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Reduction of Caveolin-1 Expression in Tumorigenic Human Cell Hybrids[1]

Toshikazu Suzuki,*,[2] Yasuyuki Suzuki,* Kentaro Hanada,* Akihiro Hashimoto,† J. Leslie Redpath,‡ Eric J. Stanbridge,§ Masahiro Nishijima,* and Takayuki Kitagawa*,[3]

*Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases, Tokyo 162-8640; †Cancer Research Laboratory, Taiho Pharmaceutical Co., Ltd., Saitama 357; ‡Department of Radiation Oncology and §Department of Microbiology and Molecular Genetics, University of California, Irvine, CA 92717, USA

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Studies on human cell hybrids of a cervical carcinoma cell line, HeLa, and normal fibroblasts have indicated that the tumorigenicity of these cells is under the control of a putative tumor suppressor on chromosome 11, although the nature of this suppressor remains unknown. We examined the expression of caveolin-1, a protein component of caveolae of the plasma membrane in these cell hybrids. The non-tumorigenic cell hybrid, CGL1, and normal fibroblast WI38 cells expressed 21-24 kDa caveolin-1, whereas in tumorigenic hybrid CGL4 as well as in the parental HeLa cells, the level of caveolin-1 was markedly reduced. Caveolin-1 expression was also reduced in γ-ray-induced tumorigenic clones (GIMs) isolated from CGL1 cells, whereas non-tumorigenic irradiated cells expressed the same level of caveolin-1 as CGL1 cells. In accordance with these changes, the cellular level of caveolin-1 mRNA was reduced in the tumorigenic CGL4 cells and GIMs without any detectable changes in the caveolin-1 gene. However, the *in vivo* tumor growth of CGL4 cells was not altered when caveolin-1 was stably overexpressed through the transfection of a human caveolin-1 cDNA. These results suggest that reduction of caveolin-1 expression is necessary but not sufficient for emergence of the tumorigenic phenotypes of HeLa cell hybrids. Possible roles of the putative tumor suppressor in the control of gene expression are also discussed.

Key words: caveolin, HeLa cell, human cell hybrids, tumorigenesis, tumor suppressor.

Human cancers develop as a consequence of genetic changes that perturb normal cellular growth control both positively and negatively (*1-3*). Studies utilizing somatic cell fusion and microcell transfer have indicated that the tumorigenesis of the cervical carcinoma cell line, HeLa, is controlled by a putative tumor suppressor gene on chromosome 11 (*4-6*). Although the function and nature of this gene remain unknown, the paired combinations of non-tumorigenic and tumorigenic HeLa-derived cell hybrids have provided a useful approach by which to examine cellular changes genetically linked to the function of this putative tumor suppressor.

One of the most striking cellular changes associated with *in vivo* tumorigenesis of human cell hybrids is ectopic expression of intestinal alkaline phosphatase, IAP (*7, 8*). The γ-ray irradiation of a non-tumorigenic cell hybrid (CGL1) induced IAP-expressing mutants, GIMs, which are tumorigenic when injected into nude mice, similar to the IAP-positive tumorigenic cell hybrid, CGL4 (*9, 10*). More recently, we also found that *N*-linked glycosylation of a facilitated glucose transporter, GLUT1, in the plasma membrane was modulated in tumorigenic HeLa cell hybrids (*11, 12*). However, the roles of these membrane changes in human malignancy and their connection with the putative tumor suppressor function are largely unknown.

Caveolin-1 is a 21-24 kDa protein, which is a component of caveolae, small (50-80 nm) invaginations of the plasma membrane, that may be involved in some aspects of vesicular transport, cellular signaling and cell surface organization (*13, 14*). A substantial part of the total caveolin is present in nonionic detergent-insoluble complexes with many receptors and associated G-proteins as well as glycosylphosphatidylinositol (GPI)-anchored proteins, including alkaline phosphatases (*13-16*). Caveolin-1 was originally discovered as a major substrate for v-src kinase in transformed chick embryo fibroblasts (*17, 18*). Recently, it was reported that caveolin-1 expression is greatly reduced in several oncogenically transformed mouse fibroblasts, suggesting a tumor-suppression function for this protein (*19*).

