Adenovirus-Mediated Calponin h1 Gene Therapy Directed against Peritoneal Dissemination of Ovarian Cancer: Bifunctional Therapeutic Effects on Peritoneal Cell Layer and Cancer Cells

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Abstract Purpose: Calponin h1 (CNh1), one of the family of actin-binding proteins, stabilizes the filaments of actin and modulates various cellular biological phenotypes. Recent studies revealed the close correlation between the invasive tumor spread and the reduced expression of CNh1 and α-smooth muscle actin in the surrounding stromal cells. The purpose of this study is to evaluate the efficacy of i.p. CNh1 gene therapy against peritoneal dissemination of ovarian cancer.

Experimental Design: We used an adenoviral vector to induce the CNh1 gene into peritoneal cells and ovarian cancer cells as a means of enhancing or inducing the expression of α-smooth muscle actin as well as CNh1. The efficacy of gene transfer was examined by in vitro cell culture and in vivo animal experiments.

Results: The formation of longer and thicker actin fibers was observed in each transfected cell line, and the localization of these fibers coincided with that of externally transduced CNh1. With respect to changes in cell behavior, the CNh1-transfected peritoneal cells acquired an ability to resist ovarian cancer-induced shrinkage in cell shape; thus, cancer cell invasion through the monolayer of peritoneal cells was inhibited. In addition, CNh1-transfected ovarian cancer cells showed suppressed anchorage-independent growth and invasiveness, the latter of which accompanied impaired cell motility. The concomitant CNh1 transfection into both peritoneal cells and ovarian cancer cells produced an additive inhibitory effect with respect to cancer cell invasion through the peritoneal cell monolayer. By in vivo experiments designed to treat nude mice that had been i.p. inoculated with ovarian cancer cells, we found that the i.p. injected CNh1 adenovirus successfully blocked cancer-induced morphologic changes in peritoneal cell surface and significantly prolonged the survival time of tumor-bearing mice. Moreover, CNh1 adenovirus could successfully enhance the therapeutic effect of an anticancer drug without increase in side effects.

Conclusions: Thus, CNh1 gene therapy against peritoneal dissemination of ovarian cancer is bifunctionally effective (i.e., through inhibitory effects on the infected peritoneal cell layers that suppress cancer invasion and through direct antitumor effects against invasion and growth properties of cancer cells).

Peritoneal dissemination is a main obstacle to improve the prognosis of ovarian cancer patients. The process to establish peritoneal implants is as follows: cancer cells are released from the primary ovarian tumor site into the abdominal cavity, attach to and invade through the peritoneal mesothelial layer, and proliferate as “implanted” tumors. For these processes, dynamic cytoskeletal remodeling is essential not only for the tumor cells but also for the peritoneal cells. A recent study analyzing several adenocarcinomas of various organs has shown that nine genes in metastatic sites were universally down-regulated compared with their primary tumor counterparts (1). Surprisingly, four of these nine genes are associated with the actin cytoskeleton [i.e., α-actin, myosin light and heavy chain kinases, and calponin h1 (CNH1)]. As such, this seems to be a reflection of the importance of actin cytoskeletal disorganization in the metastatic process.

CNH1 is a 34-kDa actin-binding protein that was originally isolated from chicken gizzard (2). CNH1 is mainly expressed in...
smooth muscle cells in contrast to other two isoforms of calponin h2 and acidic calponin, which are mainly expressed in nonmuscle cells and the brain, respectively (3–5). CNh1 has an ability to (a) bind to the thin filament of actin, tropomyosin, and calmodulin (2, 6, 7); (b) inhibit the actin-activated myosin Mg-ATPase (8); (c) inhibit Ca2+-dependent mobility of actin on immobilized myosin (9); and (d) induce conformational changes in actin filament (F-actin; ref. 10). Therefore, CNh1 is thought to play an essential role in organizing stable actin stress fibers. We have confirmed previously that (a) the expression of α-smooth muscle actin (α-SMA) decreased in blood vessels located at the proximity of ovarian cancer nests (11) and in fibroblasts and peritoneal mesothelia cultured in the presence of ovarian cancer cell–derived factors5 and (b) the decrease in CNh1 and α-SMA expression was seemingly attributed to the secreted factors, including platelet-derived growth factor derived from cancer cells (12, 13). CNh1 knockout mice showed the enhanced peritoneal dissemination and lung metastasis by malignant melanoma cells through their highly fragile peritoneum and vascular wall in contrast to the wild-type mice. However, CNh1 gene transfection into the peritoneal cells of knockout mice could successfully inhibit cancer cell invasion into peritoneal cell layer (14, 15). The efficacy of CNh1 transfection into cancer cells themselves was also reported by our colleagues; both cell growth and tumorigenicity were significantly inhibited in CNh1-transfected leiomyosarcoma cells (16) and fibrosarcoma cells (17).

