Short Communication

Antitumour effect of cyclin-dependent kinase inhibitors (p16INK4A, p18INK4C, p19INK4D, p21WAF1/CIP1 and p27KIP1) on malignant glioma cells

T Komata1, T Kanzawa1,2, H Takeuchi2, IM Germano1, M Schreiber3, Y Kondo1,2 and S Kondo*,1,2

1Department of Neurosurgery, Mount Sinai School of Medicine, New York, NY 10029, USA; 2Department of Neurosurgery, The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030, USA; 3Department of Biology, McMaster University, Hamilton, Ont., Canada L8S 4K1

Cyclin-dependent kinase inhibitors (CDKIs) are considered as novel anticancer agents because of their ability to induce growth arrest or apoptosis in tumour cells. It has not yet been fully determined, however, which CDKI is the best candidate for the treatment of malignant gliomas and whether normal brain tissues are affected by CDKI expression. Using recombinant adenoviral vectors that express CDKIs (p16INK4A, p18INK4C, p19INK4D, p21WAF1/CIP1 and p27KIP1), we compared the antitumour effect of CDKIs on malignant glioma cell lines (A172, GB-1, T98G, U87-MG, U251-MG and U373-MG). p27KIP1 showed higher ability to suppress the growth of all tumour cells tested than other CDKIs. Interestingly, overexpression of p27KIP1 induced autophagic cell death, but not apoptosis in tumour cells. On the other hand, p27KIP1 overexpression did not inhibit the viability of cultured astrocytes (RNB) nor induced autophagy. Overall, our findings suggest that gene transfer of p27KIP1 may be a promising approach for the therapy of malignant gliomas.

Keywords: cyclin-dependent kinase inhibitors; p27 KIP1; glioma; autophagy

MATERIALS AND METHODS

Cells

Malignant glioma cells (A172, GB-1, T98G, U87-MG, U251-MG and U373-MG) (Komata et al, 2000) and cultured astrocytes RNB (Kondo et al, 1996) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, GIBCO BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (GIBCO BRL), 4 mM glutamine, 100 U ml−1 penicillin and 100 μg ml−1 streptomycin.

Construction of recombinant adenoviral vectors

The recombinant AdMH4p16, AdMHp18, AdMHp19, AdMHp21 and AdMHp27 containing p16INK4A, p18INK4C, p19INK4D, p21WAF1/CIP1 and p27KIP1 were kindly supplied from Dr FL Graham (McMaster University, Ontario, Canada). As described previously (Schreiber et al, 1999), human p16 cDNA (p BluescriptSK-p16) was obtained from Dr D Beach (Cold Spring Harbor, New York, USA). pXEP21, pAdCP17 and pAdCP19 were obtained from Dr T Thompson (Baylor College of Medicine, Houston, TX, USA), and pSGLvep27 was a gift from Dr J Roberts (Fred Hutchison Cancer Research Center, Seattle, WA, USA). AdBHGΔ13 containing E1 and E3 deletions is a control recombinant adenovirus that has identical backbone sequences to the adenoviral constructs expressing CDKIs.

Adenoviral infection

The effect of CDKIs on cell viability was determined by using a trypan blue dye exclusion assay as described previously (Komata et al, 1999).
et al, 2000). To achieve the infectivity of > 90%, A172, GB-1, U87-MG, U251-MG, U373-MG and RNB cells were tested at a multiplicity of infection (MOI) of 60 PFU cell⁻¹. On the other hand, T98G cells were infected with 180 MOI. The percentage of cell viability was calculated from the mean cell viability of treated cells divided by that of cells with control treatment. To detect the expression of CDKI in infected cells, the immunoblotting assays using anti-CDKI antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were performed as described previously (Kondo et al, 1996).

Detection of apoptotic or autophagic cell death

To detect apoptosis, the terminal deoxynucleotidyl transferase (TdT)-mediated nick end labelling (TUNEL) analysis was performed as described previously (Komata et al, 2000). To detect autophagic changes in infected cells, cells were stained with acridine orange (Poly-science, Warrington, PA, USA) as described previously (Paglin et al, 2001). At 2 days after adenoviral infection, acridine orange was added at a final concentration of 1 μg ml⁻¹ for 15 min. Microphotographs were obtained with a fluorescence microscope.

