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European Journal of Cancer 38 (2002) 436–442

European Journal of Cancer

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Calponin h1 induced a flattened morphology and suppressed the growth of human fibrosarcoma HT1080 cells

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Received 1 June 2001; received in revised form 12 October 2001; accepted 7 November 2001

Abstract

Calponin h1 (CNh1) is an actin-binding protein that is expressed mainly in smooth muscle cells and is known to regulate smooth muscle contraction. Recently, re-expression of CNh1 in leiomyosarcoma cell lines is reported to suppress cell proliferation and tumorigenicity. However, little is known about the associated cellular structural and functional changes. Since CNh1 is also detected in normal fibroblasts, we hypothesised that CNh1 would also inhibit cell proliferation of the fibrosarcoma cells, HT1080, in which CNh1 is suppressed. An expression vector of human *CNh1* complementary DNA was transfected into human HT1080 cells by a calcium-phosphate precipitation method. *CNh1*-transfected cells exhibited a flattened morphology with organised actin filaments, a significant decrease in cell motility and enhancement in adhesion to fibronectin in association with an increase in integrin $\alpha5\beta1$ expression. Anchorage-independent growth and tumorigenicity in nude mice were suppressed in the *CNh1*-transfected cells. Our results suggest that CNh1 may have a role as a tumour suppressor in human fibrosarcoma by influencing cytoskeletal activities.

Keywords: Calponin h1; Fibrosarcoma HT1080; Flattened morphology; F-actin restoration; Growth suppression

1. Introduction

Calponin is an abundant actin- and calmodulin-associated protein originally isolated from chicken gizzard [1]. To date, three distinct mammalian calponin isoforms: basic (h1), neutral (h2), and acidic calponin have been identified [2]. Calponin h1 (CNh1) is known to be involved in the regulation of smooth muscle contraction by exerting an effect on the actin–myosin interaction [3]. Meanwhile, calponin was found to be localised in the actin-containing stress fibres of the central parts of the actin cytoskeleton [4], and to be downregulated in tumours such as leiomyosarcoma [5] and osteosarcoma [6]. The transfection of *CNh1* into smooth muscle cells, fibroblasts [7] or leiomyosarcoma cells [8] suppressed cell growth and tumorigenicity. Furthermore, we observed the overgrowth of fibroblasts in *CNh1*-deleted mice in the course of dermal wound healing [9]. It has been reported that CNh1 is rapidly downregulated when growth-arrested cells re-enter the G1 phase and proliferate [10]. Reports indicating an interaction of CNh1 with MAP kinase [11], rho kinase [12], protein kinase C [13] and the actin cytoskeleton [14] suggest that CNh1 may be involved in regulating cellular signal transduction pathways relating to adhesion, cytokinesis, differentiation and proliferation of the cells, in addition to the regulation of smooth muscle contractility.

Here, we transfected *CNh1* into the human fibrosarcoma cell line, HT1080, which has been reported to show flat reversion associated with suppression of cell proliferation, to investigate if *CNh1* could exhibit anti-oncogenic function by influencing the cytoskeletal system.

2. Materials and methods

2.1. Cell culture and DNA transfections

The human fibrosarcoma cell line, HT1080, was obtained from the IFO Animal Cell Bank (Osaka, Japan). The cells were cultured in Dulbecco's modified

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0959-8049/02/$ - see front matter © 2002 Published by Elsevier Science Ltd.
PII: S0959-8049(01)00390-2

medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Sigma Chemical Co., St. Louis, MO, USA) and 1% antibiotic solution at 37 °C in 5% CO_2 in air.

A human *CNh1* cDNA was cloned into a *BamHI* site of the pCMV-*neo*-Bam mammalian expression vector, and transfected into HT1080 (4×10^5 cells) by a calcium-phosphate precipitation method using the Mammalian Transfection Kit (Stratagene, La Jolla, CA). Neomycin-resistant (*neo*) clones were selected in a medium containing 600 μg/ml of G418 (Wako, Osaka, Japan) for 14 days. Two independent vector-transfected control clones (V1 and V2) and *CNh1*-transferred clones (C1 and C2) were randomly selected, and each experiment was performed separately with the two pairs, V1, C1 and V2, C2.

