Adenoviral Expression of the Cyclin-Dependent Kinase Inhibitor p27Kip1: a Strategy for Breast Cancer Gene Therapy

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Cyclin-dependent kinase inhibitors play a critical role in regulating progression of the eukaryotic cell through the cell cycle by associating with protein complexes composed of cyclins and cyclin-dependent kinases and thus down-regulating the activity of the cyclin-dependent kinases (1). Pathways involving cyclin-dependent kinase inhibitors are frequently disrupted in cancer cells, and this disruption leads to abnormal regulation of the cell cycle. Overexpression of cyclin-dependent kinase inhibitors leads to the arrest of cells at one of the checkpoints in the cell cycle (2). Therefore, using cyclin-dependent kinase inhibitors for gene therapy of cancer is intuitively attractive because it has the potential to inhibit tumor growth (3). Thus far, adenoviral vectors expressing the cyclin-dependent kinase inhibitors p16INK4A and p21waf1/cip1 have been evaluated in preclinical models for their suitability for gene therapy of cancer (3–9).

We have constructed an adenoviral vector expressing p27Kip1 and observed that it is statistically significantly better at causing growth arrest in several breast cancer cell lines than is another adenoviral vector that expresses p21waf1/cip1 (2). More interestingly, we have recently discovered that overexpression of p27Kip1 induces apoptosis in cancer cells, an activity that is not associated with p16INK4A or p21waf1/cip1 (8,10). In fact, p21waf1/cip1 has been reported to have a protective effect against apoptosis (11,12). These observations led us to examine the effects of adenoviral expression of p27Kip1 in an in vivo breast cancer model system.

We injected MDA-MB-231 human breast cancer cells into athymic nude mice to establish subcutaneous tumor nodules and then studied the effects of intratumoral injections of various adenoviral vectors on these mice and their tumors. Adβgal, a recombinant adenovirus that expresses Escherichia coli β-galactosidase, was used to establish the infectability of the MDA-MB-231 cells. As shown in Fig. 1, A, after in vitro infection with Adβgal at a multiplicity of infection of 100 plaque-forming units per cell, all cells treated with X-gal (5-bromo-4-chloro-3-indolyl β-D-galactoside) were colored blue. Thus, adenoviral vectors can transduce a transgene into this cell line with a high degree of efficiency. Next, we injected the Adβgal virus intratumorally into established MDA-MB-231 tumor nodules in athymic nude mice. At various intervals, animals were killed, and their tumors were excised and evaluated for β-galactosidase expression as described in Fig. 1. Blue-colored tumor nodules could be easily observed up to 14 days after infection (Fig. 1, B). Microscopic examination of sections from these blue tumors revealed that the blue color was present intracellularly, thus establishing that the transgene is expressed in the cells (Fig. 1, C). (All animal experiments reported in this study were carried out in accordance with institutional guidelines.)

To study the effect of adenovirus-mediated overexpression of p27Kip1, we injected subcutaneous tumor nodules four times with phosphate-buffered saline (PBS) (mock injection) or with one of the following adenoviral vectors: AdNull, a control vector that does not express any transgene; Adp27, a recombinant adenovirus that expresses p27Kip1; and Adp21, an adenoviral vector that expresses p21waf1/cip1. From our previous experience (unpublished results), we used a dose schedule of 2.5 × 10⁸ plaque-forming units of virus per dose per day for 4 days, for a total viral dose of 10⁹ plaque-forming units for all three viruses. Tumors were measured every 5 days for a period of 30 days as described previously (13). On day 0, average tumor volumes (± standard deviation) in this experiment were 18 (± 4) mm³, and on day 30, average tumor volumes were 1320 (± 387) mm³ in the Adp27-treated group compared with 4190 (± 563) mm³, 3335 (± 672) mm³, and 3117 (± 238) mm³ in the PBS-, AdNull-, and Adp21-treated groups, respectively. In other words, tumors in the Adp27-treated group were only one third as large as tumors in the mock-treated group. In this experiment, in accord with institutional guidelines, animals were killed on the day that their tumors measured more than 20 mm in one dimension; i.e., all animals were killed when the size of their tumor(s) reached 20 mm. There were no deaths from “other causes” in any group. Data from all animals were included in the calculation of average tumor volume. Data from the animals that were killed because of their large tumors were analyzed as if the animals had tumor volumes that were equal to the volume of their last measurement (20 mm in one dimension). Data from the tumor-free animals in the treated groups were analyzed as if the animals had tumor volumes equal to zero.

