Jpn. J. Cancer Res. 88, 712–717, August 1997

Retroviral Introduction of the *p16* Gene into Murine Cell Lines to Elicit Marked Antiproliferative Effects

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The *p16* gene is a candidate tumor suppressor, because mutation of the gene has been reported in many transformed cell lines and some primary tumor tissues. We have examined this possibility in murine cell lines (NIH3T3 and RSV-M) which lack *p16* gene expression. Full-length human p16 cDNA was obtained from a HeLa cell line using polymerase chain reaction amplification. We constructed two separate retrovirus vectors carrying this p16 cDNA. First, we transduced the p16 cDNA into the murine cell lines using a retrovirus vector harboring the neomycin-resistance gene. The *p16* gene-transduced cells formed no colonies after selection with G418, in contrast to the vector-transduced cells. Next, we used another retrovirus vector that expresses both the p16 cDNA and the *Lac Z* gene, which enabled us to distinguish affected cells from unaffected ones. Proliferation of the *p16* gene-transduced cells was markedly inhibited and morphological change in the cells was also observed. Thus, we concluded that the *p16* gene has an antiproliferative effect on the cell cycle and that the loss of its function may play a major role in dysregulated proliferation of the cells.

Key words: *p16* gene — Cell cycle — Retrovirus — Murine cell lines — Antiproliferative effect

In the past several years, extensive information on cell cycle mechanisms has been accumulated, and the relationship between dysregulation of the normal cell cycle and tumorigenesis has become much clearer.[1)] Recently, a number of cell cycle regulatory genes have been cloned.[2–9)] The *p16* (*MST1*, *INK4A*, *CDKN2*) gene was the first to be cloned and its function was shown to be mediated by binding to and inactivating CDK4 and CDK6 (cyclin-dependent kinases 4 and 6) in the transition from G1 to S phase, resulting in cell cycle arrest.[2,3)] This gene is mutated at high frequency in many transformed cell lines and primary tumor tissues of a wide variety of human malignancies.[10–21)] Although the mutation rate of the *p16* gene in primary malignant tissues was initially found to be much lower than that in cell lines,[22–24)] it has turned out that the *p16* gene expression is greatly reduced by hypermethylation at the 5′-CpG island in many primary malignant cells.[25–29)] Several researchers have reported that some degree of antiproliferative effect was obtained when the *p16* gene was exogenously transfected into tumor cells in which the gene is not expressed.[16,30–32)] However, they only showed that the proliferative ability of p16 transfectants was diminished as compared with that of wild-type cells.

In the present study, we analyzed the antiproliferative effect of the *p16* gene exogenously introduced by retroviral transduction into murine cell lines which do not express the gene endogenously. In addition, the antiproliferative efficiency of the *p16* gene after selection of the transformants was examined not only by counting colonies formed, but also by directly measuring the proliferative rate of *p16* gene transfectants expressing the reporter gene.

MATERIALS AND METHODS

Cell lines Human cervical carcinoma HeLa, murine fibroblast NIH3T3 and glioma RSV-M cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Hela cell line has a naive *p16* gene expression.

Cloning of *p16* gene The p16 cDNA was cloned by the reverse transcriptase-polymerase chain reaction (RT-PCR) method. Total RNA was prepared from the Hela cell line using TRIZOL Reagent (Gibco BRL, MD). In this PCR strategy, we needed to separate the sequence into two fragments at a *Kpn* I site. First, the anterior half was amplified by using the nested PCR method. The outer primer set was 5′-GGGAGAACAGACAACG-GG-3′ (sense) and 5′-CCTTCCGCGGCATCTATG-3′ (antisense). The inner primer set was 5′-AACAGACA-ACGGGCGGC-3′ (sense) and 5′-GGCATGGTTACT-GCCTCT-3′ (antisense). Secondly, the posterior half was amplified using the primer set 5′-TGGGCCATCGC-GATGTC-3′ (sense) and 5′-CCCTAGTTCACAAAA-TGCTT-3′ (antisense). The PCR cycle consisted of denaturation at 95°C for 1 min, annealing at 55°C for 2 min

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and extension at 72°C for 3 min, and PCR was run in the presence of 10% dimethyl sulfoxide for 30 cycles. Each amplified fragment was confirmed by the dideoxy sequencing method. In order to get the full p16 coding sequence, the anterior and posterior halves were subcloned into *Bam*H I/*Kpn* I and *Kpn* I/*Sal* I sites of pBluescriptII KS-, respectively. As the final step, the oligonucleotide 5′-TATGCGGCCGCGTCGACGCATGGATCCTAT-3′ harboring the initiation codon and the cloning sites was added to the plasmid vector described above at the *Not* I/*Bam*H I sites.