Statistical analysis

The data were expressed as means ± s.d. Statistical analysis was performed by using Student’s t-test (two-tailed). The criterion for statistical significance was taken as P<0.05.

RESULTS

Effect of CDKI overexpression on viability of malignant glioma cells

To investigate the effect of CDKI on malignant glioma cell lines, cell viability was determined 3 or 5 days after adenoviral infection. As shown in Figure 1, the treatment of U373-MG cells with AdMH4p16INK4A, AdMH4p18INK4C, AdMH4p19INK4D, AdMH4p21WAF1/CIP1 or AdMH4p27KIP1 for 3 days significantly inhibited the cell viability compared to that with control vector (P<0.002 to P<0.004). In U251-MG, U87-MG, A172 and GB-1, similar antitumour effects were observed with each CDKI 3 days after adenoviral infection (P<0.0002 to P<0.02). On the other hand, the effect of AdMH4p18INK4C or AdMH4p19INK4D was not significant for T98G cells (P=0.17 or P=0.71), although other CDKIs were effective (P<0.0001 to P<0.03). Among the CDKIs used in the present study, p27KIP1 was more effective for tumour cells than the other CDKIs. The number of viable cells of U373-MG treated with AdMH4p27KIP1 decreased below the initial cell number (5000): 4138 (day 5), 2555 (day 3) and 378 (day 7). This indicates that approximately half of p27KIP1-infected U373-MG cells underwent cell death by day 5. Additionally, cell number of other tumour cell lines decreased by 30–60% from the initial cell number like U373-MG cells 5 days after the treatment with AdMH4p27KIP1. These results indicate that 27KIP1 has the highest antitumour effect on all malignant glioma cells tested in the present study.

Effect of p27KIP1 in RNB cells

To investigate whether normal brain tissues are affected by p27KIP1 expression, cultured rat astrocyte, RNB cells, were treated with AdMH4p27KIP1. As shown in Figure 2, the effect of p27KIP1 overexpression was not significant for RNB cells, while the viability of U373-MG cells decreased to 15% of the control (P<0.005). This result suggested that p27KIP1 -based therapy will be selective for tumour cells.

Effect of p27KIP1 on induction of apoptosis or autophagy in U373-MG cells

To determine the type of cell death induced by AdMH4p27KIP1, we first performed the TUNEL staining. The incidence of TUNEL-positive cells in U373-MG cells treated with AdBHGΔ13 or AdMH4p27KIP1 for 3 or 5 days was less than 1%. Next, we investigated the changes in the cellular acidic compartments to detect the occurrence of autophagic cell death. Vital staining of U373-MG cells with acridine orange revealed the appearance of acidic vesicular organelles (AVO) 3 days after AdMH4p27KIP1 infection. These results indicate that the antitumour effect of p27KIP1 on malignant glioma cells was because of autophagic cell death.

DISCUSSION

In the present study, we have demonstrated that p27KIP1 shows a greater antitumour effect than the other CDKIs (p16INK4A,
p27KIP1 plays a central role in the negative control of cell growth. p27KIP1 is generally expressed at high levels in cells arrested by treatment with transforming growth factor-β, contact inhibition or serum deprivation (Kolf et al, 1993; Poljak et al, 1994). In contrast, p27KIP1-deficient mice develop a variety of abnormalities including multiorgan hyperplasia and pituitary tumours (Nakayama et al, 1996). Furthermore, the higher the levels of p27KIP1 expression, the better the prognosis with regard to human malignant gliomas (Alleyne et al, 1999), breast cancer (Porter et al, 1997) or lung cancer (Esposito et al, 1994). In contrast, p27KIP1-deficient cells are markedly hampered in their ability to translocate growth factor receptors, contact inhibition or apoptosis due to p27KIP1 (Schwartz et al, 1993; Bursch et al, 1994; Cheng et al, 1998). p27KIP1 prevents nuclear collapse or precedes it (Schwartz et al, 2001; 2003). Therefore, induction of p27KIP1 gene in tumour cells is expected to be a promising strategy to inhibit their malignant cellular proliferation.