2.2. Analyses of growth in monolayers and anchorage-independent growth

At 1, 2, 3 and 4 days after plating (4×10^4 cells/35-mm dish), cells in quartet dishes for each transfectant were trypsinised and counted on a haemocytometer. Soft agar assays were performed according to the method previously described in Ref. [8]. Twenty-one days after plating cells, the number of individual colonies larger than 0.1 mm in diameter was counted.

2.3. 2.3.In vivo *tumour growth*

Six-week-old male BALB/c nude mice were given subcutaneous (s.c.) injections of vector- or *CNh1*-transfected cells. Each injection consisted of 2×10^6 cells in 200 μl of Hank's solution. Growth of the tumours was evaluated every 3 days by calculating tumour volume according to the following formula: volume = $(\text{width})^2\times\text{length}/2$ [15]. The first sign of distress was observed in one mouse 17 days after the injection, all mice in the experiment were euthanised on the same day, their tumours excised, and fixed in Carnoy solution (60% ethanol, 30% chloroform, 10% acetic acid) for staining.

2.4. Adhesion assays

Bacterial 35-mm dishes were coated with 20 μg collagen (Type I, Collagen Corporation, Polo Alto, CA, USA), Matrigel (Collaborative Research, Fukuoka, Japan) or fibronectin (Sigma). Then transfectant cells were plated onto these dishes with 2×10^5 cells/dish in DMEM and 1 mg/ml bovine serum albumin (BSA) without 10% FBS, followed by incubation at 37 °C under 5% CO_2. The incubation times were 30 min for collagen, and 1 h for Matrigel or fibronectin. Attached cells after washing with phosphate-buffered solution (PBS) were trypsinised and the number was counted on a haemocytometer.

2.5. Motility assay using gold colloid coated plates

Random cell motility was estimated as an area of phagokinetic track on gold colloid coated glass plates previously described in Ref. [16]. The area was analysed with an ARGUS Image Processor System (Hamamatsu Photonics Co., Hamamatsu, Japan).

2.6. Stainings

2.6.1. Immunofluorescence of cells

For actin stress fibre stainings, cells were incubated with Rhodamine phalloidin (Eugene, OR, USA, 1:200) for 30 min. For α5β1, anti-human integrin α5β1 mAb (Chemicon Intl Inc., CA, USA, 1:200) and anti-mouse IgG conjugated with Rhodamine (1:500) were used. Anti-human fibronectin rabbit IgG (DAKO Co., CA, USA, 1:200) and anti-rabbit IgG conjugated with Rhodamine (1:500) were used for the fibronectin staining. Anti-human integrin α5β1 mAb (1:200) and anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) (1:500) were used for the analysis using the fluorescence activated cell sorter, FACSVantage^{TM}SE (Becton Dickinson, San Jose, CA, USA).

2.6.2. Immunohistochemistry of tumour tissues

Endogenous peroxidase activity was blocked by treatment with 0.3% hydrogen peroxide in methanol and antigen retrieval was attempted by boiling the sections in a microwave oven (600W, 12 min) in 0.01 M citric acid, pH 6.0. Incubations with mouse anti-calponin mAb (hCP, Sigma, 1:50) and anti-mouse goat IgG antibody conjugated with horseradish peroxidase (p0447, DAKO, 1:100) were followed by 3′3-diaminobenzidine HCl (DAB).

2.7. Statistical analysis

Statistical analysis was performed using two-way analysis of variance, and differences between groups within the set were analysed by Fisher's protected least significant difference test (Systat) for cell proliferation and tumour volume analyses. For the other experiments the Student's *t*-test was applied. $P<0.05$ was considered significant. All values were expressed as means-±standard error of the mean (S.E.M).

