Transduction of Antisense Cyclin D1 Using Two-step Gene Transfer Inhibits the Growth of Rat Hepatoma Cells

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ABSTRACT

Cyclin D1, one of the G₁ cyclins, is frequently overexpressed in several types of carcinomas and is thought to play an important role in tumorigenesis and tumor progression including hepatocellular carcinoma. We constructed a retrovirus vector-carrying rat cyclin D1 cDNA in the reverse orientation, resulting in expression of antisense (AS) cyclin D1 mRNA. For efficient transduction of this recombinant retrovirus, two-step gene transfer was performed. The rat hepatoma cell line (dRLh84) was infected with this recombinant retrovirus after preinfection with adenovirus expressing the retrovirus receptor. In the rat hepatoma cells, AS cyclin D1 mRNA was expressed, inducing a decrease in the expression of endogenous cyclin D1 mRNA and an inhibition of cell growth. Moreover, two-step gene transfer of AS cyclin D1 into s.c. hepatoma xenografts resulted in inhibition of tumor growth and prolonged animal survival. In the virus-infected tumor xenografts, expression of cyclin D1 was immunohistochemically inhibited, and apoptosis of hepatoma cells was detected. These findings suggest that transduction of AS cyclin D1 is useful as an adjunct to standard treatments for hepatocellular carcinoma.

INTRODUCTION

Recent genetic analysis has revealed abnormalities in cell-cycle regulators, which likely contribute to carcinogenesis via aberrant cell-cycle progression. A restriction point has been identified late in the G₁ phase, just before the G₁-S-phase transition, in which cycling is stimulated by G₁ phase cyclins. Cyclin D1 is a proto-oncogenic regulator of the G₁-S-phase checkpoint and appears to phosphorylate pRb by binding to cdk 4 or 6 (1). Phosphorylation of pRb in mid-to late-G₁ releases transcription factors bound with pRb, including members of the E2F family, and leads to stimulation of DNA synthesis (2).

Cyclin D1 is overexpressed in many cancers as a result of gene amplification or translocations targeting the D1 locus (formally designated CCND1) on human chromosome 11q13 (3). Amplification of chromosome 11q13 is frequent in a broad spectrum of common adult cancers, including squamous cell carcinoma of the head and neck, esophageal cancer, bladder cancer, primary breast cancer, small cell lung cancer, and HCC (3). In HCC, a very close relationship between the gene amplification and protein overexpression of cyclin D1 has been reported, and cyclin D1 overexpression also reflects the aggressiveness of HCC and affects its prognosis (4–7).

HCC is one of the most common cancers in the world, especially in Asia and Africa (8). With the recent advances in therapeutic modalities, the prognosis of patients with HCC has been much improved. Even so, surgical candidates are few because of widespread intrahepatic involvement or the lack of hepatic reserve resulting from coexisting advanced cirrhosis (9). In addition, clinical observations have shown that tumor recurrence rates are very high in patients with HCC who received surgical or medical treatments (10). Therefore, new treatment modalities for HCC have been investigated. One approach is gene therapy using killing or suicide genes such as the herpes simplex virus thymidine kinase or cytotoxic deaminase genes, cytokine genes, and oncosuppressor genes.

In the present study, we inserted the 0.4-kbp fragment of rat cyclin D1 cDNA in the AS orientation into a retrovirus vector and transduced rat hepatoma cells in vitro and in vivo using a two-step gene transfer method. The two-step gene transfer method is an efficient method of retrovirus-mediated gene transduction that uses preinfection with an adenovirus carrying the ecotropic retrovirus receptor (MCAT-1) gene (11). Our results demonstrate that efficient transduction of AS cyclin D1 by two-step gene transfer leads to inhibition of cyclin D1 mRNA expression and cell growth in vitro and also induces tumor growth inhibition associated with a marked decrease in cyclin D1 protein expression and an increase in apoptosis in vivo. We report the first clear evidence that two-step gene transfer of AS cyclin D1 is effective for treatment of HCC in vivo, suggesting its potential efficacy for treatment of human HCC.

