit anti-mouse VEGF antibody (Santa Cruz Biotechnology) as a primary antibody, respectively. Incubation with the primary antibody was carried out for 30 min at room temperature. Incubation with a 1:150 dilution of biotinylated anti-rat or anti-rabbit secondary antibody was carried out for 30 min at room temperature, and staining was visualized by use of the avidin-biotin-complex (ABC) system with diaminobenzidine (DAB), using a Vectastain ABC kit (Vector Laboratory). The sections were counterstained lightly with methyl green or hematoxylin.

Human umbilical vein endothelial cell (HUVEC) proliferation. The effects of HDGF on the proliferation of HUVEC were determined by measuring the changes in cell number. HUVECs were seeded at a density of 5×10^3 cells per well in DMEM with 10% FCS in 24-well plates. After 24 h, the medium was replaced with fresh DMEM-5% FCS medium containing either with or without recombinant HDGF (1, 10, 100 ng/ml). Forty-eight hours later, the medium was replaced with fresh medium with or without recombinant HDGF, and the cells were incubated for a further 48 h (the total treatment time with HDGF was 96 h). The cells were then harvested by trypsinization and counted. The experiments were performed in triplicate. The recombinant HDGF was produced as GST-HDGF fusion protein according to the method previously reported.[14,19] In brief, HDGF cDNA was subcloned into pGEX-3X vector (Amersham Pharmacia Biotech). The lysate of transformed *Escherichia coli*, which had produced the fusion protein, was incubated with glutathione-Sepharose beads at 4°C. After these beads had been washed thoroughly with PBS, the recombinant HDGF was released by Factor Xa and then purified by heparin-Sepharose column chromatography.

Tubule formation of HUVECs. The effects of HDGF on tubule formation of HUVEC were evaluated with an angiogenesis kit (Kurabo, Osaka) according to the manufacturer's protocol.[21] Various concentrations of recombinant HDGF (r-HDGF) were added to the Optimised Medium (Kurabo) for culture of HUVEC. HUVECs co-cultured with human fibroblasts were seeded in 24-well plates with or without r-HDGF for 11 days. The medium was changed every 3 days. After 11 days, HUVECs were stained with mouse anti-human CD31 antibody according to the manufacturer's protocol. The length of the tubules formation was quantified with NIH Image in 5 different fields of each well. The experiments were performed in quadruplicate.

Reporter gene assay. The *VEGF* gene promoter region was provided by Dr. Abraham (SIOS, Inc., CA). This gene was inserted upstream of the *luciferase* gene in pGVB (Toyo Ink, Tokyo). A modified luciferase reporter plasmid (1 μg) was co-transfected with pRL-SV40 (0.02 μg) and pEF-Bos-HDGF or pEF-BOS into the NIH3T3 cells by use of the "TransFast" Transfection Reagent (Promega Corp.), according to the manufacturer's protocol. After 48-h culture, the cells were lysed and the luciferase activity was measured with the Dual-Luciferase Reporter Assay system (Promega Corp.).

Neutralizing antibodies against VEGF. As described above, NIH3T3-HDGF cells (2×10^6) were inoculated subcutaneously into the flank of BALB/c *nu/nu* mice. A neutralizing anti-mouse VEGF antibody (R&D, Minneapolis, MN) or normal goat IgG (R&D) was administered via i.p. injection twice a week at a dose of 50 μg/200 μl PBS per mouse, respectively. Four animals were used in each group.

Statistical analysis. All values were expressed as the mean±SD. Student's unpaired *t* test was performed for comparison of data between two groups. ANOVA was performed to compare data among three groups. A probability value of $P<0.05$ was considered to denote statistical significance.