Construction of retroviral vectors Two retroviral vectors were constructed for the two separate experiments (Fig. 1). The p16 cDNA was subcloned into pDL+[33] carrying the neomycin-resistance gene at the *Sal* I site (Fig. 1A). Another construct was made using the *IRES* (internal ribosome entry segment) gene from EMCV (encephalomyocarditis virus) (Fig. 1B).[34–36] First, the p16 cDNA was inserted into the *Sal* I site of pTY299+ (Yoshimatsu *et al.*, unpublished results). Secondly, the *IRES* gene was obtained from pG1XENPX[35] by *Xho* I-*Nco* I digestion and this *Nco* I site was ligated to *Sal* I linkers. The *Xho* I-*Sal* I fragment of *IRES* gene was inserted into pTY299+, whose *Hin*d III site was changed to a *Xho* I site by linker ligation. Thirdly, the *Lac Z* gene was obtained from SPUD1[37] by *Hin*d III digestion and the blunt-ended *Lac Z* gene was inserted into the *Hpa* I site of pTY299+. In this vector, both the p16 cDNA and the *Lac Z* gene are driven by 5′-LTR and the translation of the *Lac Z* gene is initiated by ribosomes entering from the IRES.

Virus packaging cell line Because of the inability to obtain stable transformants of the *p16* gene due to significant growth suppression, we had to perform these experiments using a transient retroviral expression assay. In these experiments, a newly constructed virus packaging cell line, which is expected to produce high titer even in transient expression, was established (Yoshimatsu *et al.*, unpublished results). The cell line was designated as MP34.

Retroviral transduction Each transfection procedure was conducted with 2 μg of DNA and 10 μl of "LipofectAMINE" Reagent (Gibco BRL). At 48 h after transfection, the culture medium including viral particles was recovered and filtered. It was then spread onto the target cells.

Colony formation ability after selection with G418 At 24 h after viral transduction, the cells were split at a 1 : 10 dilution and the next day, the medium was changed to G418-containing (Geneticin; Gibco BRL) medium. They were maintained for 2 weeks.

Analysis of cell proliferation rate At 24, 48 and 72 h after viral transduction, X-gal staining was performed. The number of X-gal positive cells forming each cluster was counted for every experiment.

RESULTS

***p16* expression of recipients** First, we analyzed the *p16* gene expression in murine cell lines to look for suitable recipients. Two murine cell lines, NIH3T3 and RSV-M, were used as transduction recipients in the present study. Murine lung and liver were selected as positive controls. The *p16* gene expression was examined by the RT-PCR method on the basis of the previously reported murine p16 cDNA sequence.[38] As shown in Fig. 2, expression of the *p16* gene was not detected in either of the murine cell lines used. The amplified bands were confirmed by Southern blot analysis.

Effect of exogenous *p16* gene transduced into HeLa cells Prior to studying the effect of forced *p16* gene expression in murine cell lines, we analyzed the effect of exogenous *p16* gene expression in HeLa cells, in which endogenous *p16* gene is already expressed. Because the retrovirus did

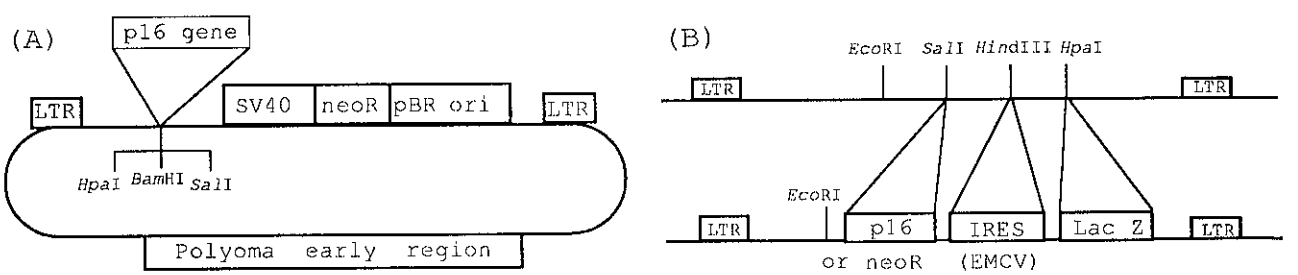

Fig. 1. Construction of retroviral vectors. A, Vector with drug-resistance gene. Cloned p16 cDNA contains the full coding sequence of the *p16* gene. The cDNA was inserted into *Sal* I sites of pDL+ harboring the neomycin-resistance gene. For negative control vectors, the same sites were replaced with no cDNA or antisense-oriented p16 cDNA. B, Vector using the *IRES* gene. p16 cDNA, IRES (from EMCV) and Lac Z were inserted into *Sal* I, *Hin*d III and *Hpa* I sites of pTY299+, respectively. Consequently, both the p16 cDNA and the *Lac Z* gene are driven by the 5′-LTR and translation of the *Lac Z* gene is initiated by the ribosomes entering from the IRES.