MATERIALS AND METHODS

Cell Culture. The Psi-2 ecotropic retrovirus packaging cells were cultured in DMEM with 10% calf serum. The 293 human embryonal kidney cells and the dRLh84 rat hepatoma cell line were maintained in DMEM with 10% fetal bovine serum.

Plasmid Constructions. To obtain the rat cyclin D1 cDNA, total RNA was isolated from primary cultured rat hepatocytes prepared from adult male Wistar rats by perfusing the liver in situ with collagenase as described previously (12). Reverse transcription-PCR was performed using a set of primers, 5'-CTGGAGCCCCTGAAGAAGAGC-3' and 5'-GAAAAGCGCTTGTGCGG-GTGACG-3', for 30 cycles of 30 s at 94°C, 15 s at 58°C, and 30 s at 72°C. The resultant 427-bp fragment, corresponding to bases 396 to 822 of rat cyclin D1 cDNA deposited with GenBank/European Molecular Biology Laboratory Data Bank under accession no. D14014 (13), was subcloned into pCR2.1 (Invitrogen); resulting in pLASCD1IN. In this construct, the cyclin D1 cDNA existed in reverse orientation, for 30 cycles of 30 s at 94°C, 15 s at 58°C, and 30 s at 72°C. The 0.4-kbp fragment of rat cyclin D1 cDNA was inserted into the AS orientation into a retrovirus vector and transduced rat hepatoma cells in vitro and in vivo using a two-step gene transfer method. The two-step gene transfer method is an efficient method of retrovirus-mediated gene transduction that uses preinfection with an adenovirus carrying the ecotropic retrovirus receptor (MCAT-1) gene (11). Our results demonstrate that efficient transduction of AS cyclin D1 by two-step gene transfer leads to inhibition of cyclin D1 mRNA expression and cell growth in vitro and also induces tumor growth inhibition associated with a marked decrease in cyclin D1 protein expression and an increase in apoptosis in vivo. We report the first clear evidence that two-step gene transfer of AS cyclin D1 is effective for treatment of HCC in vivo, suggesting its potential efficacy for treatment of human HCC.

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3 The abbreviations used are: pRb, retinoblastoma protein; AS, antisense; cdk, cyclin dependent kinase; EGFP, enhanced green fluorescent protein; HCC, hepatocellular carcinoma; MCAT-1, mouse cationic amino acid transporter-1; GAPDH, glyceroldehyde-3-phosphate dehydrogenase.
plates) were incubated with or without recombinant adenovirus, AxCAMCAT, for 1 h at a multiplicity of infection of 1. Forty-eight h later, these cells were infected with the ecotropic retrovirus, LEIN, for 4 h with 1 ml of virus supernatant (1 × 10^6 cfu/ml) plus 1 ml of fresh medium containing 8 μg/ml polybrene. Forty-eight h later, analysis of EGFP gene expression was performed using flow cytometry (COULTER EPICS XL system 8, Coulter, Tokyo, Japan).

**Northern Blot Analysis.** Total cellular RNA was isolated by the guanidinium isothiocyanate method. Both sense and AS cyclin D1 transcripts were detected using a 427-bp rat cyclin D1 cDNA described above. GAPDH cDNA was used as a control probe (17).

**Growth Inhibition of Virus-infected Hepatoma Cells.** Adenoviral infection of dRLh84 cells was carried out at multiplicity of infection of 1 for 1 h. Two days later, adenovirus-infected or noninfected cells were trypsinized and divided into four experimental groups: (a) noninfected, (b) infected with LEIN with preinfection by AxCAMCAT, (c) infected with LASCD1IN only, or (d) infected with LASCD1IN with preinfection by AxCAMCAT. Retroviral infections were carried out with 1 ml of virus supernatant (1 × 10^6 cfu/ml) plus 1 ml of fresh medium containing 8 μg/ml of polybrene for 4-h incubation. On days 1, 2, and 4 after the retroviral infection, the viable cells were enumerated.

