

Calponin h1 Suppresses Tumor Growth of Src-induced Transformed 3Y1 Cells in Association with a Decrease in Angiogenesis

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Calponin h1 (CNh1) is a basic actin-binding protein that is abundantly expressed in smooth muscle cells and involved in smooth muscle contraction by inhibiting actomyosin MgATPase. In recent studies, CNh1 was noted to suppress cell proliferation and tumorigenicity in leiomyosarcoma and tumor growth in fibrosarcoma cell lines. To further investigate the function of CNh1 as a tumor suppressor, we transfected the human *CNh1* gene into a *v-src*-transformed rat fibroblast cell line SR-3Y1. The volume of the tumors derived from one randomly selected *CNh1*-transfectant (C1) in nude mice was reduced to 34.1% of that from a randomly selected vector transfectant (V1). A similar tendency was observed in another independent pair (C2, V2). Pathological analysis showed a significant decrease in the number of mitotic cells in the *CNh1*-transfectants. Further, a marked reduction in the number of vessels in the *CNh1*-transfectant was observed. DNA synthesis under conditions without serum was significantly reduced in the *CNh1*-transfectant (C1) compared with the control transfectant (V1), while no significant difference was seen in the cellular growth in the presence of 10% serum. A slight but significant reduction in *in vitro* cellular motility in the *CNh1*-transfectant was also observed. While the suppression of growth potential and cell motility by *CNh1* transfer was significant but partial, a marked reduction in vascular endothelial growth factor (VEGF) mRNA and the secretion of VEGF protein was observed in the *CNh1*-transfectant. These results suggest that CNh1 plays a role as tumor suppressor in SR-3Y1 mainly by decreasing VEGF expression and angiogenesis *in vivo* and partially through reducing cellular proliferative potential and cell motility.

Key words: Calponin — Tumor growth — Angiogenesis — Vascular endothelial growth factor — DNA synthesis

Calponin h1 (CNh1) is a basic 34 kD actin-binding protein originally isolated from chicken gizzard smooth muscle.¹⁾ It is expressed mainly in smooth muscle cells and is known to be involved in the regulation of smooth muscle contraction through inhibiting MgATPase and in the differentiation of smooth muscle cells.^{2,3)} CNh1 also induces actin polymerization and inhibits depolymerization of actin filaments.⁴⁾ Recently, the inhibitory effects of CNh1 on cell proliferation and tumorigenicity in leiomyosarcoma were reported.^{5,6)} Further, we obtained similar results in a fibrosarcoma cell line, HT1080.⁷⁾ Various studies have suggested that CNh1 is associated with suppression of malignant or metastatic phenotypes,^{8–11)} but the mechanism is not fully clarified. In the present study, we transfected the human *CNh1* gene into a *src*-induced transformed fibroblast cell line, SR-3Y1, where the causative gene for the transformation was clearly defined, to investigate the effect of CNh1 on the cell proliferation, motility and tumor growth. It is known that *v-src* induces vascu-

lar endothelial growth factor (VEGF) expression,^{12,13)} so we also examined whether CNh1 has a suppressive effect on angiogenesis and VEGF expression.

MATERIALS AND METHODS

Cell culture and transfection Rat fibroblast cell line, 3Y1, a *v-src*-transformed 3Y1: SR-3Y1¹⁴⁾ and the following transfectants were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). A 1560 bp human CNh1 cDNA that contained the entire coding sequence for human CNh1 was inserted into the *Bam*HI site of pCMV-neo-Bam vector. This vector possesses the *neomycin acetyltransferase* gene to produce G418-resistant clones. For transfection of mock vector or human *CNh1*-inserted vector to SR-3Y1, the Calcium phosphate method using a Mammalian Transfection Kit (Stratagene, La Jolla, CA) was employed. Ten micrograms of CsCl₂-purified vector DNA was transfected into 4×10⁵ cells cultured in four 30-mm tissue culture dishes. After transfection, cells were selected in the presence of 400 μg/ml of G418 sulfate (WAKO, Osaka). Expression of CNh1

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mRNA and protein were confirmed by reverse transcriptase (RT)-PCR and western blot analysis. We obtained several independent vector-transfected clones (V1, V2...Vn) and *CNh1*-transfected clones (C1, C2...Cn) from the transfection experiments. Two randomly selected pairs of V and C clones (V1, C1 and V2, C2) were subjected to independent experiments to examine tumor growth and one pair (V1, C1) was mainly used for further analyses.