It has been controversial whether p27KIP1 expression leads tumour cells to growth arrest or cell death. Some groups demonstrate the induction of growth arrest by p27KIP1 (Sherr and Roberts, 1995; Craig et al, 1997). Others show that p27KIP1 expression induces apoptosis in several cell lines (Katayose et al, 1997; Schreiber et al, 1999), while p27KIP1 protects cells from apoptosis (Hiromura et al, 1999). In the present study, p27KIP1-induced autophagic cell death, but not apoptosis in malignant glioma cells. Recently, several groups have proposed two types of programmed cell death (Schwartz et al, 1993; Bursch et al, 2000).

In summary, p27KIP1 shows the most potent antitumour effect against malignant glioma cells, while cultured astrocytes are insensitive to p27KIP1 expression. The effect is because of autophagic cell death as well as G0/G1 growth arrest. Therefore, we expect that p27KIP1-based therapy for malignant gliomas might be a promising approach that is worth exploring further.

ACKNOWLEDGEMENTS

We thank Dr Frank L Graham for the recombinant adenoviruses (AdBHGΔI3, AdMH4p16INK4A, AdMH4p18INK4C, AdMH4p9INK4D, AdMH4p21WAF1/CIP1 and AdMH4p27KIP1). This study was supported in part by the USPHS Grants 1R01CA80233 and IR01 CA88936 awarded by the National Cancer Institute, in part by a start-up fund from The University of Texas M.D. Anderson Cancer Center, and by a generous donation from the Anthony D Bullock III Foundation (SK).
REFERENCES

Alleyne Jr CH, He J, Yang J, Hunter SB, Cotsonis G, James CD, Olson JJ (1999) Analysis of cyclin dependent kinase inhibitors in malignant astrocytomas. Int J Oncol 14: 1111–1116

Bursch W, Ellinger A, Gener C, Frohwein U, Schulte-Hermann R (2000) Programmed cell death (PCD). Apoptosis, autophagic PCD, or others? Ann NY Acad Sci 926: 1–12

Cheng M, Sexl V, Sherr CJ, Roussel MF (1998) Assembly of cyclin D-dependent kinase and titration of p27kip1 regulated by mitogen-activated protein kinase kinase (MEK1). Proc Natl Acad Sci USA 95: 1091–1096

Craig C, Wersto R, Kim M, Ohri E, Li Z, Katayose D, Lee SJ, Trepel J, Cowan K, Seth P (1997) A recombinant adenosine expressing p27kip1 induces cell cycle arrest and loss of cyclin-Cdk activity in human breast cancer cells. Oncogene 14: 2283–2289

Esposito V, Baldi A, De Luca A, Groger AM, Loda M, Giordano GC, Caputi M, Baldi F, Pagano M, Giordano A (1997) Progostic role of the cyclin-dependent kinase inhibitor p27 in non-small cell lung cancer. Cancer Res 57: 3381–3385

Guan KL, Jenkins CW, Li Y, Nichols MA, Wu X, O’Keefe CL, Matura AG, Xiong Y (1994) Growth suppression by p18, a p16INK4A/MTS1- and p14ARF/MTS2-related CDK6 inhibitor, correlates with wild-type pRb function. Genes Dev 8: 2939–2952

Hannon GJ, Beach D (1994) p15INK4B is a potential effector of TGF-beta-kinase by TGF-beta. Scienc 260: 536–539

Hannon GJ, Beach D (1994) Is p15INK4B a potent inhibitor of Gl cyclin-dependent kinases? Cell 75: 805–816

Harper JW, Adam GI, Wei N, Keyomarsi K, Elledge SJ (1993) The p21 Cdk-interacting protein Cipl is a potent inhibitor of Gl cyclin-dependent kinases. Cell 75: 805–816

Hirai H, Roussel MF, Kato JY, Ashmun RA, Sherr CJ (1995) Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases CDK4 and CDK6. Mol Cell Biol 15: 2672–2681

