Modeling mutations in the G₁ arrest pathway in human gliomas: overexpression of CDK4 but not loss of INK4a-ARF induces hyperploidiy in cultured mouse astrocytes

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Nearly all human gliomas exhibit alterations in one of three genetic loci governing G₁ arrest: INK4a-ARF, CDK4, or RB. To discern the roles of CDK4 amplification and INK4a-ARF loss in gliomagenesis, we compared the behavior of astrocytes lacking a functional INK4a-ARF locus with astrocytes overexpressing CDK4. Either a deficiency of p16INK4a and p19ARF or an increase in CDK4 allows cultured astrocytes to grow without senescence. Astrocytes overexpressing CDK4 grow more slowly than INK4a-ARF-deficient astrocytes and convert to a tetraploid state at high efficiency; in contrast, INK4a-ARF-deficient cells remain pseudodiploid, consistent with properties observed in human gliomas with corresponding lesions in these genes.

Results and Discussion

Loss of INK4a-ARF and overexpression of CDK4 both immortalize astrocyte cultures

To compare the growth properties of INK4a-ARF-deficient and CDK4-overexpressing astrocytes, we subjected appropriately selected cultures to repeated passage at standard density and counted the number of cells at each passage. Astrocytes overexpressing CDK4 were prepared by infecting primary brain cultures from Gtv-a mice carrying a tv-a transgene under the control of the astrocyte-specific glial fibrillary acidic protein (GFAP) promoter with an RCAS vector carrying the human CDK4 cDNA (RCAS-cdk4). Cultures of INK4a-ARF-deficient astrocytes were prepared by infecting primary brain cell cultures from Gtv-a transgenic; INK4a-ARF−/− mice with an RCAS vector bearing the puro-R gene (RCAS-puro) (Holland et al. 1998) and selecting for resistance to puromycin. Parallel cultures were prepared by infecting brain cells from Gtv-a transgenic mice with RCAS vectors carrying the alkaline phosphatase (AP) or the basic fibroblast growth factor (bFGF) coding sequences.

As illustrated in Figure 1A, control astrocytes underwent about three or four cell doublings, gradually entered senescence, and failed to survive beyond ~50 days. Astrocytes infected with RCAS-bFGF, which induce proliferation and migration of glial cells in vivo (Holland and Varmus 1998; also see Fig. 4, below), grew to slightly greater numbers than control astrocytes, as expected in view of the known mitogenic effects of bFGF on cultured astrocytes (Hou et al. 1995) but did not survive significantly longer. In marked contrast, INK4a-ARF−/− astrocytes exhibited no loss of growth potential with passage and could be propagated indefinitely, consistent with the behavior of mouse embryo fibroblasts (MEFs) with lesions in this locus (Alcorta et al. 1996; Nobel et al. 1996) or in ARF alone (Kamijo et al. 1997). Furthermore, astrocytes infected with RCAS-cdk4 also escaped senescence, implying that both INK4a-ARF deficiency and excess CDK4 allow immortalization of astrocytes. However, the growth rates for these two populations were markedly different; INK4a-ARF−/− cultures grew at a rapid and constant rate, whereas the CDK4-immortalized cultures initially grew slowly and then increased in rate over time.

We demonstrated that the INK4a-ARF−/− and CDK4-immortalized cultures were glia by staining cells with antibodies to GFAP (Bignami and Dahl 1976) and to nestin, an intermediate filament protein expressed in CNS progenitors (Tohyama et al. 1992). Gtv-a transgenic pri-
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CDK4 overexpression induces hyperploidy that is INK4a-ARF dependent

Tumor cells, including glioma cells, often contain an abnormal number of chromosomes (Bigner and Mark 1984), and hyperploidy occurs when mechanisms for control of cell cycle progression have been disrupted (Takahara et al. 1995). Therefore, we asked whether euploidy is maintained in cultured astrocytes with a deficiency of INK4a-ARF or an excess of Cdk4, and whether any abnormalities in ploidy are correlated with specific genetic mutations, as reported in human gliomas (van Meyel et al. 1994). Flow cytometry was used to assess DNA content in astrocytes with different passage histories after maintenance at confluence or after addition of nocodazole.