Results

Tumor formation by HDGF stable transfectants in nude mice. We generated NIH3T3 stable transfectants overexpressing myc-tagged HDGF (NIH3T3-HDGF). As reported previously, these HDGF-overexpressing stable transfectants showed increased DNA synthesis and cell proliferation compared with mock cells.[13] In six nude mice, 2×10^6 cells of an NIH3T3-HDGF clone were inoculated subcutaneously. As shown in Fig. 1, the NIH3T3-HDGF clones induced tumors within about 14 days after injection and these tumors increased in size with time. The

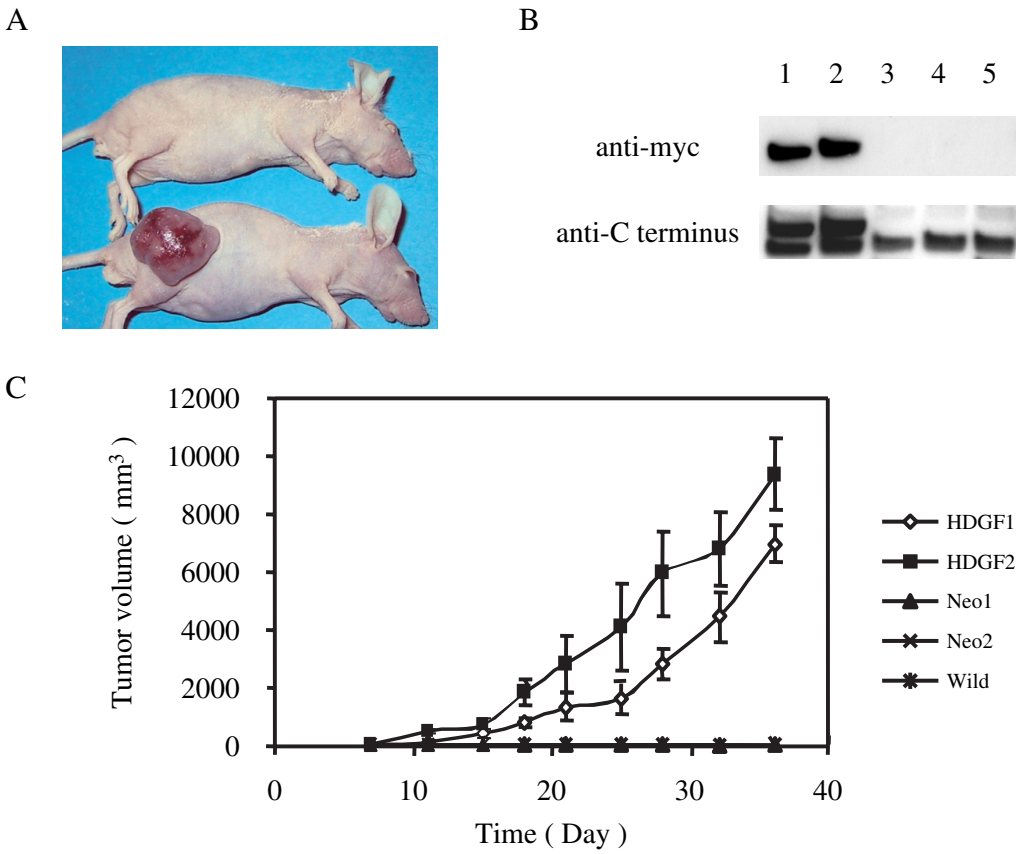


Fig. 1. Tumor formation in the nude mice by inoculation of NIH3T3-HDGF. (A) A photograph of a representative tumor that developed in a nude mouse 35 days after subcutaneous inoculation of NIH3T3-HDGF cells or NIH3T3-Neo cells. Tumor formation was observed in the nude mice inoculated with NIH3T3-HDGF cells (bottom), but not with NIH3T3-Neo cells (top). (B) Expression of HDGF protein in each clone as determined by western blot analysis with mouse monoclonal anti-c-Myc antibody and rabbit polyclonal anti-C terminus HDGF antibody. Two NIH3T3-HDGF clones expressed HDGF more abundantly than NIH3T3-Neo cells or wild-type NIH3T3 cells. 1, HDGF1; 2, HDGF2; 3, Neo1; 4, Neo2; 5, wild. (C) Tumor growth curves in nude mice after inoculation of NIH3T3-HDGF cells, NIH3T3-Neo cells and wild-type NIH3T3 cells. The more abundantly HDGF was expressed, the more rapidly NIH3T3-HDGF clones formed tumors in nude mice. Values are the means±SD (n=6) of changes in the tumor volumes in six mice in each group.