3. Results

3.1. Expression of CNh1 in HT1080 cells

The transfer of the expression vector was confirmed by the polymerase chain reaction (PCR) for the *neo* gene (sense 5′-GGCACAACAGACAATCGGCT-3′ and antisense 5′-ACTTCGCCCAATAGCAGCCA-3′;

218 bp: Fig. 1a). The expression of human *CNh1* was confirmed by reverse transcriptase (RT)-PCR (sense 5′-AAGGGCGGAACATCATTGGGCT-3′ and antisense 5′-CTCGAAGATCTGCCGCTTGGT-3′; 215 bp, using an RNA PCR Kit (Takara Shuzo Co. Ltd. Ohtsu, Japan; Fig. 1b) and Western blotting assays (hCP, 1:500 and P0447, 1:1000; 34 kDa; Fig. 1c).

3.2. Cell proliferation in monolayers and anchorage-independent colony formation

The growth rate in the *CNh1*-transfected cells in monolayers was slightly lower than for the vector-transfected cells, although the difference was not significant in either pairs, V1, C1 or V2, C2, while anchorage-independent growth was significantly suppressed by *CNh1* transfection. The number of colonies larger than 0.1 mm in diameter per field ($n=6$) were V1: 11.17 ± 0.87, C1: 5.67 ± 0.84 ($P=0.001$) and V2: 9.83 ± 1.51, C2: 6.00 ± 0.58 ($P=0.040$; Fig. 2).

3.3. Tumorigenicity assay

Expansions of tumour volumes of C1 cells s.c. injected into immunodeficient nude mice were suppressed compared with those of V1 cells, and the differences were significant at the termination of the experiment on day 17 (V1: $1.72\pm0.27\times10^3$ mm^3, C1: $1.13\pm0.28\times10^3$ mm^3, $n=6$, $P=0.013$; Fig. 3a and b), indicating that the tumour volume of the C1 cells was reduced to 65.7% of that of the V1 cells. Expression of CNh1 in the tumour derived from C1 or lack of expression in the tumour derived from V1 was confirmed immunohistochemically (Fig. 3c). The number of mitoses counted in the H&E fields ($n=10$) was slightly reduced in the tumour derived from C1 (2.80 ± 0.59) compared with that from V1 (4.50 ± 0.67; Fig. 3d). These results suggested that the tumorigenicity was suppressed by CNh1.

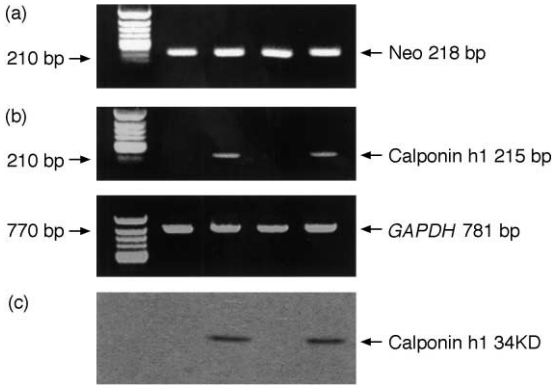

Fig. 1. Expression of *CNh1* of the two pairs, V1, C1 and V2, C2: (a) polymerase chain reaction (PCR) for vector pCMV-*neo*-Bam mammalian expression vector; (b) reverse transcriptase (RT)-PCR for *CNh1*; (c) Western blotting for CNh1. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

3.4. Flattened morphology

Cellular morphology for the transfectants in confluent stage is shown in Fig. 4a. Flattened and spread cell shape was observed in C1, while V1 exhibited a more round shape and decreased cell–cell contact. To determine the effect of CNh1 on actin stress fibre organisation, we stained the cells with Rhodamine phalloidin, which binds to filamentous F-actin, 6 h after inoculation. *CNh1*-transfected cells displayed a flattened, adhered, well-spread shape and restored an organised actin cytoskeleton (Fig. 4b). Similar results were obtained for the V2, C2 pair.

3.5. Adhesion assay

No significant differences in the adhesion to collagen or Matrigel were detected between V1 and C1, whereas in the experiment for fibronectin, more enhanced adhesion was observed in the C1 cells ($2.49\pm0.26\times10^4$) than in the V1 cells ($1.77\pm0.19\times10^4$) ($n=8$, $P=0.046$; Fig. 5a). Similar results were obtained from a repeated experiment with the other clone pair, V2 and C2 (for fibronectin: V2: $1.59\pm0.30\times10^4$, C2: $2.61\pm0.35\times10^4$, $n=8$, $P=0.043$).