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[2] Present address: Kihara Institute for Biological Research, Yokohama City University, Yokohama 244

[3] To whom correspondence should be addressed. Phone: +81-3-5285-1111 (Ext. 2128), Fax: +81-3-5285-1157, E-mail: kita@nih.go.jp

Abbreviations: CGL1, non-tumorigenic HeLa cell hybrid; CGL4, tumorigenic HeLa cell hybrid; GIM, γ-ray-induced tumorigenic mutants; CON, γ-irradiated nontumorigenic control; IAP, intestinal alkaline phosphatase; PCR, polymerase chain reaction; ECL, an enhanced chemiluminescence kit; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum.

These observations prompted us to examine changes and any functional role of caveolin-1 expression in HeLa cell hybrids, whose tumorigenicity could be controlled by a putative tumor suppressor gene. In the present paper, we demonstrate that reduction of caveolin-1 expression at both the protein and mRNA levels is well correlated with the tumorigenicity of HeLa cell hybrids. Further studies on overexpression of the caveolin-1 protein in a tumorigenic cell hybrid, however, suggested that reduction of caveolin-1 expression may be necessary but not sufficient for emergence of the tumorigenic phenotypes.

MATERIALS AND METHODS

Materials—Rabbit polyclonal antibodies against human caveolin-1 and calf IAP were purchased from Transduction Laboratories (Lexington, KY) and UCB-Bioproducts (Poole, UK), respectively. An enhanced chemiluminescence kit (ECL) and [α-^{32}P]dCTP (3,000 Ci/mmol) were obtained from Amersham Japan (Tokyo).

Cell Culture—Human cell hybrids, CGL1 (nontumorigenic) and CGL4 (tumorigenic) cells, which were derived through cell fusion between HeLaD98/AH2 and human diploid normal fibroblasts (*4-7*), γ-ray-induced tumorigenic mutants (GIMs) and γ-irradiated nontumorigenic controls (CONs), both of which were derived from CGL1 cells (*9*), and HeLa-S3 (human cervical carcinoma) cells were cultured in DMEM (Flow Laboratories, Maclean, VA) containing 5% FCS (Flow Lab.), penicillin (100 U/ml), and streptomycin (100 μg/ml), as described previously (*11, 12*). WI38 cells (human diploid normal fibroblasts) were grown in DMEM supplemented with 10% FCS, penicillin and streptomycin.

Cell Lysates and Immunoblot Analysis—Cell lysates were prepared as described previously (*11*) and their protein contents were determined using BCA reagent (Pierce, Rockford, IL) with bovine serum albumin as a standard. Sample proteins (20 μg) diluted with 2×sample buffer (4% SDS, 20% glycerol, 0.01% BPB, 100 mM dithiothreitol, 125 mM Tris-HCl, pH 6.8) were separated by 10% SDS-PAGE, and subsequently immunoblotted with a rabbit antibody against either caveolin-1 (1:1,000) or IAP (1:2,000). The washed filters were incubated with a horseradish peroxidase (HRP)-conjugated anti-rabbit IgG second antibody (Amersham), and then visualized with ECL, as described previously (*11, 12*).

PCR Cloning of Human Caveolin-1 cDNA and Transfection—Complementary DNA was prepared using poly A$^+$ RNA from CGL1 cells, as described elsewhere (*20*). The coding region of human caveolin-1 cDNA (*21*) was amplified by PCR using (AAG TCG ACG CAT GTC TGG GGG CAA ATA CGT) and (AAA AGC TTC TTG GAA CTT GAA ATT GGC ACC) as the 5′- and 3′-primers, respectively. The caveolin-1 expression vector (pSV/cav) was then constructed by ligating the *Sal*I- and *Hin*dIII-digested PCR product (0.6 kb) into pSVsport1neo.f1, which had been constructed by ligating the 2.7 kb *Cla*I-*Xmn*I fragment of pSVsport1 (GIBCO-BRL, Gaithersburg, MD) into a 2.1 kb fragment containing the f1 IG region and the Herpes simplex thymidine kinase promoter directing the neomycin-resistance gene. The nucleotide sequence of the caveolin-1 cDNA was determined by the dideoxy method using USB Sequenase® Version 2.0 (Amersham Life Science, Tokyo). CGL4 cells were transfected with the pSV/cav plasmid using the calcium phosphate method (*22*). Transfectants were selected in the presence of G418 (800 μg/ml) and cultured further with 600 μg/ml of G418.