**In Vivo Experiments.** Five-week-old male BALB/c nu/nu athymic mice were obtained from Kyushu Experimental Animal Supply (Kumamoto, Japan). The mice were maintained at constant room temperature (25°C) and provided with free access to standard diet and tap water throughout the study. Animal experiments were conducted in accordance with the highest standards of animal care as outlined in the “NIH Guidelines for Care and Use of Experimental Animals.” The protocol for these animal studies was approved by Miyazaki Medical College.

The mice were inoculated s.c. in the right thigh with 1 × 10^6 cells of dRLh84. Eleven days later, when transplanted tumors had formed s.c. nodules (100 mm^3 in size), 3 × 10^5 plaque-forming units of rat AxCAMCAT adenovirus or DMEM were directly injected into the tumors. Two days after the adenoviral infection, a single injection of 1 × 10^8 cfu of LEIN or LASCD1IN was administrated. Serial changes in tumor volume were estimated periodically after the retroviral infection, using the following formula (18):

\[ VT (\text{mm}^3) = 0.5 \times (L + W) \times L \times W \times 0.5236 \]

where \( VT \) is estimated tumor volume, and \( L \) and \( W \) are length (mm) and width (mm) of transplanted tumor, respectively.

**Detection of Cyclin D1 and Apoptosis by Immunohistochemistry.** Preformed hepatomas harvested 20 days after retroviral infection were fixed in 4% formalin, cut into 5-μm sections, and placed on poly-l-lysine-coated glass slides. After boiling in distilled water for 10 min, slides were incubated with an anti-cyclin D1 monoclonal mouse antibody (Dako Japan, Kyoto, Japan; Ref. 19) or a rabbit polyclonal antibody against single-stranded DNA (Dako Japan; Ref. 20) to detect cyclin D1 or apoptosis, respectively. Horse antimouse IgG was then applied, followed by an avidin-biotin-peroxidase complex and chromagen 3,3'-diaminobenzidine.

**Statistical Analysis.** All of the data are represented as the mean ± SE. Statistical comparison was made using Stat View software and the Mann-Whitney U test. A value of \( P < 0.05 \) was taken to indicate significance. Survival was evaluated using the Kaplan-Meier analysis, and a comparison of the survival curves was made with the log rank test.

**RESULTS**

**Efficiency of Retrovirus-mediated Gene Transfer to Rat Hepatoma Cells by Preinfection with Adenoviruses Expressing MCAT-1 (Two-step Gene Transfer).** The rat hepatoma cells (1 × 10^6 cells/well in 6-well multiplates) were incubated with or without recombinant adenovirus, AxCAMCAT, for 1 h at a multiplicity of infection of 1. Forty-eight h later, these cells were infected with the ecotropic retrovirus, LEIN, for 4 h with 1 ml of virus supernatant (1 × 10^6 cfu/ml) plus 1 ml of fresh medium containing 8 μg/ml polybrene. Forty-eight h later, analysis of EGFP gene expression was performed using flow cytometry (COULTER EPICS XL system 8, Coulter, Tokyo, Japan).

**Antisense Cyclin D1 Inhibits the Growth of Rat Hepatoma Cells Using Two-step Gene Transfer.** To evaluate the transduction efficiency of two-step gene transfer, dRLh84 rat hepatoma cells were infected with ecotropic retrovirus, LEIN, with or without preinfection of recombinant adenovirus, AxCAMCAT, and the expression of the EGFP gene was analyzed using flow cytometry (Fig. 1). Fluorescence indicating LEIN infection was detected in 32% of hepatoma cells preinfected with AxCAMCAT (Fig. 1A), whereas only 14% of cells not preinfected with AxCAMCAT expressed the EGFP gene (Fig. 1B).

