Restoration of p16INK4A protein induces myogenic differentiation in RD rhabdomyosarcoma cells

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Summary p16INK4A (p16) tumour suppressor induces growth arrest by inhibiting function of cyclin-dependent kinase (CDK)4 and CDK6. Homozygous p16 gene deletion is frequent in primary rhabdomyosarcoma (RMS) cells as well as derived cell lines. To confirm the significance of p16 gene deletion in tumour biology of RMS, a temperature-sensitive p16 mutant (E119G) gene was retrovirally transfected into the human RMS cell line RD, which has homozygous gene deletion of p16 gene. Decrease from 40°C (restrictive) to 34°C (permissive) culture temperature reduced CDK6-associated kinase activity and induced G1 growth arrest. Moreover, RD-p16 cells cultured under permissive condition demonstrated differentiated morphology coupled with expressions of myogenin and myosin light chain. These suggest that deletion of p16 gene may not only facilitate growth but also inhibit the myogenic differentiation of RD RMS cells.

Keywords: p16; rhabdomyosarcoma differentiation; cell cycle

Rhabdomyosarcoma (RMS) is a childhood malignant tumour originating from immature mesenchymal cells that rarely demonstrate myogenic differentiation (Carl et al., 1992). Alveolar RMS has been associated with a characteristic translocation, t(2;13)(q35;q14) (Turc-Carel et al., 1986), which juxtaposes the PAX3 gene known to regulate transcription during early neuromuscular development to the FKHR gene, a member of the forkhead family of transcription factors (Shapiro et al., 1993). Embryonal RMS is associated with loss of heterozygosity (LOH) at the 11p15 locus (Scrable et al., 1987), which affects the expression of insulin-like growth factor (IGF), a growth factor of RMS (El-Badry et al., 1990). Although much has been learned in the past decade regarding molecular genetic alterations associated with the development of RMS, exact mechanisms for aberrant growth without muscle differentiation are still obscure.

p16INK4A (p16) gene induces dephosphorylation of pRB by inhibiting binding of cyclin-dependent kinase (CDK)4 and CDK6 to cyclin D, resulting in G1 growth arrest (Serrano et al., 1993). The discovery that p16 gene is mutated or deleted in a striking proportion of human tumours raised the possibility that abnormalities in p16 might predispose to cancer development (Hirama et al., 1995). Of interest, homozygous deletion of p16 gene was observed only in primary tumours of lymphoid malignancies (Ogawa et al., 1994; Hattar et al., 1995), although point mutations in p16 gene were noted in many other kinds of tumours (Hussussian et al., 1994; Ranade et al., 1995). Moreover, p16 gene deletion is frequently detected in primary tumours, whereas p16 point mutations are associated with progressive cancer and established cell lines rather than primary tumours (Hirama et al., 1995). Therefore, p16 gene deletion might be related with tumorigenesis; on the other hand, p16 gene point mutations may contribute to disease progression. Frequent p16 gene deletion has been reported in RMS cells, including 100% of cell lines and 25% of primary tumours (Iolascon et al., 1996), as in lymphoid malignancies, suggesting its potential importance in tumorigenesis of RMS.

To confirm the significance of p16 gene deletion in tumour biology of RMS, p16 gene was retrovirally transfected into the human RMS cell line RD, which has homozygous gene deletion of p16 gene. Since ectopic expression of p16 suppresses cell growth, a temperature-sensitive p16 mutant (E119G) was used in this experiment. Restoration of functional p16 protein induced not only G1 growth arrest but also myogenic differentiation evidenced by morphological changes and expressions of myogenin and myosin light chain. These data, therefore, suggest that deletion of the p16 gene not only facilitates growth but also inhibits myogenic differentiation of RD RMS cells.

MATERIALS AND METHODS

Cell line and culture

RD, a cell line established from a patient with embryonal RMS (DeGiovanni et al., 1989), was purchased from American Type Culture Collection (Rockville, MD, USA) and maintained in Dulbecco’s modified Eagle medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U ml⁻¹ penicillin (Gibco), 20 mm l-glutamine, and 100 μg ml⁻¹ streptomycin (Gibco) in humidified air with 5% dioxygen and 5% carbon dioxide at 37°C. For differentiation, RD cells were cultured in 2% horse serum plus DMEM.