RNA Isolation and Northern Blot Analysis—Total RNA was isolated from the human cell hybrids using ISOGEN (Wako Chemicals, Tokyo). It was then denatured, and run on a 1.5% agarose gel containing 2.2 M formaldehyde (20 μg of RNA/lane), before being transferred to a nylon membrane (Hybond-N; Amersham). The immobilized membrane was hybridized at 42°C for 16-20 h with [α-^{32}P]dCTP-labeled human caveolin-1 cDNA. The hybridized blot was exposed to X-ray film after extensive washes at 42°C, as described previously (*23*). After removal of the caveolin-1 cDNA, the membrane was re-hybridized with a human β-actin DNA probe (Wako Chemicals, Tokyo).

DNA Isolation and Southern Blot Analysis—Genomic DNA was extracted from the human cell hybrids using a DNA isolation kit (Bio 101, La Jolla, CA), and then completely digested with *Bam*H1, *Hin*dIII, or *Eco*RI at 37°C overnight. The DNA fragments were electrophoresed in a 0.8% agarose gel, and then blotted onto a nylon membrane. Southern blot analysis was performed in 50% formamide at 42°C for 20 h, using [α-^{32}P]dCTP-labeled caveolin-1 cDNA. After washing, the hybridized blot was exposed to X-ray film at −80°C with an intensifying screen.

Tumorigenicity Testing In Vivo—The cell lines of interest were expanded and cell suspensions were prepared so that the final concentration of cells was 1×10^7/ml. Then 5×10^6 cells/0.2 ml were s.c. injected into the right and left flanks of athymic nu/nu nude mice. Eight sites (two/mouse) were injected per cell line, and the tumor formation and volumes at the injected sites were measured, as described previously (*5, 6, 9*).

RESULTS

Caveolin-1 Expression in Human Cell Hybrids—Caveolin-1 expression was determined by immunoblotting of cell lysates prepared from a tumorigenic HeLa-derived cell hybrid, CGL4, and a non-tumorigenic hybrid, CGL1. Both CGL1 and CGL4 are subclonal derivatives of the parental non-tumorigenic hybrid cells, ESH5, as shown in Fig. 1A (*4, 5*). A substantial amount of 21-kDa caveolin-1 was present in CGL1 cells and the normal diploid fibroblasts, WI38, whereas caveolin-1 expression in CGL4 cells and HeLa-S3 cells was greatly reduced (Fig. 1B). In contrast, the cell lysates prepared from CGL4 and HeLa-S3 cells were found to express IAP, whereas it was undetectable in WI38 and CGL1 cells (Fig. 1C), as previously described (*7-9, 12*). Thus, the reduced expression of caveolin-1 and the upregulation of IAP seem to be closely associated with the tumorigenic state of these cell hybrids.

Reduced Caveolin-1 Expression in γ-Ray-Induced Tumorigenic Mutants—To examine this possibility further, caveolin-1 expression was determined in γ-ray-induced tumorigenic mutants (GIMs) isolated from CGL1 cells. These GIMs were independently selected as γ-ray-induced IAP-expressing cells (*9, 10*). Control cell lines (CON2 and CON5), which were non-tumorigenic and IAP-negative γ-irradiated clones, were shown to express 21 kDa caveolin-1 (Fig. 2). In contrast, the caveolin-1 expression in three