Based on these observations, we hypothesized that CNh1 has bifunctional effects (i.e., an enhancement of peritoneal defense ability on the one hand and a direct inhibitory effect against ovarian cancer cells on the other). The purpose of this study was to examine the effects of CNh1 gene transduction into both cancer cells and peritoneal cells with respect to inhibiting peritoneal dissemination of ovarian cancers.

**Materials and Methods**

**Cell lines and animals.** Four i.p. transplantable (SHIN-3, MCAS/as, OVAS-21/om, and SKOV3i.p.1) and a poorly transplantable (SKOV3) human ovarian cancer cell lines were used in this study. SHIN-3, a serious adenocarcinoma cell line, was purchased from Scienstaff Co. Ltd. (Nara, Japan). MCAS/as and OVAS-21/om were established from ascites and omentum of nude mice i.p. inoculated by their parental cell lines of MCAS (mucinous adenocarcinoma) and OVAS-21 (clear cell adenocarcinoma), respectively. Both cell lines were generous gifts from Drs. S. Minami and Y. Yoshikawa (Department of Obstetrics and Gynecology, University of Tsukuba, Japan) and M. Noguchi (Department of Molecular Pathology, University of Tsukuba). SKOV3i.p.1 provided by Dr. I.J. Fidler (Department of Cancer Biology, M.D. Anderson Cancer Center, Houston, TX), was established as an i.p. transplantable subline from a parental adenocarcinoma cell line of SKOV3 (18). A Chinese hamster peritoneal cell line, CCL14, was purchased from American Type Culture Collection (Manasas, VA). FK is a primary culture of human peritoneal cells, which were obtained from the surgical specimen of omentum under the patient’s consent in Department of Obstetrics and Gynecology, Kyushu University Hospital. Each cell line was cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, 100 units/ml penicillin G, and 2.5 μg/ml amphotericin B at 37°C in 5% CO2 in air.

Female athymic BALB/c nu/nu mice between 6 and 8 weeks old (Charles River Japan, Atsugi, Japan) were used for in vivo experiments. The mice were maintained in a laminar-flow cabinet under specific pathogen-free conditions while receiving standard feed and water *ad libitum*. Our experiments were reviewed by the Committee of Ethics in Animal Experiments in the Graduate School of Medical Sciences, Kyushu University, and the Law (no. 105) and the Notification (no. 6) of the Government.

**Construction of recombinant adenovirus and adenosine gene transfection into cultured cells.** Recombinant adenovirus was inserted with the CNh1-green fluorescent protein (GFP) fusion gene (AdCNh1) or with the GFP gene only (AdGFP, control vector) as described previously (15). Briefly, CNh1-GFP fusion gene was produced by inserting human CNh1 gene into pEGFP-C2 (Clontech, Palo Alto, CA), and then subcloned under the transcriptional control of CAG promoter/enhancer in cosmid vector pAxCAwt (adenovirus expression kit, TaKaRa, Japan; Fig. 1). The recombinant was continued in 293 cells by four times infection and high titer virus was obtained. Adenoviral infection into the cells was carried out by incubating subconfluent cells with adenoviral vectors (2 × 109 plaque-forming units/15-mm dish) for 2 hours in RPMI 1640 at 37°C. Cells were then washed twice with PBS and the medium was changed into RPMI 1640 containing 10% fetal bovine serum. After 24 hours of incubation, cells were used for immunofluorescent cell staining and colony-forming assay. For Western blot analysis, in vivo invasion assay, and cell motility assay, cells were used after 4 days of additional incubation.