Western blot analysis Total proteins (20 $\mu\text{g}/\text{lane}$) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The membranes were incubated with anti-human calponin antibody (Sigma, St. Louis, MO). This antibody can detect rat CNh1 in addition to human CNh1. For detection, the blots were incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) antibody (DAKO, Carpinteria, CA), and developed using an enhanced chemiluminescence detection system (Amersham, Buckinghamshire, UK).

Tumor growth assay in nude mice Six-week-old female athymic BALB/c nude mice were administered s.c. injections of vector- and *CNh1*-transfected cells in the flank (C1, V1, $n=5$; C2, V2, $n=6$). The tumor growth was evaluated by calculating tumor volume from the width and length of the tumors according to the following formula: Tumor volume (mm^3) = $(\text{Length} \times \text{Width}^2) / 2$. Hematoxylin-eosin (HE) and immunohistochemical stainings using anti-human calponin antibody (DAKO) or anti-factor VIII antibody (DAKO) were performed on tumor tissues induced in nude mice. For staining of calponin, paraffin-embedded tissue sections were digested with pepsin at 37°C for 20 min, immersed in anti-human calponin antibody as the primary antibody, and incubated with anti-mouse IgG antibody conjugated with horseradish peroxidase, followed by color development using diaminobenzidine tetrahydrochloride (DAB). For staining of anti-factor VIII antibody, digestion by proteinase K for 6 min was used for retrieval of the antigen. Thereafter, the following methods were as described for calponin staining. The number of mitotic cells in each tumor section was counted on HE-stained sections in 200 high-power ($\times 200$) fields. For each section, 12 fields were randomly chosen for assessment. For quantitative analysis of vessel density, microvessels positively stained with factor VIII or lumina containing red blood cells surrounded by endothelium were counted in 400 high-power ($\times 400$) fields. In each section, 10 randomly chosen fields were used for counting. This assay was performed by two independent observers.

Cell proliferation The cells were seeded in 35-mm dishes at 4×10^4 cells/dish and cultured at 37°C in DMEM with 10% FBS under 5% CO_2 . After 1 and 4 days of incubation, each transfectant was trypsinized and counted. Cell proliferation under the low-serum condition was evaluated

using a cell count reagent (Nacalai, Kyoto) which contained tetrazolium salt as the chromatic substrate. The cells were plated at a density of 4×10^3 cells/ $100 \mu\text{l}$ into a 96-well plate. They were incubated in DMEM with 10% FBS for 24 h, then the medium was replaced with DMEM supplemented with 1% FBS and the plate was incubated for an additional 48 h. The absorbance of the wells was measured using a microplate reader at a wavelength of 450 nm.

[^3H]Thymidine incorporation DNA synthesis was measured in terms of [^3H]methylthymidine incorporation. The cells (8×10^3 cells/well) were seeded in 96-well plates in DMEM supplemented with 10% FBS for 24 h. The cells were washed with serum-free DMEM and incubated for 24 h in DMEM with 0.1% bovine serum albumin (BSA). The cells were then stimulated with or without mitogens and cytokines for 24 h in the absence of serum, and labeled with [^3H]thymidine (final concentration 10 $\mu\text{Ci}/\text{ml}$; Amersham) for 4 h. Labeled cells were trypsinized and transferred to a Unifilter plate (Packard, Meriden, CT) using a cell harvester. Twenty microliters of scintillation fluid was added, and the radioactivity was measured with a scintillation counter (Top Count, Packard).

Cell migration analysis by gold colloidal method Coverslips (10×10 mm) were coated with colloidal gold particles, and then 1×10^4 cells/ml were seeded on these coverslips, which were placed in 35-mm culture dishes. They were cultured in DMEM with 10% FBS for 11 h, fixed in 3.5% formaldehyde solution in phosphate-buffered saline (PBS), and mounted on microscope slides. The tracks made by cells were analyzed with an ARGUS Image Processor System (Hamamatsu Photonics Co., Hamamatsu).

mRNA expression of VEGF For preparation of RNA, cells from each cell line were grown to about 90% confluence in 10-cm diameter petri dishes. Total RNA was prepared by the acid guanidium-phenol-chloroform method using ISOGEN (Nippon Gene, Toyama). Northern blot analysis was performed according to the methods previously described.^{15,16} Briefly, the rat *VEGF* primer sequences used for preparation of the cDNA probe were as follows; sense, 5'-AACCATGAACTTTCTGCTCTC-3', antisense, 5'-GGTGAGAGGTCTAGTTCCCGA-3'. Total RNA (20 $\mu\text{g}/\text{lane}$) was separated on a 1% formaldehyde gel and transferred to Hybond N⁺ (Amersham) with 20 \times SSC (salt sodium citrate). The membrane was hybridized at 42°C for 16 h with [α - ^{32}P]dCTP-labeled rat *VEGF* probe. The bands for *VEGF* were detected using a luminoimage analyzer (BAS-1500, Fuji Film, Tokyo). Re-hybridization for glyceraldehyde-3'-phosphate dehydrogenase (GAPDH) was performed as an inner control. The bands were quantified by Image Gauge v.3.45 (Fuji Film).