Infection with Adp27 caused tumor regression in the majority of the animals. As shown in Fig. 2, A, 36% of the animals that received Adp27 became tumor free by day 5 after completing the injections as compared with none of the animals in the control (mock treatment) groups. This proportion steadily increased to more than 60% by day 15. Of the animals that received AdNull or Adp21, 18% became tumor free by day 15. However, all PBS-injected animals had rapidly growing tumors that necessitated euthanasia in 60% of the animals by day 30. A representative pair of animals that received AdNull or Adp27 is shown in Fig. 2, B. Similar results were obtained when this experiment was repeated. To investigate whether these Adp27 effects were a general phenomenon, we examined the effect of Adp27...
Fig. 1. MDA-MB-231 human breast cancer cells and xenografts efficiently express a transgene after recombinant adenoviral infection. A) After infection of cells with Adβgal, a recombinant virus encoding *Escherichia coli* β-galactosidase (100 plaque-forming units per cell), the cells were fixed (5 minutes, 4 °C) in 2% formaldehyde and 0.2% glutaraldehyde. Cells were then incubated in 5 mM K₄Fe(CN)₆, 5 mM K₃Fe(CN)₆, 2 mM MgCl₂, and 200 μg/mL X-gal (5-bromo-4-chloro-3-indolyl β-D-galactoside) solution (4 hours, 37 °C) and photographed (×100 magnification) by use of a phase-contrast microscope. Expression of β-galactosidase was considered positive when a blue color was detected in the cells. Uninfected cells (left), AdNull (a control virus)-infected cells (middle), and Adβgal-infected cells (right) are shown. B) Subcutaneous xenografts of MDA-MB-231 cells were established in athymic nude mice by the injection of 5 × 10⁶ cells. Tumors were injected with Adβgal (10⁹ plaque-forming units). At various times, the tumor-bearing mice were killed and their tumors were excised, fixed in formaldehyde and glutaraldehyde for 4 hours, and stained for 4 hours as described above. Tumors at the following times are shown: day 1 after AdNull infection (left), day 1 after Adβgal infection (middle), and day 14 after Adβgal infection (right). C) For histologic examination, Adβgal-infected tumors that showed β-galactosidase activity were sectioned (5-μm sections), counterstained with hematoxylin–eosin, and examined microscopically at magnifications of ×40 (left) and ×200 (right).