**Plasmid gene transfection into cultured cells.** A human CNh1 cDNA was subcloned into a pCMV-neo-Bam vector as described previously (17). Either CNh1 cDNA construct or control vector (20 μg) was transfected into SKOV3 cell line by Lipofectin method. Transfected cells were maintained at 37°C in a complete RPMI 1640. After 14 days, the G418-resistant clones were harvested and established.

**Western blot analysis.** Subconfluently growing cells were lysed in a lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 0.25 mol/L NaCl, 0.5% NP40, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin]. After centrifugation at 13,000 × g for 10 minutes to remove debris, equal protein amount of cell lysates was separated on

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5 Unpublished observations.

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Fig. 1. Construction of CNh1-GFP recombinant adenovirus vector (AdCNh1) and its control GFP vector (AdGFP).

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motility assay.

Serum was placed in the upper compartment and incubated for 24 hours. After washing, the wells were incubated with their respective secondary antibodies (Amersham, Piscataway, NJ) and analyzed with enhanced chemiluminescence system (Amersham). All primary antibodies are informed to detect CNh1 protein stably even in murine tissues.

Immunofluorescent cell staining for F-actin. Cells were washed with PBS and fixed in 3.7% formaldehyde solution in PBS for 10 minutes at room temperature. After permeabilization with 1% Triton X-100, the rhodamine-phalloidin (Molecular Probes, OR) diluted with 0.1% bovine serumalbumin in PBS was added and incubated at room temperature for 20 minutes. F-actin and GFP were observed with confocal laser scanning microscope using specific filters for rhodamine and FITC, respectively.

Soft-agar colony-forming assay. After 24 hours of infection with AdCNh1 or AdGFP, 10^5 cells were seeded into the complete medium, including 0.3% agar, and placed over a hardened 0.5% agar base layer in 60-mm dishes. Visible colonies were counted in triplicate after 14 days of incubation to evaluate the anchorage-independent cell growth.

Tumorigenicity in nude mice. Single-cell suspension containing 10^7 SKOV3 cells with or without plasmid transfection was i.m. injected into the thigh of nude mice. The mice were observed weekly, and tumor growth was evaluated by measuring the thickness of the inoculated thigh.

Cell motility assay. Cell motility was determined using Transwell chambers inserted with 8-μm pore size membrane (Costar, Corning, NY) according to the method described previously (19). Each lower compartment of Transwell chambers was filled with a conditioned medium as a source of chemoattractant, which was a supernatant of confluently cultured NIH3T3 cells in DMEM supplemented with 10% fetal bovine serum for 24 hours. Single-cell suspension containing 5 × 10^5 cells infected by AdCNh1 or AdGFP in 100 μL RPMI 1640 with 0.1% bovine serum albumin was placed in its upper compartment. The cells were incubated for 12 hours at 37°C, fixed with methanol, and stained with H&E. Cells on the upper surface of the filter were removed with a cotton swab, and cells that migrated to the lower surface were counted by six fields of a light microscope at ×200 magnification.

In vitro invasion assay. In vitro invasion assay was done using Transwell chambers with 8-μm pore membrane coated with 20 μg Matrigel (Becton Dickinson Collaborative Research, Bedford, MA) as described previously (20). Conditioned medium prepared by NIH3T3 cells was placed in the lower compartment. Single-cell suspension containing 10^5 cells in 100 μL RPMI 1640 containing 10% fetal bovine serum was placed in the upper compartment and incubated for 24 hours. The subsequent procedures were the same as those of the cell motility assay.

Assay for invasion through the peritoneal cell monolayer. Single-cell suspensions containing 10^5 cancer cells infected with AdCNh1 or AdGFP were plated on the monolayer of CCL14 peritoneal cells infected with AdGFP or AdCNh1, and cultured for additional 24 hours. The number of colonies derived from a penetrated cancer cell was counted by six fields of a phase-contrast microscope at ×400 magnification.