Immunofluorescence staining with the anti-fibronectin antibody was faint in both the C1 and V1 cells, and no significant difference was observed between them. For

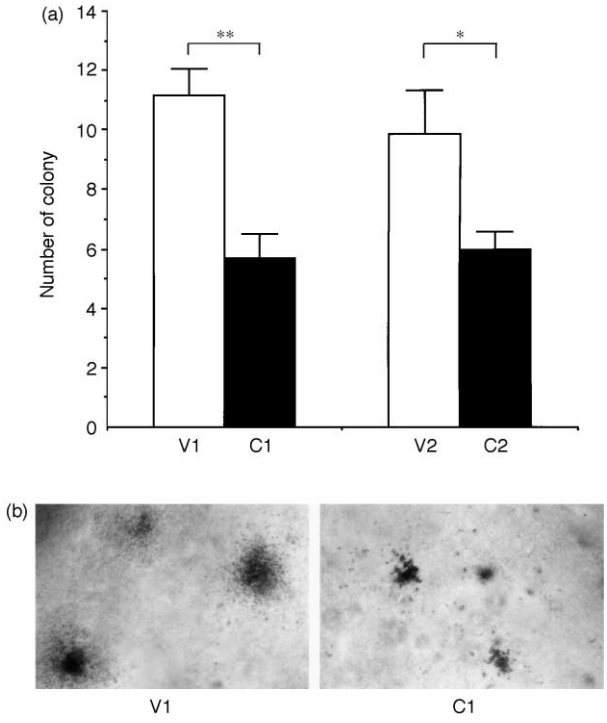

Fig. 2. Suppression of anchorage-independent growth by CNh1: (a) the number of colonies larger than 0.1 mm in diameter was counted, $^*P<0.05$, $^{**}P<0.01$; (b) phase-contrast micrographs of colonies of V1 and C1 in soft agar. Scale bar: 200 μm.

α5β1, all of the V1 cells were stained faintly. In contrast, among the C1 cells, the membranes of some of the cells were stained a little stronger (Fig. 5b). Analysis performed with FACS supported that CNh1 stimulated the expression of integrin α5β1 in *CNh1*-transfected cells. Although, V1 and C1 cells stained with an irrelevant antibody showed similar intensity (data not shown), slightly higher levels of integrin α5β1 were detected on C1 cells than V1 cells (Fig. 5c).

3.6. Motility assay by gold colloid

Cells were plated on the gold colloid-coated coverslides, and volume areas of the phagokinetic track were