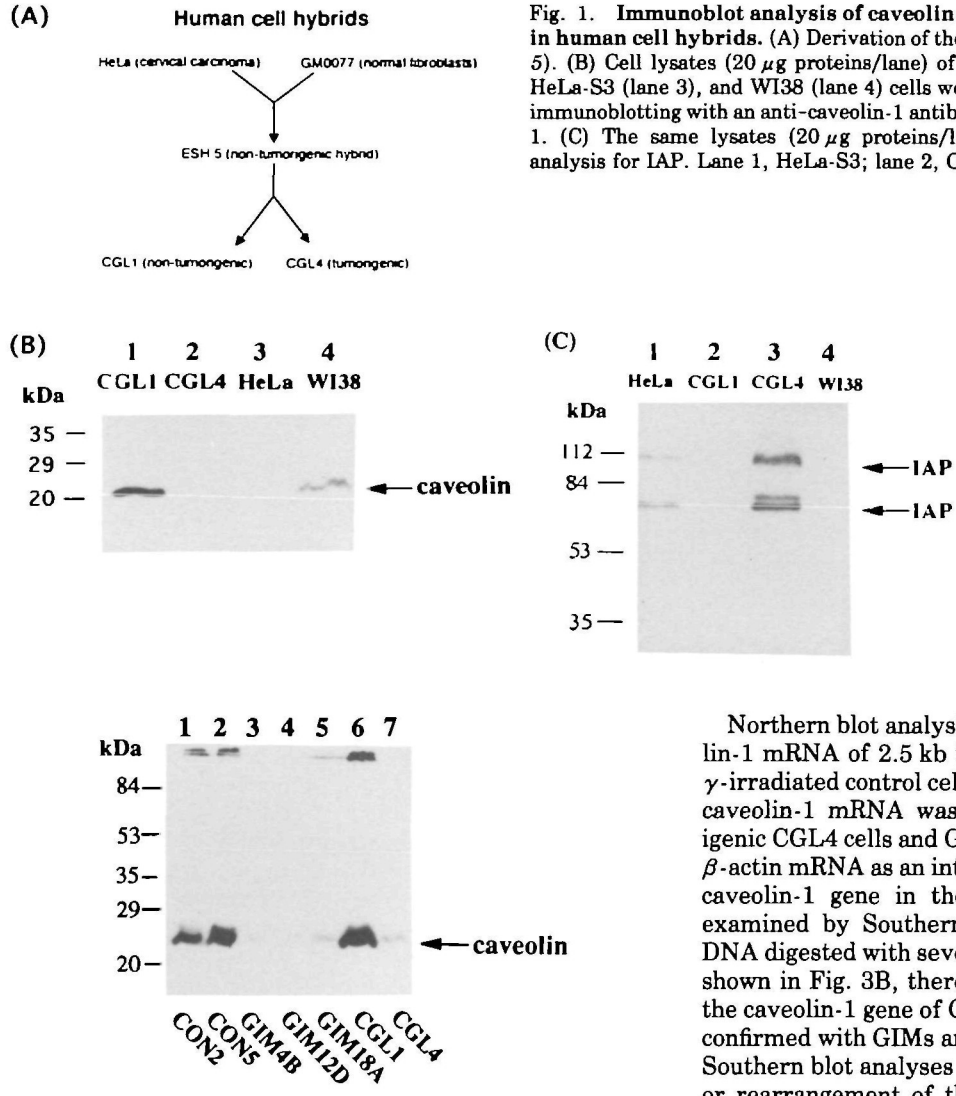

Fig. 1. **Immunoblot analysis of caveolin-1 and intestinal alkaline phosphatase in human cell hybrids.** (A) Derivation of the human cell hybrids used in this study (*4, 5*). (B) Cell lysates (20 μg proteins/lane) of growing CGL1 (lane 1), CGL4 (lane 2), HeLa-S3 (lane 3), and WI38 (lane 4) cells were separated by SDS-PAGE, followed by immunoblotting with an anti-caveolin-1 antibody. The arrow indicates 21 kDa caveolin-1. (C) The same lysates (20 μg proteins/lane) were subjected to immunoblotting analysis for IAP. Lane 1, HeLa-S3; lane 2, CGL1; lane 3, CGL4; lane 4, WI38.

Fig. 2. **Caveolin-1 expression in γ-ray-irradiated human cell hybrids.** Expression of caveolin-1 was determined by immunoblotting of cell lysates (20 μg proteins/lane) prepared from both tumorigenic mutant cells (GIM4B, GIM12D, and GIM18A) and non-tumorigenic control cells (CON2 and CON5), which were derived from non-tumorigenic CGL1 cells. Lane 1, CON2; lane 2, CON5; lane 3, GIM4B; lane 4, GIM12D; lane 5, GIM18A. As a control, lysates of CGL1 (lane 6) and CGL4 (lane 7) cells were simultaneously immunoblotted for caveolin-1.