Therapeutic experiments against i.p. inoculated ovarian cancer cells. One day after the inoculation of 10^5 cells of OVAS-21/om, the i.p. injection of AdCNh1 or AdGFP (2 × 10^5 plaque-forming units/mL) was started and repeated by every 3 days until 19 days after the inoculation. In some groups, 100 mg/kg paclitaxel was given once i.p. at 3 days after inoculation. Survival and body weight of each mouse were monitored until 5 months after the inoculation, and an autopsy was done. Paclitaxel (Taxol) was kindly provided by Bristol-Myers Squibb (Tokyo, Japan).

Scanning electron microscope. Peritoneum was resected from abdominal walls of mice at 7 days after the i.p. inoculation of SKOV3i.p.1 cancer cells followed by the adenoviral injection. Peritoneal tissues were fixed in 1% glutaraldehyde for 12 hours followed by 1-hour fixation in 1% osmium tetroxide. After dehydration in ethanol, the specimens were rinsed in isoamyl acetate and dried by a critical point drying method. The dried specimens were mounted on copper plates and coated with osmium in an osmium plasma coater (Nippon Laser and Electronics Lab, Japan). The specimens were examined with a JSM-6000F scanning electron microscope (Jeol, Pleasonton, CA) at 1.5 kV.

Statistical analysis. Mann-Whitney U test was used to assess the statistical significance of differences in soft-agar colony-forming assay, cell motility assay, in vitro invasion assay, and assay for cell invasion through the peritoneal cell layer. In vivo tumor growth assay was assessed using Fisher’s exact test. Survival curves were analyzed with Kaplan-Meier method and the difference between curves was assessed according to the log-rank test. All Ps are two-sided and considered statistically significant at <0.05.

Results

Efficacy of CNh1 gene transfection into the peritoneal cells

Expression of CNh1 and three kinds of actin proteins in the transfected peritoneal cells. Although endogenous expression of 34-kDa CNh1 protein was found in CCL14 cells infected with AdGFP or AdCNh1 (Fig. 2A) as well as noninfected CCL14 cells, exogenous CNh1 protein fused with GFP appeared additionally as a 61-kDa band in AdCNh1-infected cells (Fig. 2A). Because CNh1 is known to stabilize α-SMA, the expressions of α, β, and γ actin proteins were analyzed after CNh1 adenoviral infection. No change of expression of any actin was observed between AdGFP-infected cells and their parental noninfected cells. Although expression of β and γ actins were not changed even by AdCNh1 infection, α-SMA expression was clearly enhanced by CNh1 transfection (Fig. 2A and B).

Effects of CNh1 gene transfection into the peritoneal cells against the destabilization of their actin filaments in the presence of conditioned medium of ovarian cancer cells. Original peritoneal cells show well-developed actin stress fibers within their cytoplasm and well-stretched cell shape in normal culture medium. In the presence of ovarian cancer conditioned medium, we observed decreased actin stress fibers resulting in shrunken cell shape, intercellular dissociation, and cell detachment from culture plate, and this phenomenon was specifically accompanied by decreasing expressions of both CNh1 and α-SMA. Therefore, we examined whether CNh1 transfection into the peritoneal cells could stabilize actin filaments against the ovarian cancer-derived factors. As shown in Fig. 3A, AdGFP-infected cells in the presence of ovarian cancer conditioned medium showed smaller and shrunken cell shape with thin and short actin filaments at the cytosol and small spike-like filaments observed at the cellular margins, which is similar in appearance to the noninfected FK cells in the presence of ovarian cancer conditioned medium. Therefore, AdGFP infection could not recover the FK cells from ovarian cancer-derived morphologic changes. On the other hand, AdCNh1-infected cells maintained large and extended cell shape with thick and long filaments traversing a cell, just like untreated parental peritoneal cells, even in the presence of ovarian cancer conditioned medium (Fig. 3B).

Localization of actin stress fiber (Fig. 3B) was identical with the expression site of CNh1-GFP gene products (Fig. 3C), which was...
confirmed by the yellow fibers consisting of merged observation for both proteins (Fig. 3D). Therefore, the actin stress fiber development in AdCNh1-infected cells seemed to be due to the exogenously transfected CNh1 gene.