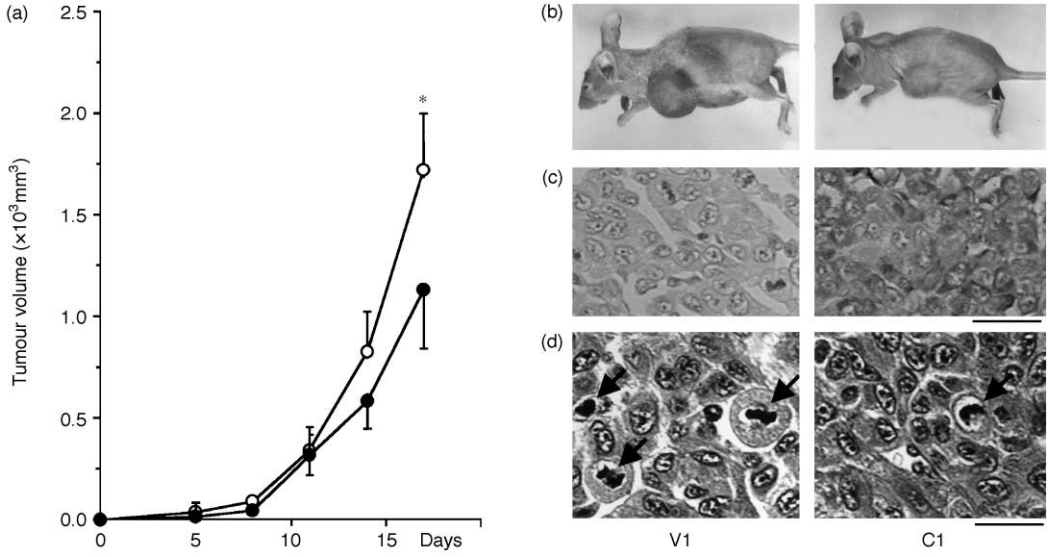

Fig. 3. Suppressed tumorigenicity in *CNh1*-transfected cells: (a) and (b) growth of the tumour derived from V1 (○) or C1 (●) in nude mice, $*P<0.05$; (c) immunohistochemical confirmation of CNh1 expression in C1-derived tumour, scale bar: 50 μm; (d) arrows indicate mitoses. Scale bar: 50 μm.

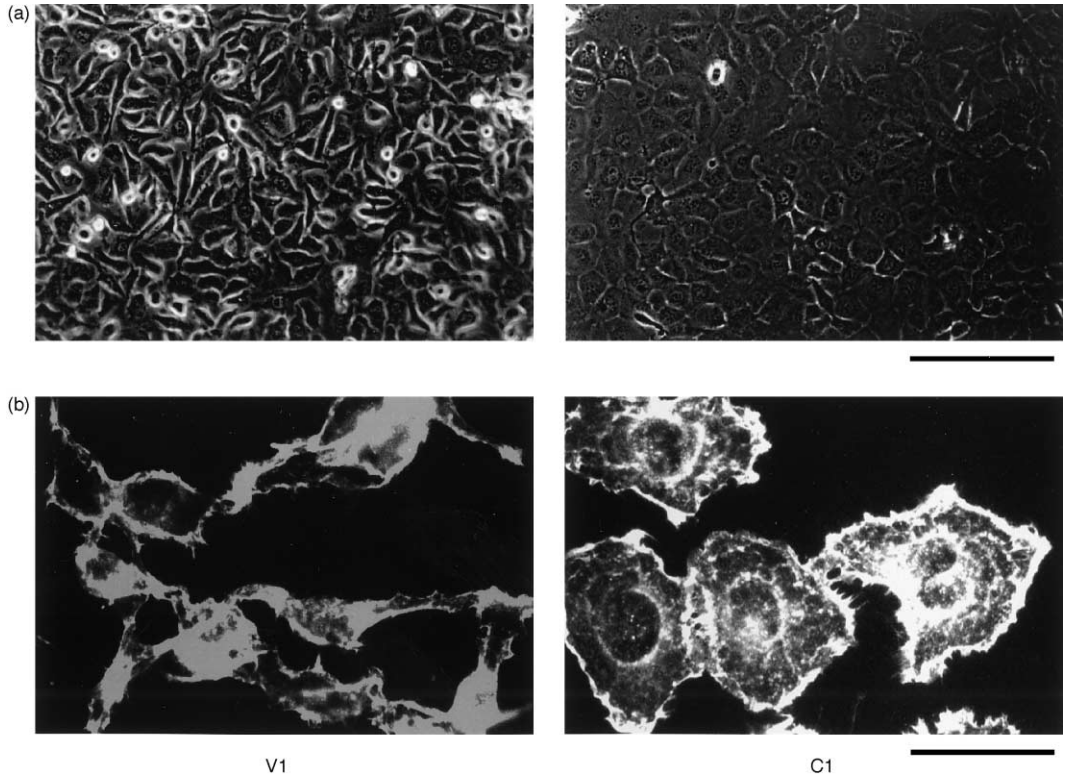

Fig. 4. Flattened morphology and restoration of actin induced by CNh1: (a) cellular morphology at the confluent stage revealed by phase-contrast microscopy, scale bar: 200 μm; (b) immunofluorescent analysis of F-actin fibres performed by Rhodamine phalloidin staining, scale bar: 50 μm.

analysed. Each phagokinetic area of coated gold colloid was V1: 11.26±0.72, C1: 7.40±0.54 ($\times 10^3$ μm^2, $n=20$, $P=0.001$) and V2: 12.08±0.83, C2: 9.41±0.78 ($\times 10^3$ μm^2, $n=20$, $P=0.025$). An inhibitory effect of CNh1 on cell motility was evident (Fig. 6).