Polymerase chain reaction

DNA was isolated from RD cells as previously described (Urashima et al., 1996a). Polymerase chain reaction (PCR) was performed on an OmniGene Thermocycler (Marsh Biomedical, Rochester, NY, USA) with 100 ng of genomic DNA, 40 pm of...
sense and antisense primers, 200 mm each of dNTP, 1 × amplification buffer, 1.5 mm magnesium chloride 0.5 ml (2.5 units) Taq polymerase and 10% dimethyl sulfoxide in a reaction volume of 25 μl.

Amplification consisted of 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 1 min. Primers for amplifying p16 exon 2 were 5'-GCT TCC TTT CGG TCA TGCCG-3' and 5'-GGAGT CAT GTC GCC CTG GCT CCT GCCTT-3'. As a control, β-globin sequences were amplified using the following oligonucleotides: sense primer 5'-AAC AGA CAT GGT GCA CC-3', and antisense primer 5'-CTA AGG TGA AGG CTC ATG GC-3'. The resulting PCR products were electrophoresed on an ethidium bromide-stained 3.0% agarose gel. The size of p16 and β-globin products are 393 base pairs (bp) and 362 bp, respectively.

Mutagenesis

The construction of p16 mutants was carried out with BioRad Mutagen Phagemid in vitro Mutagenesis System using full-length p16 cDNA including exons 1, 2 and 3 [provided by Dr Geoffrey I. Shapiro, Dana-Farber Cancer Institute (DFCI), Boston, MA, USA]. Complementary DNA strand for mutated p16 cDNA including exons 1, 2 and 3 (provided by Dr Mark Ewen, DFCI). Of the mutations attempted by cloning into EcoRI/SalI site of pBabe-puro retroviral vector (provided by Dr Mark Ewen, DFCI). The mutations attempted in previous study, a mutation at position 119 (E119G) was found to be restrictive at higher temperature and permissive at lower temperature for binding to CDK4 and CDK6, inhibiting CDK4 and CDK6 complex kinase activities, decreasing phosphorylation of pRB, and inhibiting growth (Urashima et al, 1997c).

Production of retrovirus and transfection

pBabe-puro (control) and pBabe-p16 mutated type (E119G) vector were introduced into Bing packaging cells, obtained from Dr Geoffrey I. Shapiro, Dana-Farber Cancer Institute (DFCI), using standard calcium phosphate transfection technique (Morgenstern and Land, 1990). Bing cells were cultured for 1-day post-transfection in DMEM with 10% FBS, and supernatant was exchanged with fresh media for an additional 2 days. Retroviral supernatants were then harvested post-transfection, and filtered through a 0.45 μm filter to remove living cells. RDÐRMS cells were introduced into Bing packaging cells, obtained from Dr Geoffrey I. Shapiro, Dana-Farber Cancer Institute (DFCI), for 3 h, followed by addition of fresh media (7 ml). Retroviral supernatants were then harvested post-transfection, and filtered through a 0.45 μm filter to remove living cells. RDÐRMS cells were cultured on a 100 mm tissue culture plate for 18 h prior to infection. Supernatant was exchanged with 3 ml infection cocktail consisting of fresh retroviral-containing supernatant and polybrene at a final concentration of 4.0 μg ml⁻¹ (Sigma, St Louis, MO, USA) for 3 h, followed by addition of fresh media (7 ml). Selection for mutated p16 gene transduced RD (RD-p16) cells and control vector transfectant (RD-control) was performed by culture with puromycin (2.0 μg ml⁻¹)(Sigma) (Urashima et al, 1997a). A colony expressing the highest level of p16 protein was selected at 2 weeks and amplified for a further 2 weeks at 40°C.

