Application of p27 gene therapy for human malignant glioma potentiated by using mutant p27

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Object. Malignant glioma could be an ideal candidate for local gene therapy because its invasion is local and it has little metastatic potential. A low expression level and high degradation activity of p27 are known to constitute an independent poor prognostic factor in patients with malignant glioma. In this study, the authors investigated the roles of wild-type p27 and mutant p27 on the treatment of malignant glioma.

Methods. The authors used two adenoviruses: one expressed wild-type p27 (ad-p27wt) and the other, containing a mutation at the major metabolic site, expressed mutant p27 (ad-p27mt). The antitumor effects of the two adenoviruses were compared with respect to cell growth arrest, cell cycle alteration, apoptosis induction, and in vitro tumorigenicity in three glioblastoma multiforme (GBM) cell lines and in a primary GBM cell line. Transduction with ad-p27wt or ad-p27mt induced the production of p27 and the dephosphorylation of pRB. The protein level of mutant p27 was significantly higher than that of wild-type p27. The ad-p27wt induced cell cycle arrest at the G1–S transition point, whereas the ad-p27mt induced arrest at the G2–M point. Both ad-p27wt and ad-p27mt induced a growth-inhibiting effect, apoptosis, and suppression of in vitro tumorigenicity; however, ad-p27mt displayed a stronger antitumor effect than ad-p27wt in brain tumor cell lines.

Conclusions. Gene therapy involving p27, especially mutant p27, has the potential to become a novel and powerful therapy for malignant glioma.

Key Words • adenovirus • p27 • mutant p27 • malignant glioma • gene therapy

Malignant glioma is the most common type of primary brain tumor. The current standard treatment of malignant glioma is resection followed by radiation therapy with or without adjuvant chemotherapy. Despite this multidisciplinary approach, the prognosis of this disease is very poor. Brain tumor may be an ideal target for gene therapy because it occurs in a closed space, is relatively small in size, is locally invasive in nature, and has a low metastatic potential. Many gene therapy strategies have been applied to brain tumor (for example, tumor suppressor gene therapy with p53, triggered apoptosis, and induced cell cycle arrest in brain tumor cells).

The p16 protein is a member of the WAF family of CDKIs, which induces cell cycle arrest at the G1–S transition point. Restoration of p16 to glioma cells through an adenoviral vector also has been shown to induce growth suppression and G1–S arrest. The protein p27kip1 (p27) is a member of the Cip/Kip family of CDKIs, which exerts an inhibitory effect on many steps of the cell cycle. The role of p27 in brain tumors has been investigated extensively because it is a known prognostic factor. A reduced level of p27 has been associated with a poor prognosis for malignant astrocytoma and the reduced expression of p27 in oligodendrogies has proved to be an independent poor prognostic factor. Kirla, et al., analyzed the expression of p21 and p27 in a high-grade astrocytoma and found that only p27 had an independent prognostic value, although both p27 and p21 were parallel cell cycle regulators.

The main inhibitory action of p27 arises from its binding to the cyclin E/Cdk2 complex and its dephosphorylation of pRB. Throughout the cell cycle p27 mRNA levels remain constant and p27 protein levels are mainly regulated by ubiquitin-mediated proteolysis. This regulation of p27 is linked to the phosphorylation of the protein at Thr187, followed by ubiquitination and proteasome-mediated proteolysis.

In addition to its role as a CDKI, p27 acts as a putative tumor suppressor gene. p27-knockout mice have reportedly developed multorgan hyperplasia and parathyroid tumor. Adenoviral gene transfer of p27 has been shown to induce cell cycle arrest and apoptosis in breast cancer cell lines and we have already demonstrated that the transduction of p27 via an adenoviral vector into human lung cancer
cell lines induces cell growth suppression via \( G_1 - S \) arrest.\(^9\)

Based on these two findings as they relate to the role of p27 as a tumor suppressor gene and to the process of p27 degradation at Thr187, we have also demonstrated that an adenovirus that expresses mutant p27 at Thr187 displays a stronger antitumor effect in human lung cancer cell lines and a resistance to degradation.\(^16\) To date no report has been published on the role of p27 gene therapy in brain tumor.

In this study, we applied these two p27-expressing adenoviruses to human malignant glioma cell lines to confirm the feasibility of applying p27 gene therapy to malignant glioma.