**Analysis of Cyclin D1 mRNA in Primary Cultured Rat Hepatocytes and Hepatoma Cells with and without Virus Infections.** We examined cyclin D1 mRNA in rat hepatocytes and hepatoma cells with and without virus infections by Northern blot analysis. In dRLh84 rat hepatoma cells, cyclin D1 expression was about 10-fold higher than that in primary cultured rat hepatocytes (Fig. 2A, Lanes 1 and 2). To analyze the effect of retroviral infections on the expression of cyclin D1 mRNA, dRLh84 rat hepatoma cells were infected with AxCAMCAT/LEIN, LASCD1IN alone, or AxCAMCAT/LASCD1IN. In addition, LASCD1IN-infected cells were cultured in G418 for 2 weeks, and G418-resistant pooled populations, dRLh84/LASCD1IN, in which AS cyclin D1 mRNA was strongly expressed, leading to a decreased expression of endogenous cyclin D1 mRNA (Fig. 2B, Lane 5), was established. The expression of cyclin D1 mRNA in AxCAMCAT/LEIN-infected cells was similar to that in parental dRLh84 cells (Fig. 2B, Lanes 1 and 2). In dRLh84 cells infected with LASCD1IN alone, a trace amount of 3.8-kbp exogenous AS cyclin D1 mRNA and a weak inhibition of endogenous cyclin D1 expression were observed (Fig. 2B, Lane 3). In contrast, AxCAMCAT/LASCD1IN two-step gene transfer induced an increase in the expression of AS cyclin D1 mRNA, resulting in a decreased expression of endogenous cyclin D1 mRNA (Fig. 2B, Lane 4).

**Effect of Antisense Cyclin D1 Transduction on Growth of Rat Hepatoma Cells.** To evaluate the effect of LEIN or LASCD1IN retrovirus infections on cell growth, dRLh84 cells were infected with AxCAMCAT/LEIN, LASCD1IN alone, or AxCAMCAT/LASCD1IN. Four days after the infection, although the growth of dRLh84 cells was not affected by AxCAMCAT/LEIN two-step gene transfer, AS cyclin D1 gene transduction using LASCD1IN infection or AxCAMCAT/LASCD1IN two-step gene transfer induced about 13% or 40% growth inhibition, respectively (Fig. 3).

**Effect of A5 to Cyclin D1 on Growth of Preformed Hepatomas in Athymic Mice and Animal Survival.** To analyze the in vivo effect of AS cyclin D1 transduction on tumor growth, parental dRLh84 cells were inoculated s.c. in athymic nude mice, and recombinant adenovirus or retrovirus was infected by direct injection when tumor xenografts became 100-mm^3 nodules. The growth of transplanted tumors was not affected by the treatment with AxCAMCAT/LEIN two-step gene transfer and was similar to that of tumors treated with serum-free medium alone (control). In contrast, although the LASCD1IN infection alone induced only a weak growth inhibition until 30 days after inoculation, the growth of tumor xenografts treated with AxCAMCAT/LASCD1IN two-step gene transfer was apparently

![Image](A)

![Image](B)

Fig. 1. Transduction efficiency of LEIN retroviral infection alone and AxCAMCAT/LEIN two-step gene transfer in dRLh84 rat hepatoma cells. Two days after LEIN infection with (B) or without (A) AxCAMCAT adenoviral infection, expression of EGFP was analyzed by flow cytometry as described in “Materials and Methods.”
Antisense cyclin D1 inhibits the growth of rat hepatoma

Inhibited and, in one of the six tumor xenografts, regressed completely (Fig. 4A).

In addition, we analyzed animal survival to evaluate the effect of AS cyclin D1 transduction over a longer period. Animals treated with AS cyclin D1 by two-step gene transfer survived significantly longer than did the animals treated with serum-free medium (control), as well as the animals treated with AxCAMCAT/LEIN (Fig. 4B). The survival of animals treated with AxCAMCAT/LEIN two-step gene transfer or LASCD1IN alone was only slightly longer than that of control animals treated with medium alone.

Immunohistochemical Analysis of Cyclin D1 Expression and Apoptosis in Hepatoma Cells Transplanted in Athymic Mice.