Immunoprecipitation and Western blotting

Immunoprecipitation (IP) and Western blotting (WB) were performed as previously described (Urashima et al, 1996b). For IP, cells (2 × 10⁶ cells/sample) were washed thrice with phosphate-buffered saline (PBS) and lysed for 30 min at 4°C in buffer: 1 mM Tris-HCl (pH 7.6), 150 mM sodium chloride, 0.5% Nonidet p-40, 5 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, sodium phosphate (v), aprotinin, and 1 mM NaF. Anti-p16 monoclonal antibody (Ab) (Pharmigen, San Diego, CA, USA); anti-CDK6 polyclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added for 16 h at 4°C to immunoprecipitate protein complexes. Proteins were collected using protein G sepharose (PGS). Aliquots of each lysate were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were transferred onto PVDF membrane (NEN Dupont, Boston, MA, USA), and non-specific binding was blocked by incubation with 5% skim milk. The membrane was probed with Ab followed by anti-mouse or anti-rabbit Ig Abs conjugated with horseradish peroxidase (HRP; Amersham, Arlington Heights, IL, USA). Complexes were detected using the enhanced chemiluminescence system (Amersham). To characterize differential stage of RD cells, IP and WB were performed using anti-p18, anti-p21, anti-myogenin polyclonal Abs (Santa Cruz Biotechnology); anti-Myo D monoclonal Ab (Santa Cruz Biotechnology); anti-myosin light chain monoclonal Ab (Sigma).

Immune complex kinase assays

Immune complex kinase assays were performed as previously described with modification (Urashima et al, 1997b). Briefly, cells (1 × 10⁶ ml⁻¹) were suspended in lysis buffer, centrifuged, and supernatants precipitated for 16 h at 4°C with PGS plus rabbit anti-CDK6 polyclonal Ab. Immunoprecipitated proteins on PGS were washed thrice with 1 ml of lysis buffer and twice with 50 ml mol⁻¹ HEPES (pH 7.5) containing 1 mol mol⁻¹ dithiothreitol and suspended in 30 μl of kinase buffer (50 ml mol⁻¹ HEPES, 10 ml mol⁻¹ MgCl₂, 1 mol mol⁻¹ dithiothreitol) containing substrate and 2.5 ml mol⁻¹ EGTA, 10 ml mol⁻¹ β-glycerophosphate 0.1 mol mol⁻¹ sodium orthovanadate, 1 mol mol⁻¹ NaF, 20 ml mol⁻¹ adenosine 5'-triphosphate (ATP), and 10 μCi of γ³²P-ATP (NEN Dupont, Boston, MA, USA). For CDK6 kinase assays, 1 μg of soluble glutathione S-transferase (GST)–RB fusion protein (Santa Cruz Biotechnology) was used as the substrate. After incubation for 30 min at 30°C with occasional mixing, the samples were boiled in polyacrylamide gel sample buffer and separated by SDS electrophoresis. Phosphorylated proteins were visualized by autoradiography of the dried slab gels.

Cell cycle analysis

Cell cycle distribution of RD cells was examined using propidium iodide (PI; Sigma) staining and FACS analysis, as in a previous report (Urashima et al, 1997c). Briefly, cells were collected and suspended in 0.5 ml of 3.4 mM sodium citrate, 10 mM NaCl, 0.1% NP-40 and 50 mg ml⁻¹ PI to stain nuclear DNA. Cell cycle distribution for each sample (> 10 000 cells) was determined using the flow cytometer (Ortho-Clinical Diagnostics KK, Koto-ku, Tokyo, Japan).

RESULTS

Homzygous deletion of p16 gene and ectopic expression of p16 protein in RD RMS cells

We first confirmed homozygous deletion of the p16 gene (exon 2) in RD–RMS cells using mixed primers for exon 2 of the p16 gene and β-globin gene in a PCR assay. As can be seen in Figure 1, p16 exon 2 was not detectable in genomic DNA of RD–RMS cells by...
The β-globin gene served as control. Once we confirmed homozygous deletion of p16 gene in RD cells, a temperature-sensitive mutated p16 gene (E119G) was ectopically expressed in RD cells by retroviral transfection. p16 protein was expressed in the p16 gene transfected RD (RD–p16) cells at either 40°C or 34°C, but it was not detectable within control vector transfected RD (RD–control) cells at either 40°C or 34°C (Figure 2). Expression of CDK6 protein was equivalent in these cells. However, activity of CDK6 was inhibited in cell lysates of RD-p16 cells cultured at 34°C compared with RD-control cells and RD-p16 cells cultured at 40°C.