Table 1. Anchorage-independent growth of NIH3T3-HDGF cells in soft agar

	No. of colonies
Neo	0 (0)
HDGF	0 (62.0±5.7*)
ErbB-2	309.5±57.3*

Cells were plated in 60-mm plates of soft agar and the number of colonies consisting of more than 20 cells per colony in each well was counted after 3 weeks of culture. The mean values±SD from triplicate experiments are shown. The number of small colonies consisting of not more than 20 cells per colony are shown in parenthesis. * P<0.05 vs. Neo.

parent NIH3T3 cells and vector-transfected cells (mock clones) did not generate tumors. As NIH3T3 cells are prone to transformation, one might argue that tumor formation induced by HDGF overexpression could be an artifact, but plural transfectants clones overexpressing HDGF showed evidence of tumorigenesis in nude mice. Furthermore, the more abundantly HDGF was expressed, the more rapidly NIH3T3-HDGF clones formed tumors in nude mice (Fig 1B, C). These findings suggested that the tumor formation was not an artifact, but was indeed induced by overexpression of HDGF.

Next, we investigated whether NIH3T3 stable transfectants overexpressing HDGF stimulate anchorage-independent growth in soft agar assay. Although soft agar culture of NIH3T3-HDGF clones for 3 months was performed five times, NIH3T3-HDGF stable transfectants could not form colonies of significant size in soft agar (Table 1). However, colonies consisting of foci smaller than 20 cells were more abundant in the plates seeded with HDGF stable transfectants than the mock cells. Although HDGF-overexpressing cells do not show significant colony formation in soft agar, their ability to form tumors in nude mice suggests that HDGF is critically involved in tumorigenesis *in vivo*.

Histological analysis of tumors developed in the nude mice. The tumors that developed in nude mice after the injection of NIH3T3-HDGF stable transfectants were red-colored, suggesting abundant vascular formation. Microscopic examination revealed that the tumors developed from NIH3T3-HDGF fibroblasts showed fibrosarcomatous features, and consisted of spindle-shaped cells with high chromatin contents (Fig. 2a, b). HDGF proteins were strongly expressed in the fibroblasts in the tumor (Fig. 2e, f). Moreover, microscopic examination showed abundant capillary vessel formation. These capillary endothelial cells in the tumor sections expressed the neovascularized endothelial cell marker CD31, as shown in Fig. 2c, d by immunohistochemistry. Thus, HDGF overexpression allowed NIH3T3 cells to develop tumors containing abundant new vessels in nude mice.

Angiogenic activity of HDGF. NIH3T3-HDGF stable transfectants developed tumors in nude mice *in vivo*, but did not show significant anchorage-independent growth *in vitro*. To clarify