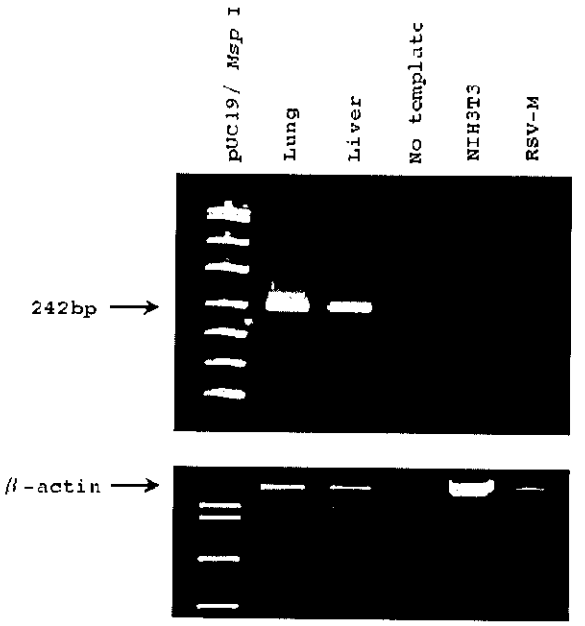

Fig. 2. Expression of endogenous *p16* gene in murine cell lines. The RT-PCR method was employed. The outer primer set: 5′-TGG AGT CCG CTG CAG ACA GAC TGG CCA G-3′ (sense), 5′-GAC CCC AGG CAT CGC GCA CAT CCA GC-3′ (antisense). The inner primer set: 5′-AGGGCCGTGTGCATGACGTG-3′ (sense), 5′-AGCCCCTGACCCGTGCAGCA-3′ (antisense). PCR conditions were as described in "Materials and Methods" with the exception of 20 cycles in the second amplification step. The 242-bp band represents the murine *p16* gene. The amplified bands were confirmed by Southern blot analysis with an oligonucleotide probe: 5′-ATTCAGGTGATGATGA-3′.

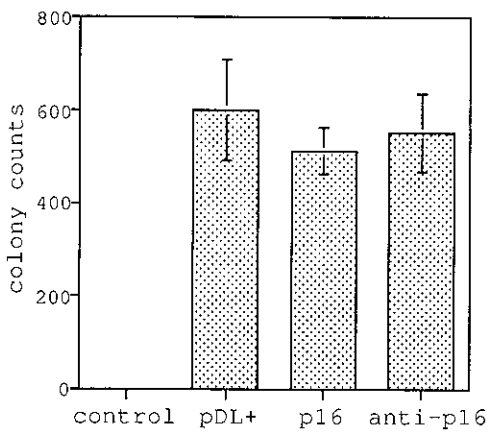

Fig. 3. Effect of exogenous *p16* gene transduced into HeLa cells. Each vector, including the neomycin-resistance gene, was transfected with "LipofectAMINE" Reagent. Forty-eight hours posttransfection, cells were split at a 1 : 10 dilution and maintained for 2 weeks in G418-containing media. The number of colonies formed per dish was counted. In the negative control dish, no colony formation was observed. Negative control: no plasmid transfection. Data are means±SD of three independent experiments.

not infect HeLa cells, the cloned p16 cDNA was transfected with "LipofectAMINE" Reagent (Gibco BRL). Since the vector contains a neomycin-resistance gene, the effect of *p16* gene transfection was examined in terms of colony-formation ability after selection with G418. The results in Fig. 3 show that no significant difference was observed in colony-formation ability between *p16* gene transfectant and vector transfectant. Therefore, we concluded that expression of the exogenous *p16* gene has no effect on the growth rate of cells originally expressing the *p16* gene.