**Efficacy of CNh1 gene transfection into ovarian cancer cells**

Expression of CNh1 and three kinds of actin proteins in the transfected ovarian cancer cells. Similar with the case of peritoneal cells, internal 34-kDa CNh1 protein was detected in SKOV3 ovarian cancer cells infected with AdGFP or AdCNh1 (Fig. 2C) as well as noninfected SKOV3 cells. After AdCNh1 infection, the band representing 61-kDa CNh1-GFP fusion protein appeared along with the induced α-SMA band (Fig. 2C). Expression of both β and γ actins was unchanged by CNh1 transfection in both peritoneal cells (B) and ovarian cancer cells (D and E). Expression of rasGAP was confirmed as an internal control.

**Fig. 2.** Western blot analysis for the changes in expressions of CNh1 and actins after CNh1 transfection. Expressions of CNh1 and three kinds (α-SMA, β, and γ) of actins were compared between AdCNh1 and AdGFP infections to CCL14 peritoneal cells (A and B) and the three ovarian cancer cell lines SKOV3 (C-E), SHIN-3 (E), and MCAS/as (E). In addition to endogenous CNh1 expression (34 kDa), external CNh1 fused to GFP gene (61 kDa) was expressed in AdCNh1-infected peritoneal cells (A) and cancer cells (C and E). Increased α-SMA expression by CNh1 transfection was confirmed in the peritoneal cells (A). Although all AdGFP-infected cancer cell lines showed an undetectable level of α-SMA expression just like their parental lines, α-SMA expression stably appeared after CNh1 transfection in each cell line (C and E). Expressions of neither β nor γ actin was unchanged by CNh1 transfection in both peritoneal cells (B) and ovarian cancer cells (D and E). Expression of rasGAP was confirmed as an internal control.
Changes of actin filaments and cell shapes occurred in CNh1-transfected ovarian cancer cells. Similar to noninfected SKOV3 cell line, AdGFP-infected SKOV3 cells showed weakly and diffusely stained actin filaments (Fig. 3E), the location of which was not identical with that of exogenously transduced GFP (Fig. 3F and G). AdCNh1 infection induced thick and long actin stress fibers and changed the cell shape into a flat and extended phenotype (Fig. 3H). The localization of the developed actin stress fibers was identical with the GFP expression sites, except the nuclear area (Fig. 3I and J), indicating that the exogenously introduced CNh1-GFP gene products produced the stable actin fibers. Similar results were observed in other AdCNh1-infected ovarian cancer cell lines (data not shown).

Decreased growth property in CNh1-transfected ovarian cancer cells. We confirmed that CNh1 transfection changed neither cell growth rate nor colony-forming ability of ovarian cancer cells when growing as a monolayer culture system (data not shown). Therefore, we evaluated anchorage-independent cell growth by soft-agar colony-forming assay and found that not shown). Therefore, we evaluated anchorage-independent cell growth by soft-agar colony-forming assay and found that all ovarian cancer cell lines infected with AdCNh1 formed markedly fewer colonies compared with the case of AdGFP infection (Fig. 4A).

Effects of CNh1 transfection on tumor growth were evaluated by i.m. inoculation of parental SKOV3 cells and their subclones transfected with human CNh1 plasmid (SKOV/CNh1) or mock vector (SKOV/mock). As shown in Fig. 4B, the CNh1-transfected SKOV/CNh1 cells showed significantly retarded growth, whereas the SKOV/mock cells showed almost the same growth rate as parental SKOV cells.

Decreased invasiveness and cell locomotion in CNh1-transfected ovarian cancer cells. We evaluated the effect of CNh1 transfection on the invasiveness by an in vitro assay. Invasion of AdCNh1-infected SKOV-3, SHIN-3, and MCAS/as cells were all significantly decreased by 67.6%, 43.1%, and 27.6%, respectively, in contrast to AdGFP-infected controls (Fig. 5A). Because the change of cell morphology strongly correlates with cell locomotion, we examined the effects of AdCNh1 on cell motility using Matrigel-uncoated Transwell chambers. The cell motility of AdCNh1-infected each cell lines was significantly decreased by 50.5%, 44.3%, and 29.1%, respectively, in contrast to each AdGFP-infected control (Fig. 5B).