4. Discussion

In this study, we observed flat reversion of HT1080 by *CNh1* gene transfection. This reverted flatter morphology was associated with organised actin filaments, a decrease in cell motility, an enhancement in adhesion to fibronectin in association with increased integrin α5β1 expression, and suppressed growth both in soft agar and tumorigenicity. These results were consistent with previously reported phenomenon induced by the treatments of HT1080 with styryl diphenylamine derivative [17] or suicide techniques [18].

While adhesion to the substratum is required for the proliferation of most mammalian cell types, fibronectin on the cell surface disappears upon oncogenic transformation [19]. According to Akamatsu and colleagues [20], overexpression of fibronectin in HT1080 cells restored the pericellular fibronectin matrix, induced a more flattened morphology, reduced motility, and suppressed growth both in soft agar and tumorigenicity. Therefore, we examined the possibility of an increase in fibronectin levels by CNh1 transfer; however, this was not the case when analysed by immunofluorescent staining. However, in *CNh1*-transfected cells, the immunofluorescent staining of integrin α5β1 was stronger on the cell membrane than in vector-transfected cells. Since stable overexpression of the α5 subunit was reported to result in decreased DNA synthesis in HT1080 cells [21], the increase in integrin α5β1 caused by *CNh1*-transfection may be involved in the suppression of transformation through stabilisation of the actin cytoskeletal system. Besides fibronectin, slightly enhanced adhesion to collagen and Matrigel was also observed in *CNh1*-transfected cells, although it was not statistically significant. Since collagen and laminin, ingredients of Matrigel, have RGD peptides, as well as

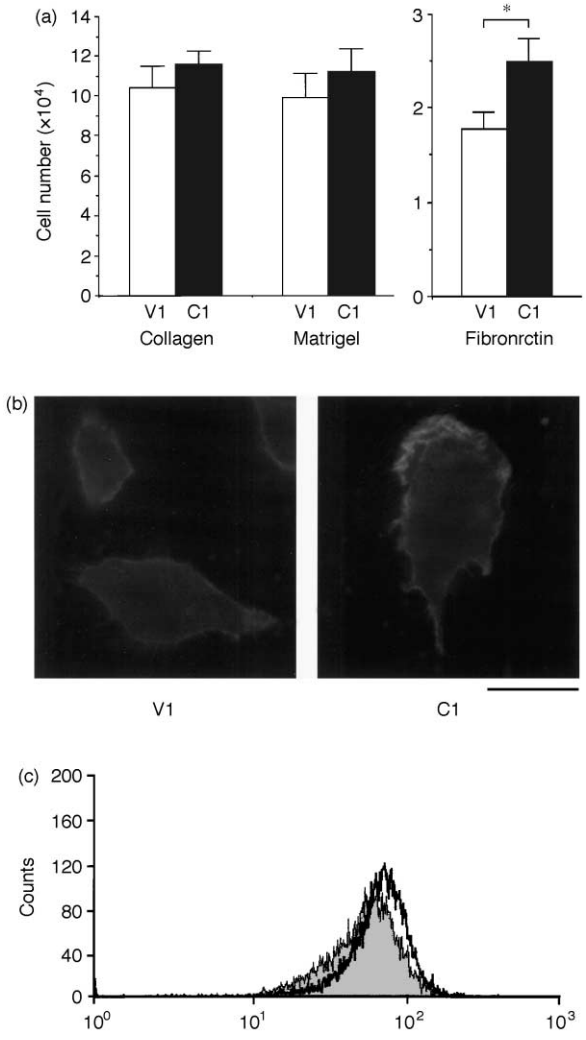

Fig. 5. Enhanced cell adhesion by CNh1: (a) numbers of attached cells 30 min (collagen) or 1 h (Matrigel or fibronectin) after inoculation were counted with a haemocytometer, $*P<0.05$; (b) immunofluorescence of integrin α5β1, scale bar: 50 μm; (c) expression of integrin α5β1 was analysed with fluorescence activated cell sorter (FACS). V1, filled curve; C1, solid line.