***p16* gene transfer into murine cell lines** To study the antiproliferative effect of the *p16* gene expression, we introduced the gene into two murine cell lines, NIH3T3 and RSV-M cells, which were shown to lack *p16* gene expression (Fig. 2). In the present experiment, we used both the vector alone and the vector with antisense-oriented p16 insert, designated as anti-p16, as control vectors. To evaluate the colony-formation ability after selection with G418, each transformant was maintained in G418-containing medium for 2 weeks. The medium was changed every 3 days. After the 2-week culture, the number of colonies formed was counted. In the case of NIH3T3 cells, while the transformants with vector alone or anti-p16 formed 184.5±55.9 or 112.0±29.3 colonies/dish, which corresponds to a retroviral titer of $1.845\pm0.559\times10^5$ or $1.120\pm0.293\times10^5$ colonies/ml, respectively, the one with the sense-oriented *p16* gene formed no colonies (Fig. 4A). This result suggests that introduction of the *p16* gene into cell lines lacking *p16* gene expression resulted in marked growth suppression, as compared to HeLa cells (Fig. 3). To demonstrate directly the growth arrest of the *p16* gene transformant, we used the vector simultaneously expressing the *Lac Z* gene and examined the number of X-gal-stained cells in each transformant. Although the number of the X-gal-stained cells with the control vectors increased day by day after the viral transduction, that in the case of the p16 sense-oriented vector did not change from day 1 to day 3 (Fig. 4B). These results clearly revealed the marked inhibition of proliferation caused by the introduction of the *p16* gene. In addition, morphological change was observed. The X-gal-stained cells with the p16 sense-oriented vector all shrank, as compared to the normal shape of those transduced with control vectors (Fig. 4C).

DISCUSSION

In the present study, we constructed two separate retroviral vectors containing the full coding sequence of