VEGF secretion To measure the cell-secreted VEGF protein, sandwich enzyme-linked immunosorbent assay

(ELISA) was performed using Quantikine M (R&D, Minneapolis, MN), a kit for mouse VEGF immunoassay that was confirmed to have 70% cross-reactivity with rat VEGF. Briefly, after culture of the cells in serum-free culture medium for 48 h, the cell culture supernatants were applied to 96-well plates coated with anti-mouse VEGF polyclonal antibody. Another peroxidase-linked polyclonal antibody specific for mouse VEGF was added as a second antibody. After removal of the unbound second antibody, the substrate solution was added and optical density was assessed using the microplate reader set to 450 nm.

Statistical analysis The significance of differences between the *CNh1*-transfectants and the mock vector transfectants in the quantitative data from cell proliferation and tumor volume were statistically evaluated using Fisher's protected least significant difference test (Systat). For the other experiments, Student's *t* test was applied. $P < 0.05$ was accepted as statistically significant. All values were expressed as the mean \pm SE.

RESULTS

Expression of CNh1 in 3Y1, SR-3Y1 and transfectants

Expression of CNh1 protein (34 kD) in 3Y1 and SR-3Y1 was examined by western blot analysis. The 3Y1 cells showed much higher expression of rat CNh1 than did the SR-3Y1 cells (Fig. 1A). Expression of CNh1 protein in *CNh1*-transfected cells (C1, C2) was also confirmed by western blot analysis (Fig. 1B); densitometrically C2 was about 88% of C1 (data not shown). We confirmed the expression of exogenous human CNh1 mRNA corresponding to the above western blot analysis by RT-PCR using a specific primer for human CNh1.⁷⁾

Suppression of tumor growth in nude mice To investigate whether CNh1 has a tumor-suppressive effect on SR-3Y1, we examined the tumor growth caused by *CNh1* and mock transfectants by inoculating cells subcutaneously into nude mice. The volume of the tumors on day 17 derived from *CNh1*-transfectants was 34.1% (C1) compared with that of mock transfectant (V1) (Fig. 2A). The suppression of tumor growth by *CNh1* transfer was also confirmed in an independent pair of transfectants (C2:V2=76.1:100). Immunohistochemistry revealed positive staining for CNh1 in tumors derived from C1 (Fig. 2B). Positive staining for CNh1 was also seen in clone C2, though it was less than in the case of C1 (data not shown). On HE-stained sections, the cellular arrangement of *CNh1*-transfectants in the tumor resembled the morphology seen in cultured normal fibroblast cells (in which elongated, spindle-shaped cells usually grow in parallel to their major axes), whereas vector transfectants *in vivo* exhibited an irregular pattern with nuclear atypia (Fig. 3A). Further, the number of mitotic cells in the tumor from a *CNh1*-transfectant (C1) was decreased to 11% of the

vector control (V1) (Fig. 3B). The number of mitotic cells in the tumor from C2 was also decreased to 62% of the vector control (V2) ($P < 0.01$, data not shown). There was no difference in the number of infiltrated cells between tumors of *CNh1*-transfectants (C1, C2) and vector controls (V1, V2), respectively. Also, we examined the apoptosis of tumor cells in nude mice by the deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) method. There was no significant difference in the number of apoptotic cells between *CNh1*-transfectants and vector controls (C1, V1, $n=5$; C2, V2, $n=4$) in our study (data not shown). These results suggest that CNh1 has a suppressive effect on the tumor formation of SR-3Y1 cells *in vivo*.

Reduction in cell motility To examine the difference in the character of cells between *CNh1*-transfectants and control cells *in vitro*, we chose clones C1 and V1, which showed differences in tumor growth. First, we performed migration analysis using the gold colloid method. The migration area of the *CNh1*-transfectant (C1) was significantly reduced to 78% of the control (V1) (Fig. 4). In contrast to our previous findings in HT1080 cells, *CNh1*-transfectants of SR-3Y1 and vector control cells did not show apparent differences in morphology, including actin stress fiber organization, *in vitro* (data not shown).