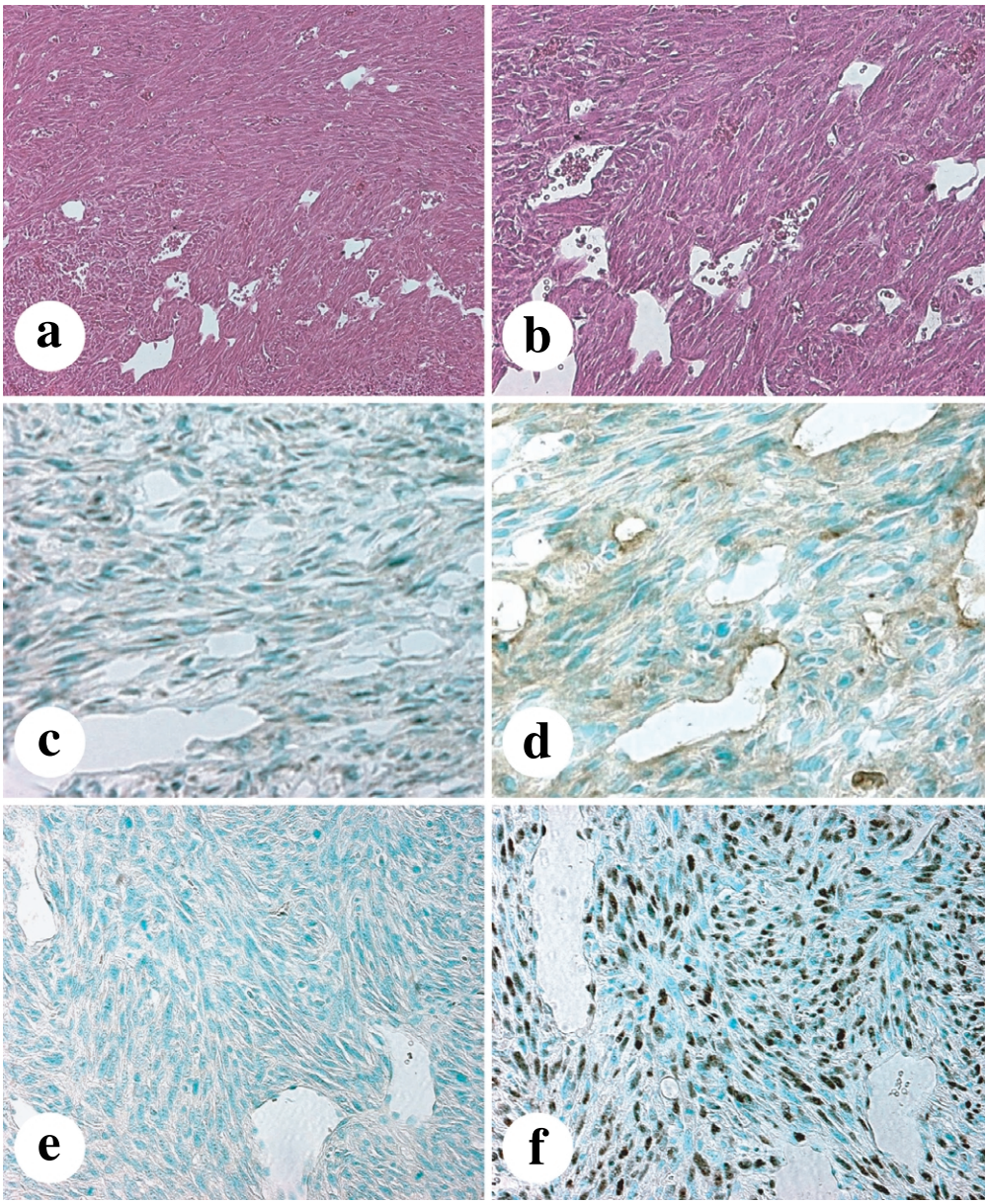


Fig. 2. Histological analysis of tumors developed in the nude mice. Hematoxylin and eosin staining of tumor cells at ×100 (a) and ×200 (b) original magnification. Immunohistchemical staining of CD31 in the tumor cells at ×200 original magnification, (c) without primary antibody for the control, or (d) with rat anti-mouse CD31 antibody. Immunohistchemical staining of HDGF in the tumor cells at ×200 original magnification, (e) without primary antibody for the control, or (f) with rabbit polyclonal anti-C terminus HDGF IgG.

the dissociation between these data, we investigated the angiogenic activity of HDGF. HDGF stimulated the proliferation of HUVECs in a dose-dependent manner, affording an increase of about 70% over the control at the dose of 100 ng/ml (Fig. 3A). Furthermore, HDGF significantly stimulated HUVEC tubule formation in an angioigenesis assay using a co-culture system with fibroblasts (Fig. 3B, C). These findings suggest that HDGF has direct angiogenic activity.

HDGF induces VEGF. The angiogenic activity of HDGF itself was demonstrated by an *in vitro* assay using HUVEC, but the vessel development in the tumors in nude mice seemed to be more prominent than would be expected from the angiogenic potential *in vitro*. Therefore, we investigated whether HDGF induces other angiogenic factors, and found that a potent angiogenic factor, namely, VEGF, was significantly induced by the transient expression of HDGF, as demonstrated by western blotting (Fig. 4A). However, basic FGF and Cox-2 were not induced (data not shown). In the immunohistochemical study, VEGF protein was demonstrated in the cytoplasm of the sarcomatous fibroblasts in tumors formed in the nude mice (Fig. 4B). The reporter assay using *VEGF* gene promoter region indicated that HDGF expression significantly increased the luciferase activity about two-fold over the control in NIH3T3 cells (Fig. 5). These findings suggested that HDGF induced *VEGF* gene expression. Furthermore, the administration of anti-VEGF neutralizing antibody significantly, though not completely, suppressed the tumor growth of HDGF-overexpressing NIH3T3 cells in nude mice (Fig. 6). Thus, HDGF may stimulate angiogenesis both directly and via induction of the potent angiogenic factor, VEGF.