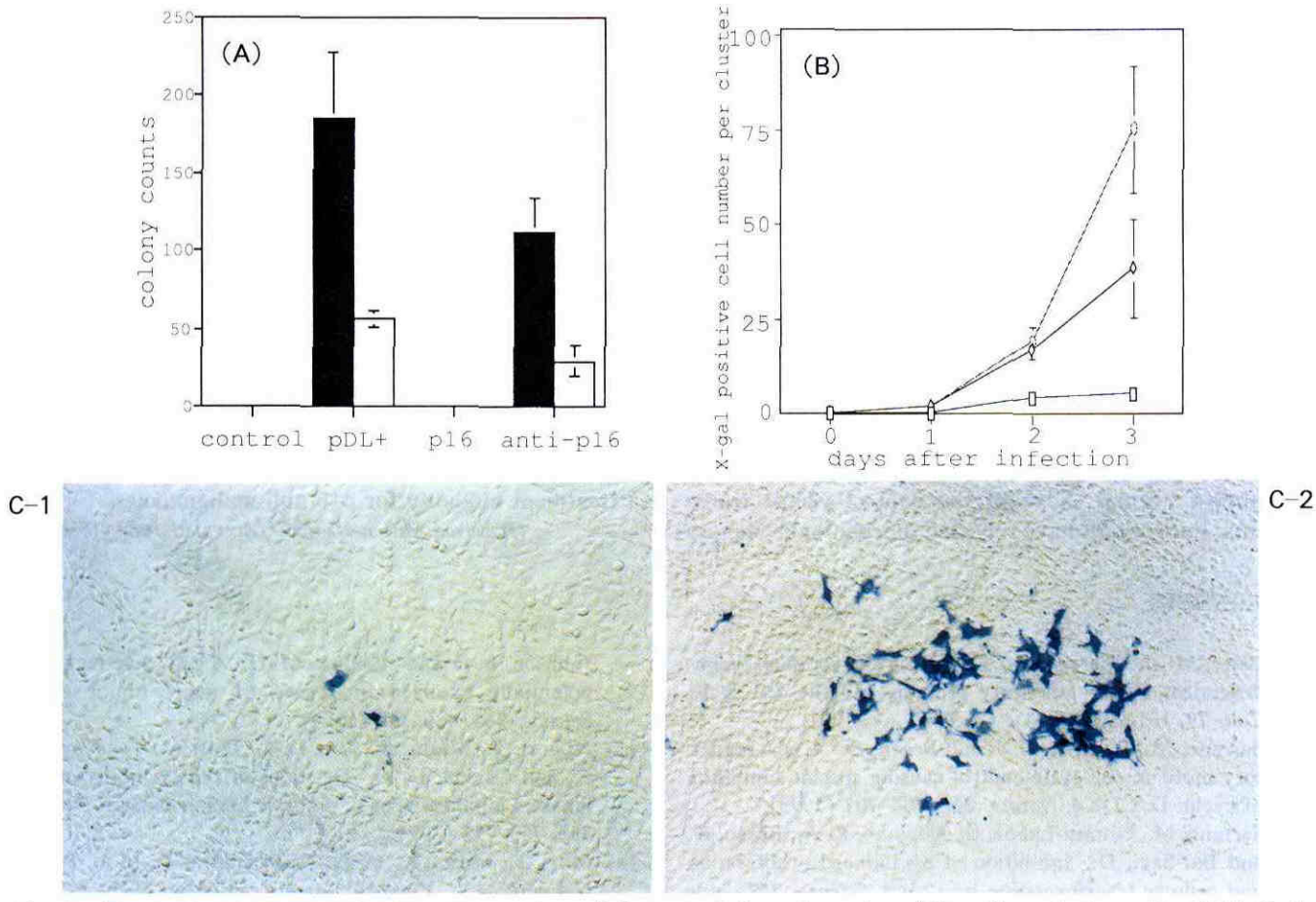

Fig. 4. Effect of *p16* gene transfer into two murine cell lines. A, Colony formation ability after selection with G418. Each vector, including the neomycin-resistance gene, was introduced by retroviral transduction. The number of colonies formed was counted after G418 selection. No colonies were observed in the transformant with p16 sense-oriented cDNA or no vector. Negative control: no plasmid transfection. Data are means±SD of three independent experiments. NIH3T3 (■), RSV-M (□). B, Analysis of cell proliferation rate. Each vector, with the *Lac Z* gene driven by IRES, was introduced by retroviral transduction. On days 1, 2 and 3 after viral transduction, the number of X-gal-stained cells forming each cluster was counted and average values are shown. The proliferation of the p16 sense-oriented cDNA transformant was markedly inhibited. Data are means±SD of three independent experiments. □ p16, ◇ anti-p16, ○ neoR/Lac Z. C, Typical photographs. The cells of the p16 sense-oriented cDNA transformant shrank. Magnification×100. C-1, p16 sense. C-2, p16 antisense.

wild-type *p16* gene. It has been reported that the introduction of a wild-type *p16* gene into p16-null transformed cell lines resulted in specific growth suppression. For example, Arap *et al.* observed specific growth suppression of p16-negative human glioma cells after calcium phosphate transfection of an exogenous *p16* gene.[30] Further, the induction of negative cell growth regulation by adenovirus-mediated *p16* gene transfer into p16-null lung cancer cell lines was demonstrated by Jin *et al.*[32] But these experiments only examined the effect of the *p16* gene in the bulk population of the transfectants. No marker gene was employed to trace the *p16* gene transfectants. In the present experiment, we created a visually traceable system by using the *Lac Z* gene with IRES in the retroviral vector. It has been shown that when two genes are connected by IRES, expression of the two genes is coordinately regulated.[35, 36] In addition, this system made it possible to discriminate the *p16* gene-introduced cells from the non-introduced ones. By applying this system, the morphological difference between affected and unaffected cells could also be examined.

Recently, other investigators have cloned murine p16 cDNA.[38] They reported that NIH3T3 lacked *p16* gene expression. We have clarified for the first time that *p16* gene expression is deleted in the RSV-M murine glioma cell line.

Here, we have also shown that introduction of exogenous *p16* gene by the retroviral vector results in specific growth suppression in murine p16-null cell lines. In contrast, transfection of additional copies of the *p16* gene into HeLa cell line, which expresses endogenous p16, did not affect the growth ability. These results are in agreement with previous reports by other investigators[16, 30–32] and support the hypothesis that the *p16* gene plays a key role in suppressing deregulated cell growth. As regards the control vectors constructed in the present study, moderate growth suppression was caused by the vector harboring antisense-oriented p16 cDNA in both experimental systems. We can not explain this phenomenon at present. Focusing on the morphologic change in the affected cells, introduction of the *p16* gene appears to cause shrinkage of the affected cells in comparison with the almost normal shape of the control vector transfectants. Fueyo *et al.* have reported morphologic changes in p16-null glioma cell lines after an adenoviral-mediated transfection of the wild-type *p16* gene. They mentioned that the glioma cells became flattened and enlarged upon restoration of the wild-type *p16* gene.[31] The morphologic changes we observed in the present study may result from the forced braking of the natural cell cycle by the exogenously introduced wild-type *p16* gene.

In conclusion, introduction of the wild-type *p16* gene into p16-null murine cell lines suppressed growth ability and influenced cell morphology. Additional studies utilizing an amphotropic virus packaging system for introduction of the gene into human glioma cell lines will be required for detailed evaluation of the gene function. Although retroviral transduction has yielded only low transduction efficiency so far, much higher titer vectors might make it possible to introduce wild-type *p16* gene as a treatment modality for p16-null malignancies.

(Received March 10, 1997/Accepted June 10, 1997)

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