Suppression of DNA synthesis and cell proliferation under a low-serum condition Next, we examined the growth rate of the *CNh1*-transfected cells (C1) and control cells *in vitro*. There was no significant difference between *CNh1*-transfectant (C1) and control cells (V1) in cellular growth under regular culture conditions, in the presence of

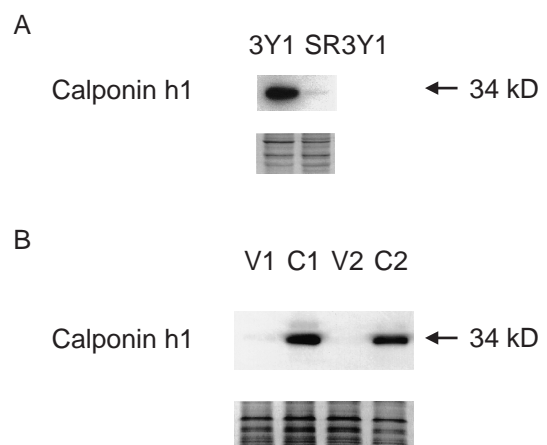


Fig. 1. (A) Western blot analysis for calponin h1 (CNh1) protein in 3Y1 and SR-3Y1. (B) Western blot analysis for CNh1 protein in clonal *CNh1*-transfectants (C1, C2) and mock vector transfectants (V1, V2). The monoclonal anti-human CNh1 is known to react with rat CNh1 as well as human CNh1.