To confirm whether AS cyclin D1 gene transduction could inhibit the expression of cyclin D1 in preformed s.c. tumors, immunohistochemistry was performed. Nuclear cyclin D1 expression was detected homogeneously in tumors without retrovirus infection (Fig. 5A, part a). However, in tumors treated with AS cyclin D1, cyclin D1 expression was partially inhibited, because direct injection of virus vectors did not achieve gene transfer into the entire tumor (Fig. 5A, part b).

Next, we analyzed the apoptosis in AS cyclin D1-treated tumors. Apoptotic cells were detected in the virus-infected areas of tumor xenografts treated with AxCAMCAT/LASCD1IN two-step gene transfer, in which cyclin D1 expression was also inhibited, whereas apoptosis was not induced in tumors treated with AxCAMCAT/LEIN (Fig. 5B).

**DISCUSSION**

The overexpression of cyclin D1 and its prognostic value have been intensively investigated in several types of carcinoma (21–23). In HCC, cyclin D1 overexpression was detected in approximately 30% of HCC tissues (7), and a positive relationship between the cyclin D1 overexpression and pRb expression indicates that cyclin D1 might play a significant role in cell-cycle progression by inactivation of pRb. Although cyclin D1 forms a complex with cdk4 or 6 and works for the phosphorylation of pRb, cdk4 and 6 gene mutations are rare in cancer of any origin (24). Three major cdk inhibitors, p16/INK4, p21/WAF1, and p27 (Kip1), can prevent pRb from being phosphorylated. p16/INK4 binds to the cyclin D1-cdk4 complex, inhibiting the formation of the heterodimer. Recently, Matsuda et al. (25) found a loss of p16/INK4 expression in approximately half of HCC tissues by immunohistochemistry and found the high levels of CpG methylation in the p16/INK4 gene promoter, leading to the inactivation of the gene, in HCC with negative p16/INK4 expression. Unlike the p16/INK4 gene, the p21/WAF1 and p27 (Kip1) genes are rarely mutated in carcinomas (26, 27). Therefore, inhibition of cyclin D1 or induction of p16 expression is supposed to be effective in inhibiting G1-S progression, and Arber et al. (28) recently demonstrated that stable expres-
sion of AS cyclin D1 induced not only a decreased expression of the endogenous cyclin D1 protein but also a reduction of cdk4 kinase activity.

Given that cyclin D1 overexpression, although less frequent than p16/INK4 inactivation in HCC, is critical to the progression of HCC (4–7), we hypothesized that suppression of cyclin D1 protein expression would lead to tumor growth inhibition. To test this hypothesis, we cloned a 0.4-kbp fragment of cyclin D1 cDNA into a retroviral vector in the AS orientation and analyzed whether AS cyclin D1, transduced by two-step gene transfer, inhibited cyclin D1 expression and growth of hepatoma cells in vitro and in vivo. Our results showed about 40% reduction of proliferation in AS cyclin D1-transduced cells compared with non- or EGFP-transduced cells in vitro 4 days after retrovirus infection. The expression of cyclin D1 mRNA was significantly decreased in AS cyclin D1-transduced cells as compared with control cells. Furthermore, our results revealed that transduction of the AS cyclin D1 into preformed s.c. tumors in athymic mice also inhibited tumor growth, resulting in prolonged animal survival.