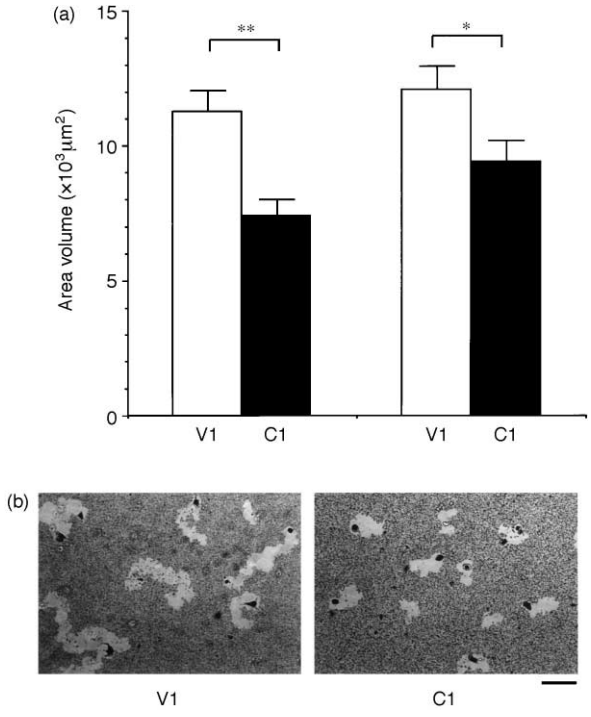

Fig. 6. Inhibitory effect of CNh1 on cell motility: (a) volume areas of phagokinetic track of cells plated on the gold colloid coated coverslides were analysed 18 h later, $*P<0.05$, $**P<0.01$; (b) phagokinetic tracks of V1 and C1. Scale bar: 100 μm.

fibronectin, alteration of the integrin superfamily caused by CNh1 is also suggested. Previously, we reported the fragility of blood vessels and the consequent enhancement of cancer metastasis in *CNh1*-deleted mice [22], in which, suppression of calponin h1 was assumed to destabilise actin filaments leading to weakened cellular adhesion molecular mechanisms. In our present study, stabilisation of actin filaments and enhanced cellular adhesion caused by transfected *CNh1* were demonstrated.

Recent studies have reported that deletion of the CH region of *vav-3* leads to actin relocalisation and induction of stress fibres, lamellipodia and membrane ruffles in NIH3T3 cells [23] and activates rhoA and rac-1 followed by an activation of focus formation [24]. indicating that the CH domain might inhibit the basal activity of vav-3 for the rho family signalling pathway. It has been reported that transient expression of activated ras, rac or rhoA in cells results in the formation of actin stress fibres, and rac-1 [25] and rhoA [26] are necessary for ras malignant transformation. However, chronic stimulation of these pathways, as seen in ras-transformed cells, results in inhibition of stress fibre formation [27]. Actually, dissolution of actin microfilaments was observed in the malignant transformant HT1080 cells [28]. There are studies indicating that restoration of stress fibres weakens malignancy in transformed cells. Namely, dominant-negative rac protein in HT1080 cells caused a partial restoration of stress fibres and a flatter shape [29]. Dominant-negative rhoA inhibited focus formation induced by H-ras in NIH 3T3 cells, and stress fibre formation that was inhibited in H-ras lines was restored by the coexpression of dominant-negative rhoA [26]. In our study, similar results to dominant-negative rac or rho in these experiments were obtained by CNh1. Our results suggest that CNh1 containing the CH domain may have inhibited the rho/rac signalling pathway, restored organised F-actin associated with flat reversion and suppressed tumorigenicity in HT1080 cells which have constitutively activated rhoA and rac [29]. We concluded that CNh1 suppressed the growth of human fibrosarcoma HT1080 cells by stabilizing F-actin and enhancing adhesion related to integrin $\alpha 5\beta 1$, and probably influencing rho/rac signaling pathway.

Acknowledgements

This work was supported by grants from Scientific Research from the Ministry of Education, Science and Culture of Japan (09254101, 10877038). We thank Mr K. Kametani, Mrs M. Watanabe, Miss T. Nishizawa and Miss K. Suzuki for their assistance in the staining preparations. We are also grateful to Dr K. Uemura for the use of the FACS.

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