Effects of CNh1 gene transfection into both peritoneal cells and ovarian cancer cells

Bifunctional inhibitory effects of CNh1 transfection against the invasion of cancer cells through peritoneal cell layer. Figure 5C shows the effect of CNh1 transfection into cancer cells and/or peritoneal cells on the cancer invasion into peritoneal cell monolayer. When either cancer cells or peritoneal cells were infected by AdCNh1, cancer cell invasion through peritoneal cell monolayer was significantly suppressed. The concomitant AdCNh1 infection into both cells produced additive and marked inhibitory effects on the cancer cell invasion.

Effects of AdCNh1 infection on morphologic changes of peritoneal cell surface induced by ovarian cancer cells. Scanning electron microscope showed that the i.p. inoculation of ovarian cancer cells changed the surface of murine peritoneal cells from a smooth phenotype without spikes (Fig. 6A) into an “edgy” one with numerous small spikes. This microrill-like cell surface change was unavoidable by i.p. infection of AdGFP following cancer cell inoculation (Fig. 6B). However, i.p. injection of AdCNh1 with cancer inoculation could avoid this change,

Fig. 3. F-actin staining in peritoneal cells and ovarian cancer cells and its relative localization with CNh1-GFP fusion gene products. A to D, human peritoneal FK cells infected with AdGFP (A) and AdCNh1 (B–D) were cultured in the presence of the conditioned medium prepared from SKOV3 ovarian cancer cells. To examine F-actin distribution by confocal laser scanning microscope, cells were fixed and stained with Alexa 568-phalloidin. Although shrunken cell shape and poor development of F-actin were observed in AdGFP-infected cells (A) as well as noninfected parental cells, marked development of actin stress fiber was observed in AdCNh1-infected cells with a stretched cell shape (B). The localization of external CNh1-GFP gene products observed through a FITC filter for GFP (C) corresponded to that of actin stress fibers, which was confirmed by the merged observation for F-actin and GFP localization (D). Bar, 5 μm. E to J, F-actin and GFP localization in SKOV3 ovarian cancer cells infected with AdGFP (E–G) or AdCNh1 (H–J). Although poor development of F-actin was observed mainly in perinuclear area of AdGFP-infected cells (E) as well as noninfected cells, the stable actin stress fibers appeared in AdCNh1-infected cells with a stretched cell shape (H). The localization of external GFP gene product (F) did not correspond to F-actin localization, which was confirmed by the merged observation for F-actin and GFP (G). On the contrary, the localization of external CNh1-GFP fusion gene products (I) obviously corresponded to F-actin localization (J). Bar, 5 μm.
resulting in a flat and smooth surface of peritoneal cells (Fig. 6C) as well as a normal surface observed in the absence of cancer cells (Fig. 6A).

In vivo treatment for i.p. inoculated ovarian cancer cells using i.p. AdCNh1 injection with or without an anticancer drug. Because all of the aforementioned results suggested that CNh1 suppressed ovarian cancer activity and enhanced peritoneal defense mechanisms, the potential for bifunctional therapeutic effects of AdCNh1 was investigated by treating nude mice inoculated i.p. with ovarian cancer cells. A preliminary experiment showed at least 3 days of effectiveness of i.p. injected adenovirus by monitoring i.m. transplanted tumor size using SKOV3 cells and their plasmid transfectants. CNh1-transfected SKOV/CNh1 (○) showed retarded tumor growth compared with parental SKOV3 cells (●) or control plasmid-transfected SKOV/mock (▲). Points, mean (n = 6 mice); bars, SD. *, P < 0.05, SKOV/CNh1 versus SKOV/mock in each measurement point.

Fig. 4. Changes of growth property after CNh1 transfection into ovarian cancer cells. A, in vitro cell growth was evaluated by soft-agar colony-forming assay using three ovarian cancer cell lines infected by AdGFP (black columns) or AdCNh1 (white columns). CNh1 transfection significantly reduced anchorage-independent growth of all ovarian cancer cell lines. Columns, mean of triplicate determinations; bars, SD. *, P < 0.05. B, in vivo tumor growth was evaluated by monitoring i.m. transplanted tumor size using SKOV3 cells and their plasmid transfectants. CNh1-transfected SKOV/CNh1 (○) showed retarded tumor growth compared with parental SKOV3 cells (●) or control plasmid-transfected SKOV/mock (▲). Points, mean (n = 6 mice); bars, SD. *, P < 0.05, SKOV/CNh1 versus SKOV/mock in each measurement point.