Previous reports (28–30) have described that AS cyclin D1 induced the inhibition of tumor growth and a complete loss of tumorigenicity in nude mice transplanted with human colonic, esophageal, and pancreatic cancer cells, which were selected with G418 and stably expressed AS cyclin D1. In the present study, although cyclin D1 transcripts were significantly decreased in dRLh84/LASCD1IN cells, which were G418-resistant pooled populations of LASCD1IN-infected hepatoma cells, moderate inhibition of cyclin D1 expression was achieved in AS cyclin D1-transduced hepatoma cells using two-step gene transfer. In addition, inhibition of cyclin D1 expression was detected locally in virus-injected s.c. tumors. Therefore, AS cyclin D1 transduced by two-step gene transfer caused incomplete growth inhibition of hepatoma cells, and the antitumor effect obtained with this new approach is as yet unsatisfactory. One approach by which to enhance the tumor shrinkage would be based on repeated injections of retrovirus during MCAT-1 expression, because even when MCAT-1 is expressed, retrovirus infection occurs only in cells actively replicating at the time of injection. The more expanded the areas in s.c. tumors transduced with AS cyclin D1 are, the more enhanced would be the antitumor effects. Another approach to enhance the antitumor effect is a combination with transduction of AS cyclin D1 and chemotherapeutic agents. Kornmann et al. (30, 31) have reported that the inhibition of cyclin D1 enhances sensitivity to multiple chemotherapeutic agents through the altered expression of several chemoresistance genes, in addition to suppressing the growth of pancreatic cancer cells. However, it has not yet been clarified whether the increased chemosensitivity is induced not only in AS cyclin D1-transduced cells but also in surrounding nontransduced cells.

Fig. 5. Immunohistochemical analysis of s.c. tumor xenografts with and without AxCAMCAT/LASCD1IN two-step gene transfer, respectively. Preformed tumor xenografts in athymic mice were harvested 20 days after the two-step gene transfer, and immunohistochemical analysis was performed using anti-cyclin D1 (A) or anti-single-stranded DNA (B) antibodies as described in “Materials and Methods.” A, part a, s.c. tumors treated with vehicle alone; part b, treated with AxCAMCAT/LASCD1IN two-step gene transfer. Arrowheads, areas in which cyclin D1 expression was markedly inhibited; ×100. B, part a, s.c. tumors treated with AxCAMCAT/LEIN; part b and part c, treated with AxCAMCAT/LASCD1IN. Arrows, apoptotic cells; ×200 (part a and part b) and ×1000 (part c).

viruses-mediated gene transduction using adenovirus carrying an ectropic receptor, MCAT-1, gene (11). The transduction efficiency of two-step gene transfer in rat hepatoma cells was approximately 2-fold higher than that of retrovirus infection alone. Yamaguchi et al. (36) reproducibly achieved a 5–20-fold increase in efficiency of amphotropic retrovirus-mediated gene transfer by using adenovirus vectors to express gibbon-ape leukemia virus receptor-2, a receptor for amphotropic retrovirus. We have reported previously (11) efficient ectropic retrovirus-mediated gene transfer to human cells, in addition to rodent cells, using adenovirus-expressing MCAT-1. Even in the case of a retrovirus receptor transduced by adenovirus, active proliferation of target cells is required for retroviral infection (37). Because cell proliferation in the tumorous tissue of HCC is higher than that in the nontumorous tissue of liver, two-step gene transfer would be suitable to target HCC cells. Furthermore, two-step gene transfer using a tissue-specific promoter, which we reported previously (11), may result in effective and tissue-specific enhancement of transgene expression, exclude the possibility of genomic DNA alteration in nontarget cells by proviral DNA integration, and lead to protection of the hepatic reserve.

As reported recently (33), we directly injected viral vectors into preformed s.c. tumors. This more closely recreates the clinical situation than do experiments documenting tumor shrinkage after ex vivo therapy. HCCs can be visualized by ultrasonography and computed tomography, and percutaneous needle biopsy and ethanol injection therapy targeting HCCs are now well-established techniques. Therefore, direct injection could become the preferred route for administering viral vectors into HCC.
In conclusion, we demonstrated that AS cyclin D1 transduction using two-stage gene transfer efficiently inhibited cyclin D1 expression and induced apoptosis, leading to growth inhibition of HCCs in vitro and in vivo. Therapeutic modalities for HCC are restricted by the number, location, and size of tumors and by the hepatic reserve of coexisting cirrhosis. Even if AS cyclin D1 treatment does not cause a tumor to vanish, sufficient shrinkage would contribute to the effectiveness of standard treatments such as ethanol injection, microwave coagulation, radiofrequency ablation, and surgery.

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REFERENCES

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