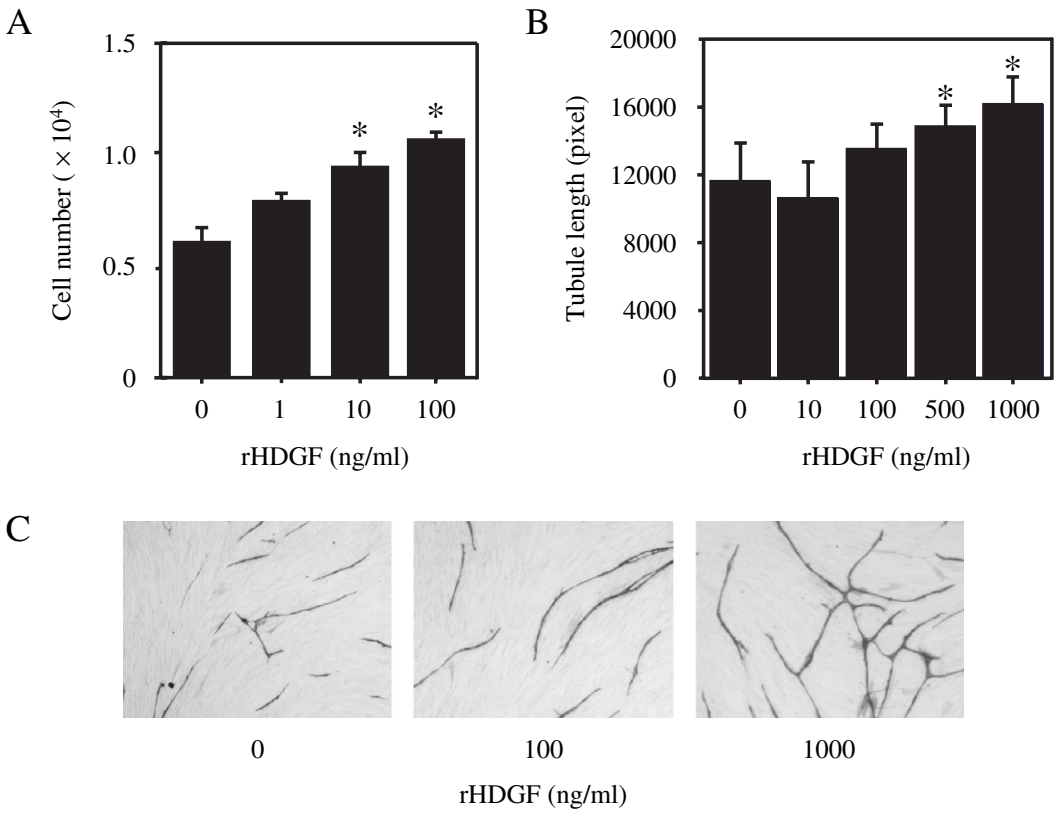


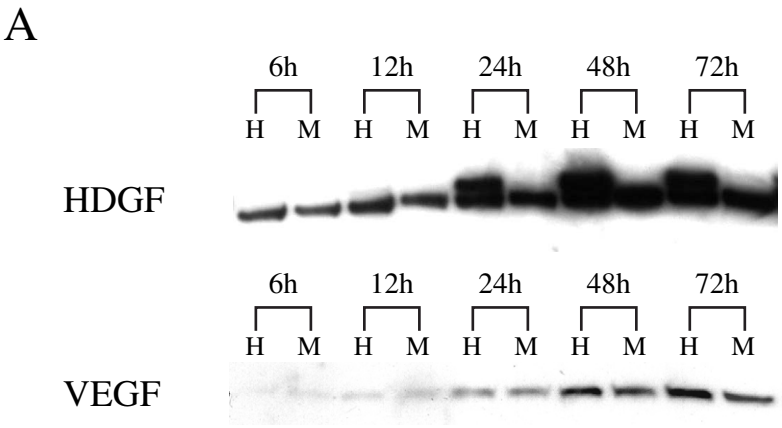
Fig. 3. HDGF stimulates the proliferation and tubule formation of HUVECs. (A) Effects of HDGF on the cell proliferation of HUVEC. HUVECs (5×10^3) were plated in 24-well plates in DMEM with 10% FCS. After 24-h culture, the medium was changed to DMEM-5% FCS with or without the indicated concentrations of recombinant HDGF. The number of viable cells in each well was counted 96 h later. The experiments were performed in triplicate. Values are means±SD (n=3). * P<0.05 vs. control. Effects of HDGF on the tubule formation of HUVEC were evaluated with an angiogenesis kit according to the manufacturer's protocol. HUVECs co-cultured with human fibroblasts were cultured in 24-well plates in medium with or without the indicated concentrations of recombinant HDGF. After 11 days, HUVECs were stained with mouse anti-human CD31 antibody. (B) Tubule lengths were measured quantitatively with NIH Image in 5 different fields of each well. The experiments were perfomed in quadruplicate. Values are means±SD (n=4). * P<0.05 vs. control. (C) Representative photographs of the tubule formation of HUVEC.

Discussion

In a previous study, HDGF was found to be expressed ubiquitously in normal tissues, and high expression was also demonstrated in fetal tissues, including the cardiovascular system, liver and kidney.[14, 15, 17, 18] These results suggested that the normal *HDGF* gene plays a very specific role in cell physiology, perhaps during embryonic development. However, HDGF was more abundantly expressed in malignant tumors than in normal tissues.[11, 20] Exogenous HDGF stimulates proliferation of several tumor cell lines, and its overexpression enhanced proliferation of HepG2 cells, while suppression of the endogenous HDGF production by antisense oligonucleotides or cDNA inhibited the proliferation of cells expressing HDGF.[10, 13, 19] Moreover, HDGF stimulated proliferation of endothelial cells, including those of the kidney and cardiovascular system, suggesting its possible involvement in angiogenesis.[14, 15] The investigation of temporally and spatially inappropriate expression of HDGF may provide clues to its physiological function. The present study shows that HDGF overexpression results in tumorigenesis of NIH3T3 murine fibroblasts by stimulating angiogenesis both directly and via induction of VEGF *in vivo*.