Materials and Methods

Cell Lines

Three human GBM cell lines (T98G, U373MG, and U87MG) were purchased from American Type Culture Collection (Manassas, VA) and the Korean Cell Line Bank (Seoul, Korea). One primary GBM cell line was established from a surgical specimen at the Department of Neurosurgery, Seoul National University Hospital. Cells were maintained in RPMI-1640 medium containing 10\% FBS and penicillin/streptomycin. The p27 status of the three long established cell lines was normal; however, we do not know whether that was the case with the primary GBM cell line.

Construction of Ad-p27wt and Ad-p27mt

The construction of adenoviruses p27wt\(^{11,12}\) and that of p27mt\(^{11,12}\) have been previously described. These vectors are replication-defective E1-deleted adenoviruses with an immediate-early CMV promoter. Briefly, the complementary DNA of human p27 was subcloned into the KpnI and BamHI sites of the polylinker of the adenoviral shuttle vector pAC CMV pLpA (kindly provided by Robert Gerard, University of Texas Southwestern Medical Center, Dallas, TX). The resulting pAC CMV-p27wt and p1M17 were cotransfected into 293 cells and maintained until the onset of a cytopathic effect. The generating adenovirus was confirmed by DNA sequencing of viral DNA and by a Western blot analysis of p27. The p27mt, with a mutation of Thr187/Pro188 (ACGCCG) to Met187/Ile188 (ATGATC), was used for the construction of ad-p27mt. A recombinant adenovirus expressing \( \beta \)-galactosidase (ad-lacZ) and an adenovirus containing no recombinant genes (ad-null) were used as control viruses in all experiments.

Transduction of Malignant Glioma Cells With Ad-p27(wt/mt)

Malignant glioma cells, which were growing exponentially, were transduced with ad-p27wt or ad-p27mt at 20 MOI for 1 hour, follow-
in vitro tumorigenicity, a soft agar clonogenic assay was performed. Briefly, tumor cells (U373MG and U87MG) were transduced with 20 MOI of ad-p27wt or ad-p27mt and then detached and plated in 0.2% agarose with a 1% agarose underlayer (5 × 10^3 cells/plate). Colonies whose extension was greater than 125 μm were counted after 3 weeks by using a calibrated graticule.

**Results**

*Transduction With Ad-p27wt and Ad-p27mt Produced p27 Protein and Induced Dephosphorylation of pRB*

A Western blot assay performed to detect p27 among proteins extracted from ad-p27wt– and ad-p27mt–transduced brain tumor cells showed an overexpression of p27 protein. Wild-type p27 and mutant p27 could not be distinguished by using the Western blot assay, but the amount of mutant p27 was significantly higher than that of wild-type p27. Both wild-type and mutant p27 induced pRB dephosphorylation (Fig. 1).

*A More Potent Effect by Ad-p27mt on the Growth Arrest of Human Malignant Gliomas*

In the four GBM cell lines, including one primary GBM cell line, ad-p27mt induced a stronger growth suppression than ad-p27wt, which indicates that mutant p27 more potently induces growth arrest in malignant glioma cell lines (Fig. 2).

*Different Patterns of Arrest Induced by Ad-p27wt and Ad-p27mt*

Both ad-p27wt and ad-p27mt induced growth arrest in two brain tumor cell lines (U373MG and U87MG) but the patterns of this arrest differed. The Ad-p27wt induced arrest at the G1–S transition point, which was manifested by a decrease in the proportion of cells in the S phase and an increase in the proportion of cells in the G1–G0 phase compared with control groups. On the other hand, ad-p27mt induced arrest at the G2–M transition point, as shown by a decrease in the proportion of cells in the S phase and an increase in the proportion of cells in the G2–M phase (Fig. 3 and Table 1).

*More Potent Induction of Apoptosis by Ad-p27mt Than by Ad-p27wt*

The Annexin V assay showed that both ad-p27mt and ad-p27wt induced apoptosis, although their effects were not strong. Nevertheless, ad-p27mt proved to be the more effective inducer of apoptosis. The proportions of apoptotic cells (annexin V–positive and propidium iodide–negative cells) in the U373MG cell line were 2.84% of the nontransduced cells, 4.02% of the ad-lacZ–transduced cells, 8.46% of the ad-p27wt–transduced cells, and 12.9% of the ad-p27mt–transduced cells. In the T98G cell line, cells displaying early apoptosis accounted for 2.96% of the nontransduced cells, 1.84% of the ad-lacZ–transduced cells, 4.86% of the ad-p27wt–transduced cells, and 8.78% of the ad-p27mt–transduced cells. Although both ad-p27wt and ad-p27mt only induced weak apoptosis, ad-p27mt was the more potent of the two adenoviruses (Fig. 4).