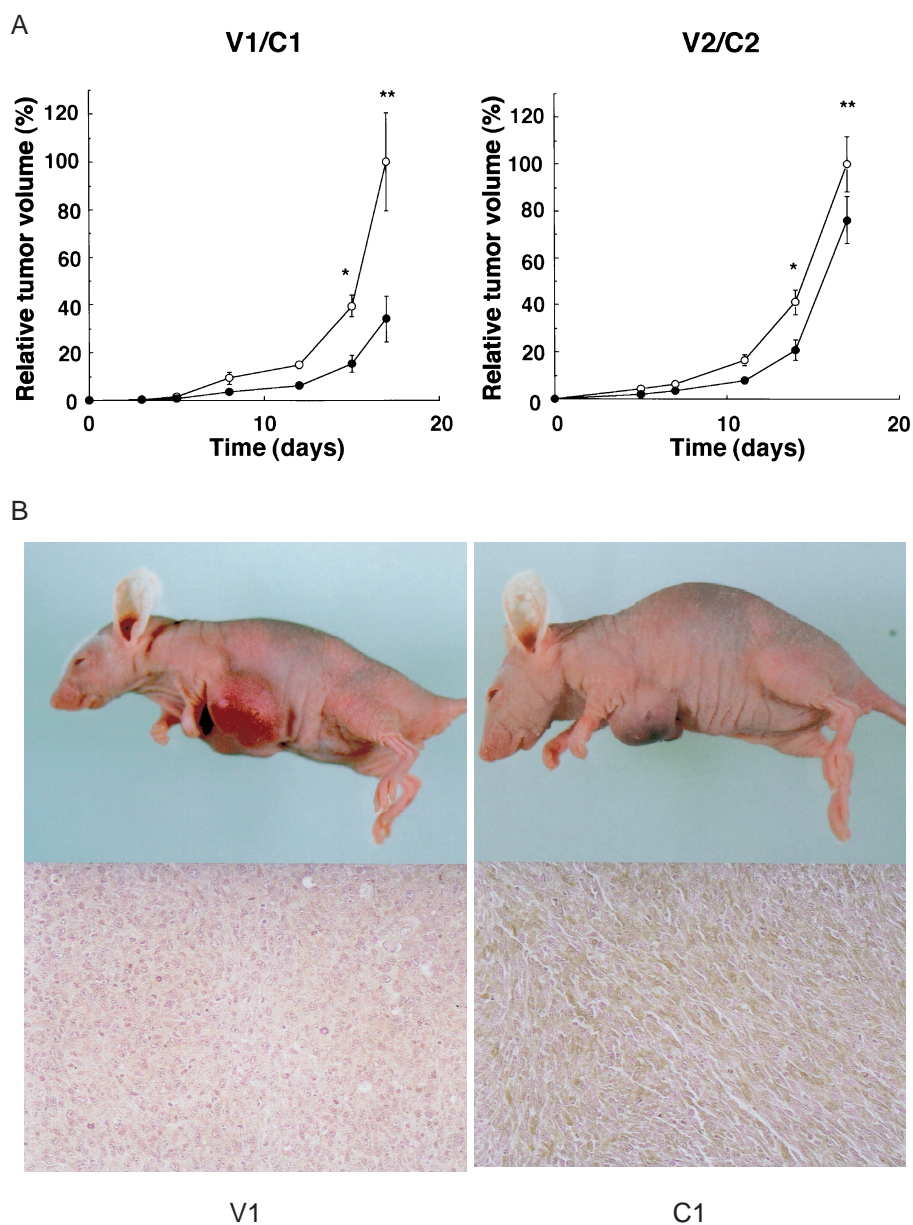


Fig. 2. (A) Tumor growth in nude mice of *CNh1*-transfectants (C1, C2; ●) and mock transfectants (V1, V2; ○). Tumor size was normalized to the average volume of V1- and V2-derived tumors on day 17, respectively in several experiments. *, $P < 0.05$; **, $P < 0.01$. (B) Tumors derived from V1 or C1 (upper panel) and immunohistochemistry using anti-human calponin antibody to confirm *CNh1* expression in C1-derived tumor (lower panel). Scale bar: 100 μm .

10% FBS (Fig. 5A). Anchorage-independent growth evaluated according to the previously described method⁶) also showed no significant difference (data not shown). However, cell proliferation in the low serum condition (1% FBS) was slight but significantly ($P < 0.05$) decreased in the case of the *CNh1*-transfectant (data not shown). Fur-

ther, DNA synthesis of the *CNh1*-transfectant (C1) was reduced to 47% of that of control cells (V1) in [³H]thymidine incorporation analysis in the presence of 0.1% BSA (Fig. 5B). Although the *CNh1*-transfectant (C1) had a slight suppressive effect on cell proliferation *in vitro*, this was not as prominent as the suppression of tumor growth

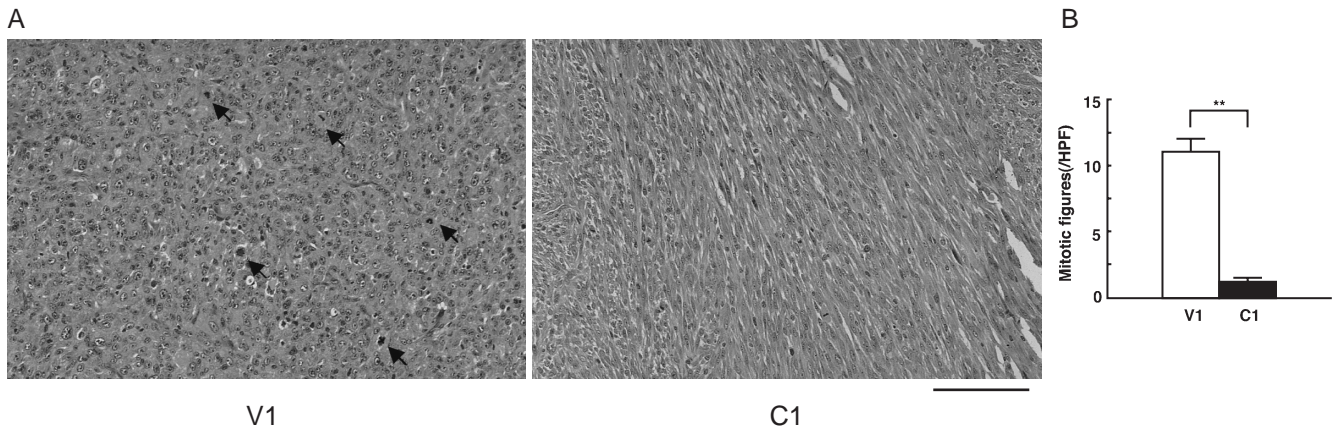


Fig. 3. (A) HE staining of the tumors derived from mock vector transfectant (V1) and *CNh1*-transfectant (C1). Arrows indicate mitoses. Scale bar: 100 μ m. (B) Number of mitotic figures in the tumor from vector controls (V1) and *CNh1*-transfectants (C1). **, $P < 0.01$.

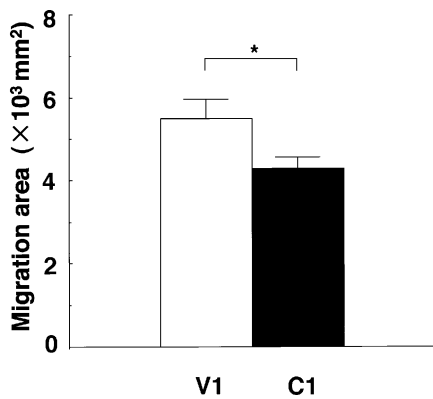


Fig. 4. Migration analysis of *CNh1*-transfectant (C1) and control cells (V1) using gold colloidal method. *, $P < 0.05$.