Tumors developed in nude mice by injection of murine fibroblasts overexpressing HDGF looked red-colored, and histological examination revealed a rich vasculature. Indeed, staining with the neovascularized endothelial cell marker CD31 demonstrated increased numbers of vessels in the tumors that developed from NIH3T3-HDGF stable transfectants. It is now well established that tumor growth beyond 1–2 mm^3 requires new vessel formation and cellular proliferation.[22] Evidence from numerous studies has shown that angiogenic activity is important for tumor growth, and that it is activated from the early preneoplastic stages of development to progression in the advanced stage of oncogenesis.[23] The present study showed that HDGF is able to stimulate tubule formation and extension of human endothelial cells in the angiogenesis assay as well as their cellular proliferation, which suggested that the HDGF molecule itself had a direct angiogenic activity in the tumors.

Nevertheless, the potential of HDGF for inducing angiogenesis *in vitro* appears to be small in contrast to its angiogenic potential *in vivo*, at least in the assay using HUVEC. Several protein factors showing angiogenic potential have been reported.[23] Recently, several studies have revealed that VEGF is one of the most important angiogenic factors, being involved especially in tumor growth.[22–24] VEGF transfection of tumor cells led to increased vascularity in tumor tissues and enhanced tumor growth, in terms of both size and frequency, *in vivo*.[25] VEGF enhanced tumor formation in nude mice by cooperating with other oncogenic proteins.[26–28] The loss of tumorigenic VEGF expression caused decreases in vascular density and permeability, and an increase in tumor cell apoptosis.[29] Although not oncogenic itself, VEGF aids in tumor proliferation through stimulation of angiogenesis and the consequent increase in the oxygen and nutrient supply *in vivo*.[23] In experimental models, VEGF has been proved to be necessary for *in vivo* tumor formation induced by several oncogenes, including ras protein.[29–31]