resulted in 22 days of the median survival in untreated mice. As shown in the survival curves (Fig. 6D), the median survival of AdCNh1- or AdGFP-treated mice was 91 or 49 days, respectively, and percent increase in life span (median survival of
treated mice — median survival of untreated mice / median survival of untreated mice × 100) of AdCNh1-treated mice was 314%. We could obtain the significantly prolonged survival by AdCNh1 injection into the murine abdominal cavity (P = 0.002, AdCNh1 versus AdGFP). No body weight loss was observed in either adenoviral administration.

To examine the effects of AdCNh1 combined with the administration of paclitaxel, a highly effective anticancer drug of the present mainstream for ovarian cancer treatment, 100 mg/kg paclitaxel was i.p. injected 3 days after cancer cell inoculation. As shown in Fig. 6E, although AdGFP failed to prolong the survival of paclitaxel-treated mice, additionally prolonged survival was obtained in the combined therapy by AdCNh1 and paclitaxel (P = 0.04, AdCNh1 + paclitaxel versus paclitaxel alone). No aggravation was observed in toxicity by either virudal administration to paclitaxel-treated mice, which transiently showed 3% body weight loss on an average.

Autopsy was done as early as possible after each mouse died from peritoneal dissemination. All of dead mice showed the distended abdomen by a massive bloody ascites and the disseminated numerous implants on the surface of i.p. organs and peritoneum (Fig. 6F). In contrast, one of six mice treated by AdCNh1 and three of five mice treated by AdCNh1 plus paclitaxel survived even 147 days after the cancer cell inoculation and showed no i.p. implants confirmed by slaughter (Fig. 6G).

Discussion

When intracellular globular actin is polymerized to form F-actin and the filaments of F-actin are bundled, their configurations are (a) big stress fiber traversing a cell, (b) loose and thin gelatinal filaments, and (c) filaments thickly lining up in microspike and lobopodium of cell periphery. It is thought that short actin filaments forming microspikes increase inversely with a decrease of long actin stress fibers, accompanying the reduction of CNh1 expression (21). We also observed that ovarian cancer-derived inhibition in CNh1 expression resulted in both decrease of actin stress fibers and formation of microspikes in peritoneal cells, both of which could be avoided by exogenous CNh1 transfection (Figs. 3A-D and 6A-C). CNh1-transfected peritoneal cells did not change their diamond-shaped configuration in spite of the presence of ovarian cancer conditioned medium (Fig. 3B). These protective effects of CNh1 against ovarian cancer-derived factors enhanced the "defense" ability (i.e., resistance of peritoneal cells), which was shown by reduced cancer cell invasion through mesothelial cell layer (Fig. 5C) and by our reported data that the fragility against peritoneal dissemination of cancer observed in the CNh1-deficient knockout mice was rescued by the exogenous introduction of the CNh1 gene into mesothelial cells (15). CNh1 brought about therapeutic effects not only for host cells but also for ovarian cancer cells as well. Forced CNh1 expression in ovarian cancer cells developed actin stress fibers