in vivo. This result suggested that there may be external factors corresponding to the inhibitory effects on the tumor formation of CNh1. We examined the effects of several growth factors and mitogens on [³H]thymidine incorporation in *CNh1* and control transfectants. Transforming growth factor β_1 (TGF- β_1) did not alter [³H]thymidine incorporation in *CNh1*-transfectant (C1), while the inhibitory effect was significant ($P < 0.01$) in vector control cells (V1) in a dose-dependent manner (data not shown). PDGF (platelet-derived growth factor)-AA, PDGF-AB, PDGF-BB, FGF (fibroblast growth factor), EGF (epidermal growth factor), IFN (interferon) γ , heparin and histamine did not show significantly different effects on [³H]thymidine incorporation between *CNh1* and control transfectants (data not shown). To clarify whether CNh1 alters the expression of cell surface TGF- β receptor I,

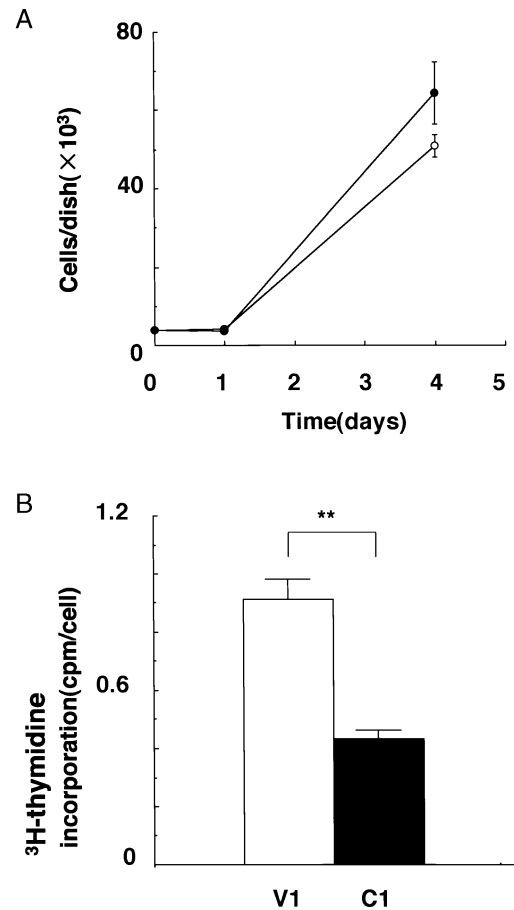


Fig. 5. (A) Growth curves of *CNh1*-transfectant (C1; ●) and vector control (V1; ○) cultured in DMEM with 10% FBS. (B) [³H]Thymidine incorporation analysis of *CNh1*-transfectant (C1) and vector control (V1) in the presence of 0.1% BSA. **, $P < 0.01$.

analysis using the fluorescence activated cell sorter was performed. However, there was no significant difference (data not shown).