Effect of functional p16 protein on proliferation of RD cells

RD-control and RD-p16 cells were cultured for 72 h at either 40°C or 34°C, and cell cycle distribution was determined by PI staining (Figure 3A). At 40°C, the percentage of cells in G1 phase was similar in RD–control cells (47%) and RD–p16 cells (51%). In contrast, the percentage of RD-p16 cells in G1 phase at 34°C increased to 73%, although cell cycle distribution of RD-control cells did not significantly vary with culture temperature. Moreover, sub G0 population including apoptotic and dead cells was not observed in all conditions. Viable cell number did not...
Effect of functional p16 protein on morphology and differentiation of RD cells

Under phase contrast microscopy, RD–p16 cells cultured at 34°C demonstrated a differentiated morphology, with elongated myotube-formation and multinuclei, whereas RD–p16 cells cultured at 40°C, as well as RD–control cells cultured at 40°C and 34°C, demonstrated round form or fibroelastic morphology (Figure 4). Although RD cells cultured in differentiation medium (2% horse serum) at 37°C showed differentiation tendency (Figure 4), effect of active p16 expression at 34°C did not enhance the differentiation phenotype significantly (data not shown).

RD–p16 cells were cultured under either restrictive (40°C) or permissive (34°C) condition for 7 d and RD cells were cultured in differentiation medium for 7 d to examine for expressions of p18, p21, Myo D, myogenin, and myosin light chain proteins (Figure 5). Expressions of p18, p21, and Myo D proteins were unchanged in RD–control cells and RD–p16 cells cultured at either 40°C or 34°C, and were lower than in RD cells cultured in differentiation medium. On the other hand, expression level of myogenin protein was low in RD–control cells cultured at either 40°C or 34°C and RD–p16 cells cultured at 40°C, whereas it was significantly higher in RD–p16 cells cultured at 34°C and in RD cells cultured in differentiation medium. Myosin light chain protein was also strongly expressed in RD–p16 cells cultured at 34°C and RD cells cultured in differentiation medium, whereas it was not detectable in RD–control cells cultured at either 40°C or 34°C and RD–p16 cells cultured at 40°C.

DISCUSSION

In the present report, we demonstrated that lack of p16 gene may both facilitate cell proliferation and inhibit myogenic differentiation using RD–RMS cells. We first confirmed homozygous deletion of p16 gene exon 2 in RD cells by PCR, and lack of p16 protein by IP and WB. An alternative RNA transcript for p16 (p16β) has been identified (Mao et al, 1995; Stone et al, 1995), and is composed of exon 1β, upstream from exon 1α of p16, spliced onto the remaining exons 2 and 3 of p16. Although p16β transcript was not studied in this experiment, homozygous deletion of p16 exon 2 may suggest lack of the p16β transcript. Next p16 protein was expressed ectopically in RD–RMS cells using retroviral transfection in order to investigate the role of homozygous deletion of p16 gene in growth and differentiation of RMS cells. However, growth inhibition in RD cells ectopically expressing p16, coupled with the outgrowth of clones that express low levels of this protein, complicate this approach. To overcome this limitation, we attempted to create p16 temperature-sensitive mutants (Urashima et al, 1997).

In previous reports, function of p16 is believed to associate with growth inhibition, cell mortality and senescence (Serrano et al, 1996, 1997). However, in this study, restoration of functional p16 protein in RD cells also induced myogenic differentiation, associated with morphological changes and with expression of myosin light chain protein, which is specifically expressed in differentiated myocytes and syncytiotubules but not in proliferating myoblasts (Andres and Walsh, 1996). Moreover, p21 is expressed low in proliferating myoblasts but high in differentiated myocytes (Havery et al, 1995; Parker et al, 1995). In this study, p21 and p18 expression increased slightly in RD cells cultured in differentiation medium, whereas they were not altered in RD cells with

increase in RD–p16 cells cultured at 34°C, but increased in RD–control cells cultured at 40°C and 34°C and RD–p16 cells cultured at 40°C (Figure 3B). Percentages of dead cells counted with trypan blue were less than 10% in all conditions.
activated p16. In a normal murine myoblast model, myogenic differentiation was inhibited by forced expression of cyclin D1; in contrast, myoblast differentiation was enhanced by transfection with p16 (Skapek et al, 1995). p16, therefore, may be associated contrast, myoblast differentiation was enhanced by transfection differentiation was inhibited by forced expression of cyclin D1; in activated p16. In a normal murine myoblast model, myogenic ACKNOWLEDGEMENT

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