*Stronger Suppression of Tumorigenicity in Malignant Glioma Cell Lines Following Transduction With Ad-p27mt*

Transduction with ad-p27wt or ad-p27mt almost completely abolished the clonogenic ability of U373MG cells in soft agar. In U87MG cells, however, ad-p27mt induced a stronger suppression than ad-p27wt, although both ade-
noviruses significantly suppressed in vitro tumorigenicity (Fig. 5).

Discussion

In contrast to other neoplasms, brain tumors occur in a closed space, are relatively small, and have a low metastatic potential. These characteristics make brain tumors an ideal target for direct-acting gene therapy. Nevertheless, current gene therapy trials have thus far failed to show clinically significant responses.

The p27 gene is a member of the multifunctional universal CDKI family and a putative tumor suppressor gene that is rarely mutated in tumors.2,14,21 The reduced expression of p27 in a brain tumor is associated with a poor prognosis,2,14,21 as is the case with other tumors.22 In colorectal cancer and in non–small cell lung cancer, a reduced level of p27 expression is related to proteasome-mediated proteolysis.8,17 In the human glioma cell lines T98G and NAC6, which display contact inhibition within in vitro cultures, the expression of p27 was markedly enhanced; however, no enhancement was found in glioma cell lines without contact inhibition, such as A172 and U251 cells.11

A high p27 degradation activity was found in highly ma-
p27 gene therapy in malignant glioma

Lignant gliomas with low-to-absent p27 expression. In the same study the proteasome inhibitor, LLnL, abolished enhanced p27 degradation in a malignant glioma. The overexpression of p27 delivered through adenoviral gene transfer suppressed breast cancer cell growth regardless of the presence of p27 mutation. In a gliosis model, but not in a brain tumor model, p27 overexpression due to delivery of p27 through an adenoviral vector, inhibited astrocyte proliferation and was accompanied by a downregulation of cyclin A. This finding indicates a potential role for p27 in cases of malignant glioma.

In this study, we attempted to develop an ad-p27 gene therapy for malignant glioma and to potentiate its antitumor effect by manipulating the p27 metabolic pathway. As we described, the metabolism of p27 depends on its phosphorylation at Thr187 and subsequent ubiquitination. Based on observations of p27 metabolism and the nature of the p27 tumor suppressor gene, we previously demonstrated...
the stronger antitumor effect of ad-p27mt compared with that of ad-p27wt in a lung-cancer model, despite the fact that both vectors produced distinct antitumor effects.18,19 A Western blot assay for p27 showed that the transduction of both adenoviruses induced p27 protein overexpression and pRB dephosphorylation in a GBM cell line. Furthermore, the amount of mutant p27 was significantly higher than that of wild-type p27 protein, indicating that mutant p27 has a long half-life, because the infection doses (MOIs) of both adenoviruses were identical. In addition, in previous work we showed that mutant p27 is more stable than the wild type by using a “S-pulse-chase experiment.”

The p27 protein is a CDKI that affects the cell cycle in multiple steps. Wild-type p27 produced by ad-p27wt induced G1–S arrest as expected; however, mutant p27 induced G1–M arrest and increased cellular DNA content. In a previous experiment in which we used lung cancer cell lines, both types of p27 were found to induce G1–S arrest.18 We do not have any explanation for this discrepancy; however, it may be related to tissue specificity.

In the present study, the growth assay showed that ad-p27mt caused more growth suppression than ad-p27wt in all the GBM cell lines that were tested (T98G, U373MG, and U87MG) as well as in a primary GBM cell line, even though both types of vectors effectively blocked cancer cell growth.

A soft agar clonogenic assay also produced the same result, especially in U87MG cells. The clonogenicity of the GBM cell lines was more strongly suppressed by ad-p27mt than by ad-p27wt.

The antitumor effect of ad-p27 was not influenced by the p53 status of the cell line. Both U87MG (wild-type p53) and U373MG (mutant p53) cells were suppressed by p27 gene therapy. More importantly, a primary GBM cell line, established from a specimen obtained at surgery, was also greatly inhibited. This finding strongly indicates the clinical potential of p27 gene therapy in malignant glioma.

In conclusion, this study demonstrates, for the first time, the potential of p27 gene therapy via an adenoviral vector in malignant glioma and a means of enhancing the antitumor effect of p27 by using an adenovirus that expresses a mutant p27 in which the mutation is located at a major metabolic site.

References

K. Park, et al.