Fig. 2. Intratumoral injection of Adp27, a recombinant adenovirus encoding the cyclin-dependent kinase inhibitor p27kip1, results in complete remissions of breast cancer xenografts. A) To the left, MDA-MB-231 human breast cancer cells (5 × 10⁶ cells) were injected subcutaneously into 6-week-old athymic nude mice. After 3–6 days, palpable tumors formed in the mice. Viruses (Adp27, Adp21 [encoding the cyclin-dependent kinase inhibitor p21Waf/Cip1], or AdNull [a control virus]) were injected directly into the tumors (2.5 × 10⁸ plaque-forming units) in 100 μL of phosphate-buffered saline (PBS). Eleven animals were in each group. Tumor size was measured every 5 days. To arrive at the percentages plotted, the numerator was the number of animals bearing tumors as recorded at each time point and the denominator was the total number of animals with tumors at the beginning of the experiments. Because animals were killed only when their tumors reached a maximum size (20 mm) and there were no deaths from other causes, there are no “missing animals” as such. On the right, MDA-MB-468 human breast cancer cells (3 × 10⁶ cells) were injected subcutaneously into 6-week-old athymic nude mice. After 6 days, palpable tumors were found. Viruses (Adp27, Adp21, or AdNull) were injected directly into the tumors (2.5 × 10⁸ plaque-forming units) in 100 μL of PBS. Tumor size was measured every 5 days. Ten animals were in each group. B) Photographs of representative animals from the groups (MDA-MB-231 model) that received AdNull or Adp27 on day 15 are shown. Note a large tumor in the animal that received AdNull (left) but the absence of tumors in the animal that received Adp27 (right). C) Induction of apoptosis in the tumor cells injected with Adp27 is shown. MDA-MB-231 tumors were injected with a single dose (2.5 × 10⁸ plaque-forming units in 0.1 mL) of AdNull (left) or Adp27 (right). The tumor-bearing animals were killed 48 hours later, and their tumors were excised, 48 hours later, fixed, and stained for apoptotic cell by TUNEL (terminal deoxynucleotidyltransferase-mediated digoxigenin-uridine triphosphate nick-end labeling) assays as described previously (10). Apoptotic cells are stained brown. A higher magnification view (×630) of single cell undergoing apoptosis is shown in the inset in the right panel (note the presence of condensed nuclei undergoing fragmentation). A higher magnification view (×630) of a control cell showing the lack of DNA fragmentation is in the inset in the left panel.
in another human breast cancer model system that used MDA-MB-468 cells. Our laboratory had shown previously that these cells can also be infected with recombinant adenoviral vectors (14). MDA-MB-468 cells were injected subcutaneously into nude mice (3 x 10^6 cells per animal), and the established tumors were injected with Adp27 or AdNull by use of the dose schedule described above for the MDA-MB-231 model system. In the MDA-MB-468 system, we also found that Adp27 infection induced tumor regressions in the majority of the animals (Fig. 2, A). Thus, by day 30 in the mock-infected group, 100% of the mice were tumor bearing; in the Adp27-treated group, 67% of the mice were tumor free; and in the AdNull-treated group, only 25% of the mice were tumor free. These results clearly indicate the efficacy of Adp27 in the MDA-MB-468 model system.

We also investigated whether injecting Adp27 directly into the tumors can induce apoptosis of the tumor cells in vivo as documented in in vitro studies (10). Adp27 was injected into MDA-MB-231 tumors; 48 hours later, the tumors were examined to determine whether apoptotic cells were present. As shown in Fig. 2, C, tumors that received a control virus had a minimum number of cells that were undergoing apoptosis, but tumors that received Adp27 had many more cells that appeared to be undergoing apoptosis. It is likely that Adp27-mediated apoptosis might be playing a crucial role in the therapeutic effect of Adp27 observed in this breast cancer model.

In this study, we examined the effects of adenovirus-mediated overexpression of p27Kip1 on the growth of MDA-MB-231 and MDA-MB-468 human breast cancer xenografts in nude mice. After four doses of Adp27, a complete response rate of about 60% was observed. However, it is somewhat puzzling to us why the remaining 40% of the tumors evaded the tumoricidal effect of Adp27. Perhaps the injection technique was not completely standardized and some tumors did not receive the proper dose, perhaps the vector was poorly distributed in the tumors, or perhaps there were biologic variations among the animals. Nevertheless, a 60% response rate is comparable to our unpublished results and the experience of other groups with adenovirus-mediated overexpression of human wild-type p53 in similar animal models (13,15,16) and substantially better than the results of overexpression of p21waft/cip1 or p16INK4A (3–9). This difference may be accounted for by the induction of apoptosis after p27Kip1 overexpression, a property it shares with p53 but not with p21waft/cip1 and p16INK4A. However, it should be noted that p16INK4A in combination with wild-type p53 has been demonstrated to induce apoptosis in cancer cells (9).

Adenoviral vectors expressing human wild-type p53 are currently being evaluated in many clinical studies in patients with advanced cancer (16). From the encouraging results presented here, gene therapy of cancer with adenoviral vectors expressing p27Kip1, alone or combined with other tumor suppressor genes, or other strategies of cancer gene therapy appears to be a promising treatment option.

REFERENCES


NOTE

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