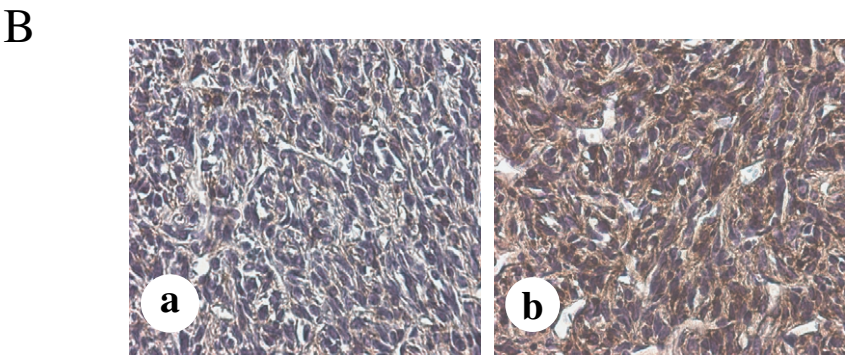


Fig. 4. HDGF induces VEGF protein. pEF-Bos-HDGF or pEF-BOS (mock) was transfected onto the NIH3T3 cells. The cells were harvested and lysed after 6, 12, 24, 48 and 72-h culture. Proteins samples (15 μg) were loaded in each well to analyze HDGF and VEGF protein levels by western blotting. (A) Changes of HDGF (top) and VEGF (bottom) protein levels in cells transfected with pEF-Bos-HDGF or with pEF-Bos vector only (H, HDGF; M, mock). (B) Immunohistochemical staining of VEGF in the tumor cells at ×400 original magnification, (a) without primary antibody for the control, or (b) with rabbit anti-mouse VEGF antibody.

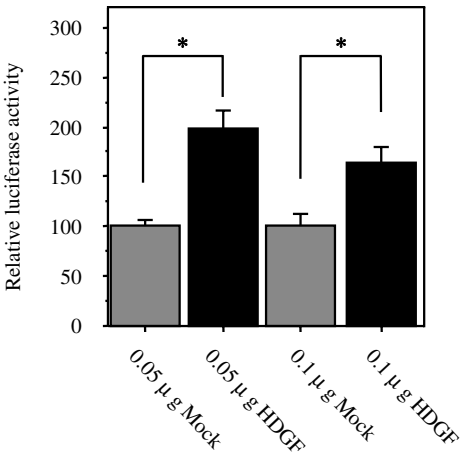


Fig. 5. HDGF overexpression increased luciferase activity in the reporter assay using VEGF gene promoter. The *VEGF* gene promoter region was inserted upstream of the *luciferase* gene in pGV-B. A modified luciferase reporter plasmid was co-transfected with pRL-SV40 and pEF-Bos-HDGF (HDGF) or pEF-BOS (mock) into NIH3T3 cells. The data are expressed as a percentage of those in pEF-BOS-transfected control cells. The experiments were performed in triplicate. Values are means±SD (n=3). * P<0.05 vs. mock.

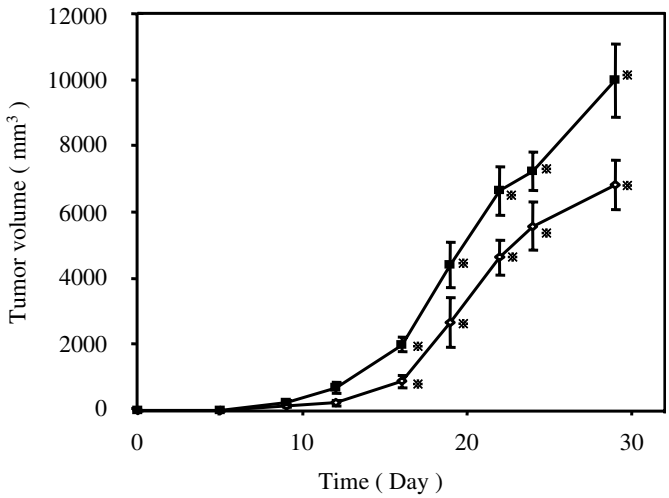


Fig. 6. Anti-VEGF antibody inhibited HDGF-induced tumor growth in nude mice. A neutralizing anti-mouse VEGF antibody (◇) or normal goat IgG (■) was administered via i. p. injection twice a week, respectively (50 μg/200 μl PBS per mouse) after inoculation of NIH3T3-HDGF cells into nude mice. Anti-VEGF antibody inhibited HDGF-induced tumor growth. Values are the means±SD (n=4) of changes in the tumor volumes in four mice in each group. * P<0.01 vs. normal IgG.