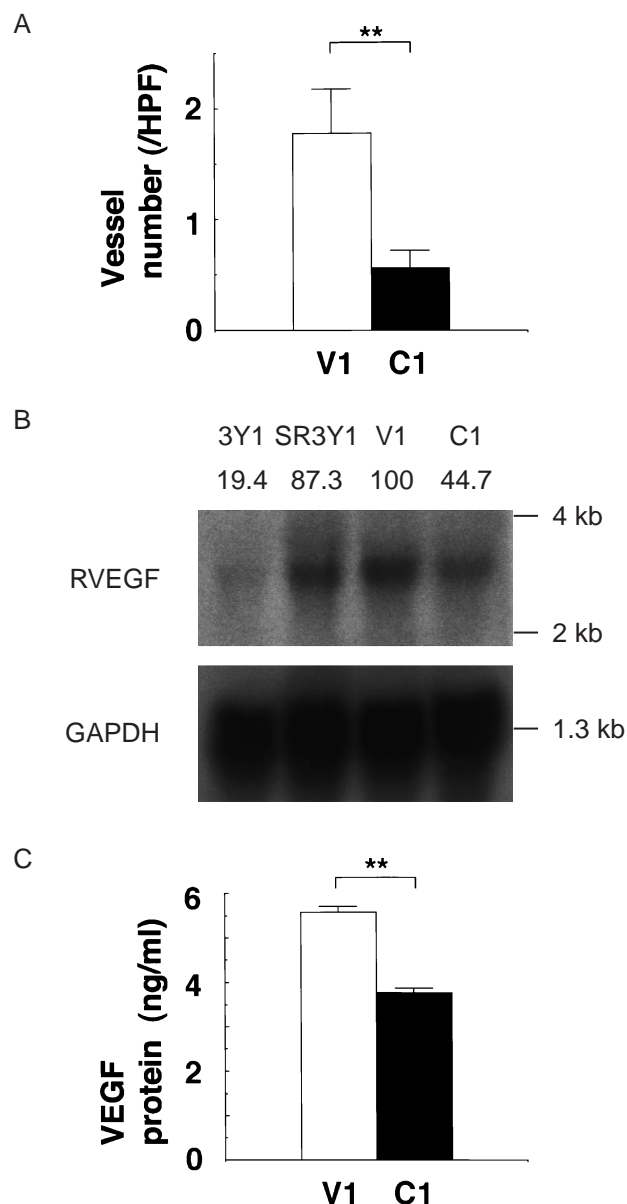


Fig. 6. (A) Number of vessels in the tumor from *CNh1*-transfectant cells (C1) and vector controls (V1). (B) Northern blot analysis of VEGF mRNA in cultured *CNh1*-transfectant (C1) and vector control (V1). The numbers above the figure indicate the VEGF mRNA index. The VEGF mRNA index was calculated as follows: VEGF mRNA Index=(VEGF mRNA level/GAPDH mRNA level)×100. (C) VEGF protein secretion of *CNh1*-transfectant (C1) and vector control (V1) measured by ELISA. **, $P<0.01$.

Decreased angiogenesis and VEGF expression in *CNh1* transfectant Another possibility is that CNh1 reduces tumor angiogenesis, resulting in the suppression of tumor growth. The number of blood vessels in the tumors derived from the *CNh1*-transfectant (C1) was about one-third of that in the case of the control transfectant (V1) (Fig. 6A). While a similar tendency was observed in another pair (V2, C2), the difference was not so great as seen in the pair of C1 and V1 ($P>0.05$, data not shown), indicating that the suppression of angiogenesis depended on the expression of exogenous CNh1. In northern blot analysis, SR-3Y1 cells showed a 4.5-fold higher expression of VEGF mRNA than 3Y1 cells. Further, the cultured *CNh1*-transfectant (C1) exhibited a reduced expression of VEGF mRNA compared with the control transfectant (V1:C1=100:44.7) (Fig. 6B). ELISA assay showed that VEGF protein secretion was also suppressed by CNh1 (Fig. 6C).

DISCUSSION

CNh1 is an actin-, tropomyosin- and calmodulin-binding protein which is expressed mainly in smooth muscle cells. It is involved in smooth muscle contraction, smooth muscle differentiation and actin bundle formation. Moreover, a role of CNh1 as a tumor suppressor has been noted recently. Down-regulation of the *CNh1* gene is associated with the malignant or metastatic phenotype of several human malignant tumor cells, including leiomyosarcoma⁵ and osteosarcoma.⁸ Previous studies revealed that *CNh1* gene transfection into fibroblast, leiomyosarcoma, and fibrosarcoma cell lines resulted in a reduction of cell proliferation or tumor growth.^{6,7,10} However, the mechanism of the tumor-suppressive effect of CNh1 remains to be determined. In the present study, we transfected human CNh1 cDNA into an *src*-transformed fibroblastic cell line SR-3Y1 and showed that CNh1 suppressed the tumor growth in association with a decrease in VEGF expression and angiogenesis and also in part with a reduction in cell proliferative potential and cell motility.

Src tyrosine kinase is a membrane-anchored nonreceptor protein kinase, and the proto-oncogene *c-src* is reported to be involved in cell motility and metastasis.¹⁷ *V-src* is an oncogenic form of *c-src*, with the Src tyrosine kinase constitutively activated. We previously showed that SR-3Y1 cells had lost the actin cable-like structures, in contrast with 3Y1 cells which showed bundles of actin filaments.¹⁸ Motile fibroblasts contain fewer stress fibers than nonmotile counterparts.¹⁹ Danninger and Gimona showed that CNh1 localized to actin stress fibers and stabilized them, followed by a decrease of cell motility.²⁰ Our previous study⁷ on HT1080 cells afforded similar results. In this study, *CNh1*-transfected SR-3Y1 cells exhibited a slight reduction in cell motility, but no marked

change in cell morphology occurred *in vitro*. Integrin $\alpha 5\beta 1$ expression, which was increased in *CNh1*-transfected HT1080 cells,⁷⁾ did not change in SR-3Y1 cells transfected with CNh1. The mechanism of the suppression of cell motility in *v-src* transformed cells remains to be examined, with attention to the regulation system of the actin-cytoskeleton, such as the Rho signaling pathway.