GIM clones (4B; 12D and 18A) was greatly reduced, supporting a nice correlation to tumorigenic phenotypes.

Northern and Southern Blot Analyses for the Caveolin-1 Gene—To determine the caveolin-1 gene, the full-length caveolin-1 cDNA in CGL1 was cloned by the PCR procedure. The 0.6 kb PCR product contained an open reading frame encoding a protein of 178 amino acids. The deduced amino acid sequence revealed that this cDNA was 98.9% identical to that of the previously reported human caveolin-1 (*21*), although two amino acids, His^{82} and Thr^{144}, were substituted with Asp^{82} and Ile^{144}, respectively. Since these two amino acids, Asp^{82} and Ile^{144}, of caveolin-1 are conserved in dog, mouse, rat, and chicken caveolin-1 (*18, 24, 25*), we used this cDNA as the human caveolin-1 cDNA.

Northern blot analysis with this cDNA revealed a caveolin-1 mRNA of 2.5 kb in non-tumorigenic CGL1 cells and γ-irradiated control cells (Fig. 3A). In contrast, the level of caveolin-1 mRNA was significantly decreased in tumorigenic CGL4 cells and GIMs when compared to the levels of β-actin mRNA as an internal standard. The presence of the caveolin-1 gene in these human cell hybrids was also examined by Southern blot analysis using the genomic DNA digested with several specific restriction enzymes. As shown in Fig. 3B, there was no fundamental difference in the caveolin-1 gene of CGL4 and CGL1 cells. This was also confirmed with GIMs and CONs (Fig. 3C). These results of Southern blot analyses ruled out the possibility of deletion or rearrangement of this gene in tumorigenic HeLa cell hybrids.

Transfection of Human Caveolin-1 cDNA into a Tumorigenic Hybrid Cell—To search for a likely role of caveolin-1, the caveolin-1 cDNA ligated into an expression vector was transfected into a tumorigenic CGL4 clone, CGL4.1. Among the G418-resistant clones examined, a significant increase in 21-kDa caveolin-1 expression was detected in CGL4.1/cav8, being similar to that seen in CGL1 cells (Fig. 4). A small increase in this protein was also noted in CGL 4.1/cav2 and CGL4.1/cav7. We then determined the *in vivo* tumorigenicity of the transfected cells by s.c. inoculation into athymic nude mice, and the results are summarized in Table I. With CGL4 cells, tumors typically arise rapidly and grow to a large size (>800 mm^3) within 3 weeks after injection, as reported previously (*5, 9*). Two caveolin-1 expressing transfectants, CGL4.1/cav2 and CGL4.1/cav8, similarly grew into large tumors at all injection sites within 3 weeks. However, in no case did any tumors arise on inoculation of CGL1 cells, even though the inoculation period was extended to 5 weeks.