Hiromura K, Pippin JW, Fero ML, Roberts JM, Shankland SJ (1999) Modulation of apoptosis by the cyclin-dependent kinase inhibitor p27kip1. J Clin Invest 103: 597–604

Katayose Y, Kim M, Rakkar AN, Li Z, Cowan KH, Seth P (1997) Promoting apoptosis: a novel activity associated with the cyclin-dependent kinase inhibitor p27. Cancer Res 57: 5441–5445

Koff A, Ohtsuki M, Polvak K, Roberts JM, Massague J (1993) Negative regulation of G1 in mammalian cells: inhibition of cyclin E-dependent kinase by TGF-beta. Science 260: 536–539

Komata T, Kondo Y, Koga S, Ko SC, Chung LW, Kondo S (2000) Combination therapy of malignant glioma cells with 2-5A-antisense telomerase RNA and recombinant adenosine virus p53. Gene Therapy 7: 2071–2079

Kondo S, Morimura T, Barret M, Kondo Y, Peterson JW, Kaakaji R, Takeuchi J, Toms SA, Liu J, Werbel B, Barna BP (1996) The transforming activities of MDM2 in cultured neonatal rat astrocytes. Oncogene 13: 1773–1779

Lee MH, Reynisdottir I, Massague J (1995) Cloning of p57kip2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. Genes Dev 9: 639–649

Morgan DO (1995) Principles of CDK regulation. Nature 374: 131–134

Nakayama K, Ishida N, Shirane M, Inomata A, Inoue T, Shishido N, Horii I, Loh DY, Nakayama K (1996) Mice lacking p27kip1 display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. Cell 85: 707–720

Nourse J, Firpo E, Flanagan WM, Coats S, Polvak K, Lee MH, Massague J, Crabtree GR, Roberts JM (1994) Interleukin-2-mediated elimination of the p27kip1 cyclin-dependent kinase inhibitor prevented by rapamycin. Nature 372: 570–573

Paglin S, Hollister T, Delohery T, Hackett N, McMahill M, Sphicas E, Domingo D, Yahalom J (2001) A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. Cancer Res 61: 439–444

Polyak K, Kato JY, Solomon MJ, Sherr CJ, Massague J, Roberts JM, Koff A (1994) p27kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. Genes Dev 8: 9–22

Porter PL, Malone KE, Heagerty PJ, Alexander GM, Gatti LA, Firpo EJ, Daling JR, Roberts JM (1997) Expression of cell-cycle regulators p27kip1 and cyclin E, alone and in combination, correlate with survival in young breast cancer patients. Nat Med 3: 222–225

Reed SI, Bailly E, Dulic V, Hengst L, Resnitzky D, Slingerland J (1994) Gl control in mammalian cells. J Cell Sci 108(Suppl): 69–73

Schreiber M, Muller WJ, Singh G, Graham FI (1999) Comparison of the effectiveness of adenosine vectors expressing cyclin kinase inhibitors p16INK4A, p18INK4C, p19INK4D, p21WAF1/KIP1 and p27kip1 in inducing cell cycle arrest, apoptosis and inhibition of tumorigenicity. Oncogene 18: 1663–1676

Schwartz LM, Smith SW, Jones ME, Osborne BA (1993) Do all programmed cell deaths occur via apoptosis? Proc Natl Acad Sci USA 90: 980–984

Serrano M, Hannon GJ, Beach D (1993) A new regulatory motif in cell-cycle control in mammalian cells. Nature 366: 704–707

Sherr CJ (1994) Gl phase progression: cycling on cue. Cell 79: 551–555

Sherr CJ, Roberts JM (1995) Inhibitors of mammalian Gl cyclin-dependent kinases. Genes Dev 9: 1149–1163

Sherr CJ, Roberts JM (1999) CDK inhibitors: positive and negative regulators of Gl-phase progression. Genes Dev 13: 1501–1512

Toyoshima H, Hunter T (1994) p27kip1, a novel inhibitor of Gl cyclin-Cdk protein kinase activity, is related to p21. Cell 78: 67–74

Xiong Y (1996) Why are there so many CDK inhibitors? Biochim Biophys Acta 1288: 01–05