In the present study, we demonstrated the increased expression of VEGF protein in HDGF-overexpressing cells, and further confirmed significant induction of the *VEGF* gene by reporter assay using the *VEGF* gene promotor region. VEGF transcription was regulated by hypoxia and the MAP kinase signaling cascade via hypoxia inducible factor-1 (HIF-1) in a hypoxic condition and Sp1/AP2 transcriptional factor complexes in a normoxic condition.[32] It is considered that HDGF might stimulate VEGF promoter activity by recruitment of Sp1/AP2 transcriptional factors through the MAP kinase pathway, or that HDGF might increase VEGF production by direct binding to the Sp1/AP2 and/or AP1 binding motifs. However, the mechanism through which HDGF stimulates the VEGF promoter activity remains to be clarified. VEGF induced by HDGF may cooperate with HDGF to enhance the tumorigenicity of NIH3T3-HDGF stable transfectants *in vivo*. However, no in-

duction of the other potent angiogenic factor, basic FGF, was detected in HDGF-overexpressing cells by western blotting. Another angiogenesis-inducing factor, Cox-2, was not induced by HDGF overexpression, either.[33, 34] The present findings suggested that HDGF induced tumor formation in the nude mice both directly through its own angiogenic potential and via induction of *VEGF* gene expression. Anti-VEGF neutralizing antibody only partially suppressed the tumor growth of HDGF-overexpressing cells in nude mice, supporting the idea that the tumor growth of HDGF-overexpressing cells is partly due to the angiogenic activity of HDGF itself.

It is noteworthy that NIH3T3-HDGF stable transfectants induce tumor formation in nude mice, but do not show significant anchorage-independent colony formation in soft agar. Generally, most oncogenic proteins induce not only tumorigenesis in nude mice, but also colony formation in soft agar. However, some molecules can induce tumor formation in nude mice without anchorage-independent potential in soft agar *in vitro*.[35, 36] Other cancer-derived cell lines showed no or low anchorage-independent growth activity, although they can induce significant tumorigenesis in nude mice, and they acquired more potent transforming activity if other factors, such as TGF-α, FGF-8 and lipoxygenase, were overexpressed.[37–39] Furthermore, some genes such as *FGF-3* and *rsc* (rabbit squamous cell carcinoma) have potent oncogenic activities in nude mice, but do not induce anchorage-independent growth in the semi-solid media.[36, 40, 41] HDGF seems to belong to this group. Although NIH3T3-HDGF stable transfectants could not form large colonies in semi-solid media, but they did form more small colonies than the mock or parent cells in the soft agar assay. These findings showed that HDGF really has transforming activity, but with a relatively low potential *in vitro*. We hypothesize that, at least for NIH3T3 cells, the growth ability in soft agar is probably due to accumulation of additional genetic alterations. HDGF itself may nevertheless have a transforming activity and be involved in tumorigenesis *in vivo*, because the *HDGF* gene was quite potent in the nude mouse tumorigenesis assay. Thus, for detection of transforming activity, tumor formation assay in the nude mice should be more sensitive than the assay of anchorage-independent growth in soft agar. From a different standpoint, this dissociation between the different transforming properties should be useful to investigate the functions of the signal transduction pathways involved.

In summary, overexpression of HDGF induces tumorigenesis *in vivo* through the combination of intrinsic angiogenic activity and induction of VEGF, without causing significant anchorage-independent growth in soft agar. It is important to investigate the upstream mechanism of *HDGF* gene induction, and the signal transduction pathways from this gene. Clarification of the upstream and downstream activation mechanisms should provide important clues to understand the development and progression of human cancer.

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