DISCUSSION

The present results suggest that the reduced expression of

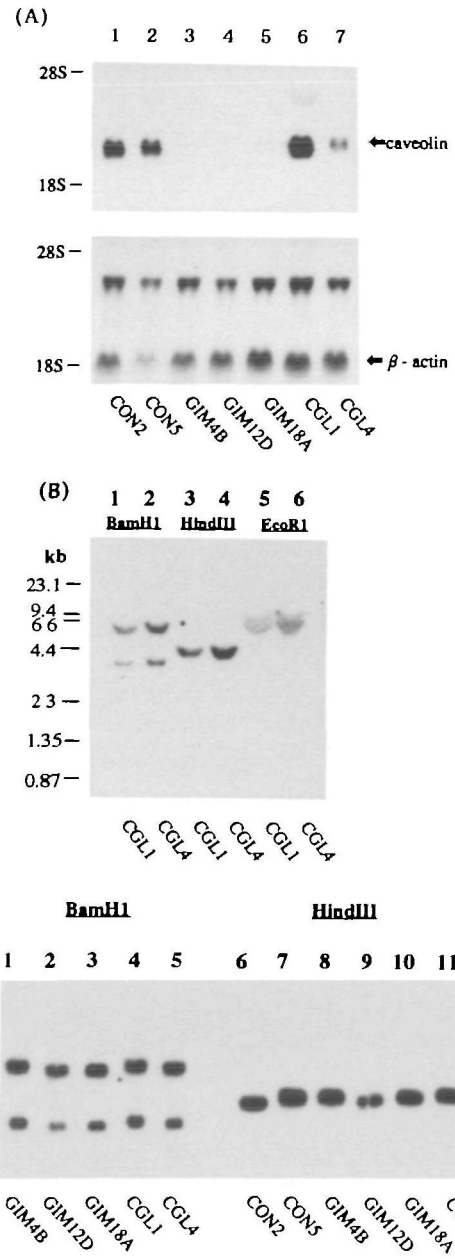

Fig. 3. **Northern and Southern blot analyses of the caveolin-1 gene in human cell hybrids.** (A) Total RNA was extracted from the cells and the caveolin-1 mRNA level was determined by Northern blot analysis. Lane 1, CON2; lane 2, CON5; lane 3, GIM4B; lane 4, GIM12D; lane 5, GIM18A; lane 6, CGL1; lane 7, CGL4. The RNA sizes were estimated by acridine orange staining (0.3 mg/ml) of the agarose gel, with 28S and 18S rRNA as markers. An arrow indicates the 2.5 kb caveolin-1 mRNA. The RNA-blotting membrane was rehybridized with a human β-actin DNA probe (0.4 kb), as an internal standard. (B) Genomic DNA was extracted from CGL1 (lanes 1, 3, and 5) and CGL4 (lanes 2, 4, and 6) cells, and then digested with either *Bam*H1 (lanes 1 and 2), *Hin*dIII (lanes 3 and 4), or *Eco*R1 (lanes 5 and 6), followed by Southern blot analysis. Arrows indicate molecular markers of λ-DNA fragments in kb. (C) Southern blot analysis was also performed with genomic DNA of GIMs and CONs digested with either *Bam*H1 (lanes 1-5) or *Hin*dIII (lanes 6-12). Lanes 1 and 8, GIM4B; 2 and 9, GIM12D; 3 and 10, GIM18A; 6, CON2; 7, CON5. As a control, genomic DNA isolated from CGL1 (lanes 4 and 11) and CGL4 (lanes 5 and 12) cells was simultaneously examined.

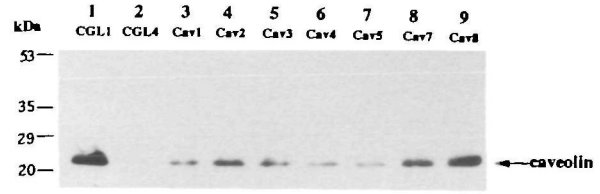

Fig. 4. **Overexpression of caveolin-1 cDNA in a tumorigenic cell hybrid.** A human caveolin-1 cDNA inserted into a mammalian expression vector, pSVsport1neo.f1, was transfected into a tumorigenic CGL4 clone, CGL4.1. After incubation in the selection medium, G418-resistant clones (CGL4.1/cav) were isolated. Cell lysates prepared from the indicated clones were subjected to immunoblotting analysis for caveolin-1, as described in Fig. 1. Lane 1, CGL1; lane 2, CGL4.1; lane 3, CGL4.1/cav1; lane 4, CGL4.1/cav2; lane 5, CGL 4.1/cav3; lane 6, CGL4.1/cav4; lane 7, CGL4.1/cav5; lane 8, CGL 4.1/cav7; lane 9, CGL4.1/cav8. Each lane contained 20 μg protein of cell lysate.