Fig. 6. Effects of AdCNh1 injection into mice inoculated i.p. with ovarian cancer cells. A to C, morphologic changes in the cell surface of peritoneum were observed by scanning electron microscope after the i.p. inoculation of SKOV3.ip.1 cancer cells. The surface of peritoneal cells changed from the ordinary flat and smooth cell surface (A) into a "fluffy" phenotype accompanied by many microspikes, and this change was not avoided by AdGFP (B). However, AdCNh1 could maintain almost normal cell surface against the ovarian cancer-derived influence (C). Bar, 10 μm.D and E, therapeutic effects of AdCNh1 i.p. injection combined with or without paclitaxel were evaluated by the survival of treated mice. AdCNh1 or AdGFP was repeatedly injected i.p. after the i.p. inoculation of OVAs-21/on cancer cells (D). Significantly prolonged survival was observed in AdCNh1-treated mice (○) compared with AdGFP-treated mice (●, P = 0.002). Paclitaxel was i.p. injected 3 days after tumor inoculation to examine the effects of AdCNh1 combined with anticancer drug (E). Additionally prolonged survival was obtained in the combined therapy by AdCNh1 and paclitaxel (○) compared with the therapy by paclitaxel alone (△, P = 0.04). F and G, i.p. appearances after the adenosalve treatment were shown by representative mice. An AdGFP-treated mouse, died 49 days after the inoculation, showed peritonitis carcinomatosis with massive ascites accompanied by numerous disseminated implants on the surface of i.p. organs and peritoneum (F). In contrast, an AdCNh1-treated mouse, which survived even 147 days after the inoculation, showed neither ascites nor i.p. implants (G). As for the side effects of AdCNh1 treatment, neither body weight loss nor intra-abdominal adhesion was observed. © 2006 American Association for Cancer Research.
accompanying α-SMA induction (Figs. 2C-E and 3H-I), which resulted in retardation of growth and invasiveness (Figs. 4 and 5A). As for growth-inhibitory effects mediated by CNh1, in vitro anchorage-independent cell growth (Fig. 4A) and in vivo tumor growth (Fig. 4B) were both suppressed, although in vitro growth was not inhibited when cells were cultured as monolayers. As for CNh1-inhibitory effect on invasiveness, cell motility was significantly retarded (Fig. 5B). These results would seem reasonable, considering that CNh1-induced stabilization of actin stress fibers inhibits both three-dimensional cell growth and cell locomotion, for which dynamic state of actin filaments is thought to be essential. In vivo efficacy of CNh1 specific to ovarian cancer cells was confirmed by CNh1-plasmid transfectants of SKOV3ip.1, which survived much longer than the mock transfectant (data not shown).

Cotransfection of CNh1 into both peritoneal cells and ovarian cancer cells resulted in additive inhibition of cancer cell invasion through peritoneal cell layer compared with the inhibitory effect of CNh1 transfection into either cell population alone (Fig. 5C). In vivo therapeutic effects of CNh1 were confirmed by the i.p. administration of CNh1 adenoviral vector into nude mice bearing i.p. inoculated ovarian cancer cells (Fig. 6D). We observed that ovarian cancer cells seemed to easily invade through the peritoneal layer, which consisted of retracted and dissociated mesothelial cells with poor actin stress fiber development. This may be due to the down-regulation of CNh1 and α-SMA induced by ovarian cancer cell–derived secretory factor(s), including platelet-derived growth factor. Effects of AdCNh1 used to infect both mesothelial cells and ovarian cancer cells are summarized as follows: (a) peritoneal cells with developed actin stress fiber had a greater ability to act as a barrier against cancer cells, avoiding ovarian cancer-induced suppression of CNh1 and α-SMA expression, and (b) ovarian cancer cells impaired their growth and invasion properties by induced actin stress fibers.

As such, adenovirus-mediated CNh1 gene therapy against peritoneal dissemination of ovarian cancer may be considered as a potentially novel therapeutic intervention, whereby one and the same gene has two distinctive major effects: one is to control the cancer cells and the other is to bolster a host defense (anti-invasive) mechanism.

Considering clinical application of this therapy, preventive administration of AdCNh1 just after the optimal surgery would be desirable for effective inhibition of peritoneal dissemination. At least, it seems to be difficult for this therapy to eradicate the established and large peritoneal implants. Because AdCNh1 could successfully enhance the therapeutic effect of paclitaxel, without increase in side effects (Fig. 6E and G), CNh1 gene therapy may be safely repeatable and hence potentially to improve patient prognosis especially when used in combination with conventional postoperative chemotherapy. Gene therapy using i.v. given adenovirus can be compromised in situations where the injected adenovirus is easily and rapidly washed out from the tumor site by bloodstream. However, adenoviral gene therapy using i.p. injection has an advantage in that the adenovirus can stay at the site of target cells (cancer cells and peritoneal cells), owing to the closed space of the “abdominal cavity.” With respect to the problem of decreasing efficacy in repeating adenovirus gene therapy by appearance of human antibody against adenovirus, we would like to expect developing more suitable i.p. vector system or finding a nontoxic small molecule that can effectively enhance CNh1 expression after i.p. administration.

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References

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