In cell proliferation analysis *in vitro*, the cell proliferation was not inhibited by CNh1 in a high-serum (10%) condition, while a significant decrease in proliferation of the *CNh1*-transfectant was observed under a low-serum (1%) condition. [³H]Thymidine incorporation analysis also revealed a reduction in DNA synthesis caused by CNh1 in hypoalimentation states. Clinically, benign tumors halt their growth at a certain size, while malignant tumors continue to grow with no limit. The difference between malignant and benign tumors may arise from the nature of their responses to a hypoalimentation state. Our data suggest that CNh1 may inhibit tumor growth in the hypoalimentation state.

Although the point in the cell cycle at which CNh1 functions has not been determined yet, *CNh1* gene expression was reported to be down-regulated when primary rat aortic smooth muscle cells begin to pass through the G₁/S checkpoint of the cell cycle and proliferate.²¹⁾ As cell proliferation differed only slightly between *CNh1*-transfectants and vector controls *in vitro*, we speculate that external factors differently affect the tumor growth between *CNh1*-transfectants and control cells. Heparin is reported to induce CNh1 and cell cycle inhibitor p27, inhibiting the cell proliferation of uterine smooth muscle cells.²²⁾ Although several growth factors and mitogens, including the above, were tested, we could not find any factor which can explain the suppression of tumor growth of *CNh1*-transfectants. However, an interesting result on [³H]thymidine incorporation was obtained in the presence of TGF- β_1 . TGF- β_1 reduced DNA synthesis dose-dependently in vector control cells (V1), while it did not show a suppressive effect on *CNh1*-transfectant cells. A similar result was previously reported by Fukuda *et al.* in 3Y1 and SR-3Y1 cells.²³⁾ Expression of the receptor of TGF- β_1 was not significantly different between *CNh1*-transfectant and control cells. These results suggest that CNh1 may alter responsiveness to TGF- β_1 downstream of the TGF- β_1 receptor and somewhat "normalize" SR-3Y1 cells.

Angiogenesis is essential for tumor growth.²⁴⁾ VEGF is a ~43 kD secreted glycoprotein that has been shown in bioassays to induce endothelial proliferation, angiogenesis, and capillary hyperpermeability.²⁵⁾ It is considered as a major positive regulator of tumor angiogenesis.^{26, 27)} Hypoxia is known to induce VEGF expression through activation of c-Src.¹²⁾ However, *v-Src* could induce VEGF expression in the absence of hypoxia.^{12, 13)} Based upon these reports, and as the number of vessels was decreased in the

tumor derived from *CNh1*-transfectant, we consider that CNh1 suppresses VEGF expression in SR-3Y1. Our data revealed higher expression of VEGF mRNA in *v-src* transformed SR-3Y1 cells compared to 3Y1 cells. Further, we showed that *CNh1*-transfection into SR-3Y1 cells resulted in a decrease in the expression of VEGF mRNA and protein. These data indicate that CNh1 suppresses tumor angiogenesis by reducing VEGF expression. Although VEGF is known to decrease apoptosis, thereby increasing tumorigenicity of tumor cells,^{28, 29)} the number of apoptotic cells in the tumors was not significantly different between *CNh1*-transfectants and control cells in this study. In recent reports, VEGF stimulated proliferation of tumor cells by activating mitogen-activated protein kinases (MAPKs) and *c-fos*, implying mitogenic effects of VEGF in accordance with our results.^{30, 31)} Thus, CNh1 may suppress this VEGF-induced mitogenic effect. VEGF has been demonstrated to be an autocrine as well as a paracrine growth factor in tumor cells.^{30, 32)} Further, extracellular matrix components (ECM) were reported to be required for VEGF-induced mitogenic effects.³¹⁾ Speculating from these reports, the different effects of CNh1 on morphology *in vitro* and *in vivo* might be derived from the paracrine effect of VEGF and/or involvement of ECM in addition to the abundance of cytokines *in vivo*. V-Src is known to up-regulate VEGF through several signaling pathways such as Stat3 (signal transducer and activation of transcription 3) and HIF-1 (hypoxia-inducible factor 1), and these signals are also reported to be involved in apoptosis.^{33, 34)} As CNh1 transfer did not appear to affect apoptosis of tumor cells, and Stat3 and HIF-1 are reported to correlate with apoptosis, CNh1 may suppress VEGF expression downstream of Stat3 and HIF-1.

In conclusion, CNh1 exhibits various functions to suppress malignant phenotypes; the induced inhibitory effect on tumor growth of *v-src*-transformed 3Y1 cells is due mainly to antiangiogenic activity. Further experiments remain to be done to see how CNh1 is involved in various signaling pathways.

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