TABLE I. ***In vivo* tumorigenicity of human hybrid cells and caveolin-1 transfectants.**

Cell line	Tumorigenicity[a]	Tumor volume (mm^3)[b]
CGL1	0/8	None
CGL4.1	8/8	809.0±209.5
CGL4.1/cav2	8/8	1,288.2±374.4
CGL4.1/cav8	8/8	815.0±259.6

[a]No. of tumors formed per no. of injected sites detected 5 weeks after each injection. The indicated cells were injected s.c. at two sites/mouse. [b]Each value indicates the tumor size on day 21 after injection. There was no difference in the rate of *in vivo* tumor growth between CGL4.1 cells and the transfectants during the assay.

caveolin-1, a principal component of the caveolae structure in the plasma membrane (*13-16*), is closely associated with the tumorigenicity of HeLa cell hybrids. Decreases in expression of the caveolin-1 mRNA and protein were evident in three γ-ray-induced tumorigenic mutants (GIMs) isolated from non-tumorigenic CGL1 cells (Figs. 2 and 3). In these GIMs large-scale damage to chromosome 11 was evident, while no large-scale damage to the control chromosome 13 was detected (*10*). In contrast, none of the CONs have lost a complete copy of either chromosome 11 or chromosome 13 (*10*). These results as well as those of previous studies (*4-9*), implied a nice correlation between the neoplastic transformation and loss of the putative tumor suppressor locus in this cell system. Although it has yet to be proven that an additional locus is also commonly deleted or damaged in these tumorigenic cells, no major damage to the caveolin-1 gene was detected in the tumorigenic GIMs and CGL4 cells on Southern analysis (Fig. 3). These facts taken together strongly suggest tight association of the reduced expression of caveolin-1 with a loss or inactivation of the putative tumor suppressor gene in chromosome 11. This reduction of caveolin-1 expression may be mainly regulated at the transcriptional level, since transfection of an expression vector, pSVneo, containing caveolin-1 cDNA into CGL4 cells resulted in overexpression of the protein (Fig. 4).

Then, we examined how caveolin-1 expression is involved in the tumorigenesis of human hybrid cells. However, the *in vivo* tumor growth of CGL4.1 cells was unaffected when caveolin-1 was stably overexpressed (Fig. 4 and Table I). These results suggest that vector-induced caveolin-1 expression alone may be insufficient for sup-

pression of the *in vivo* tumor growth of these hybrid cells. Although more studies are needed for clarification of the function and localization of caveolin-1 within the cells, preliminary experiments suggest that caveolin-1 is similarly localized within detergent-insoluble complexes in both non-tumorigenic and tumorigenic cell hybrids (unpublished data), as described for other cell systems (*13-16*).

Reduction of caveolin-1 has previously been reported in mouse fibroblasts (NIH 3T3) transformed with various types of oncogenes, such as v-*abl*, H-*ras* and, middle T antigen (*19*). More recently, it was also demonstrated that expression of the caveolin-1 protein in v-*abl*-transformed and H-*ras*-transformed NIH3T3 cells using an inducible-expression system abrogated anchorage-independent growth in soft agar (*26*). Although these results clearly indicate a suppressive effect of the caveolin-1 protein on the anchorage-independent growth of mouse fibroblasts, it remains unclear whether or not caveolin-1 acts as a tumor-suppressive protein in other cell systems, such as in the *in vivo* tumor growth of HeLa cell hybrids. The discrepancy between these observations and our results may be due to differences in the cell types, tumorigenic states and expression system as well as in the assay system. While both tumorigenic and non-tumorigenic HeLa cell hybrids are able to grow in soft agar (*4, 5*), the growth of mouse fibroblasts in soft agar is characteristic of oncogenically transformed cells (*19, 26*).

In addition, the opposite effects of the putative tumor suppressor gene on the expression of IAP and caveolin-1 are worth noting. As described, there is a specific correlation between the ectopic expression of IAP and tumorigenicity as well as loss of the putative tumor suppressor locus on chromosome 11 in these cell hybrids (*7-10*). These facts imply that the putative tumor suppressor may function as a *trans*-regulator for the expression of specific cellular genes at the transcription level both positively and negatively, as suggested for other tumor suppressors such as p53 (*2, 3, 27*). Although the promoter regions of both the IAP gene (*28*) and the caveolin-1 gene (*29*) have been characterized, it remains uncertain whether these genes have any common features. Further studies on caveolin-1 expression in this cell system may lead to useful approaches for understanding the cellular function as well as the molecular mechanism(s) which underlies the putative tumor suppressor function involved in human tumor formation.

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