By these measures, six of six astrocyte cultures independently infected with RCAS-cdk4 were mostly converted to tetraploid status within 15–20 population doublings (PD), at a rate approaching 10% of cells per generation (Fig. 2A). After 75 PDs, virtually all CDK4-expressing cells had twice the normal amount of DNA at confluence, and nearly half of the nocodazole-treated cells were arrested in G2/M, but <10% of the cells had an 8N DNA content (Fig. 2B, right). These findings were confirmed by direct inspection of metaphase chromosomes (Fig. 2D); chromosome numbers in metaphase spreads from INK4a-ARF-
deficient cells were -40 and thus diploid or pseudodiploid, whereas CDK4-overexpressing cells had approximately twice the normal number of chromosomes.

The number of recognized genetic alterations capable of immortalizing cells in culture is relatively small; and of those, only loss of p53 function and hyperproduction of Myc protein have been reported previously to induce hyperploidy. The mechanism by which elevated levels of Cdk4 induce hyperploidy is unknown but in some way must result from endoduplication of chromosomes and aberrant cell cycle arrest in G2.

We next examined the DNA content of INK4a-ARF+/−; Gtv-a astrocytes infected with RCAS-cdk4 and producing at least 10-fold more Cdk4 than normal cells (Fig. 1E). Surprisingly, these cells maintained pseudodiploidy, implying a requirement for one or both of the products of the INK4a-ARF locus for induction of inappropriate rounds of DNA replication by Cdk4. p19ARF functions by binding to Mdm2 and thereby inactivating p53 (Kamijo et al. 1997; Pomerantz et al. 1998; Zhang et al. 1998) and is involved in both G1 and G2 arrest (Quelle et al. 1997). Therefore, it may be a more likely candidate to promote Cdk4-induced hyperploidy than p16INK4A, which is known only to inhibit Cdk4 and block passage from G1 to S phase. Overexpression of CDK4 in astrocytes derived from mice with targeted mutations specific for p16INK4A or p19ARF would help identify the INK4a-ARF product required for Cdk4-induced hyperploidy.

Curiously, although mutations in p53 lead to aneuploidy in culture, a mutation that specifically eliminates the production of p19ARF results in pseudodiploid immortalized cells (Kamijo et al. 1997). It is not known whether the shift in ploidy seen in p53−/− cells is dependent on the presence of wild-type p19ARF or p16INK4A as appears to be the case for the ploidy shift due to cdk4 overexpression.

Cdk4-induced immortalization and hyperploidy can occur independent of p53 mutations

Mutations in p53 are associated with loss of growth control and chromosomal instability (Levine 1993). Thus, secondary mutations of p53 in astrocytes infected with RCAS-cdk4 could be responsible for the properties described above. To address this possibility we analyzed two independent populations of RCAS-cdk4-infected Gtv-a astrocytes that had been maintained in culture for 3 months. Both populations (cdk4.1 and cdk4.2) showed elevated levels of Cdk4 by Western analysis (Fig. 1D). Flow cytometry of these two populations (performed on day 6 of the experiment shown in Fig. 3) demonstrated that cdk4.1 cells are mostly tetraploid and cdk4.2 cells mostly octaploid.

We judged p53 status using three criteria: immunohistochemical staining with antibodies specific for the mutant conformation of p53; the level of total p53 by Western blot analysis; and the induction of p21 after DNA damage. By all three criteria the cdk4.1 population showed no evidence of mutant p53 (Fig. 3). These data imply that in the cdk4.1 culture, immortalization and hyperploidy occurred in the presence of wild-type p53 protein and normal p53 function.

The cdk4.2 culture, in contrast, stained positively with the antibody for the mutant p53 and failed to induce p21 after camptothecan treatment. The cdk4.2 population was maintained in culture for an additional 6 months (cdk4.2∗) and analyzed for ploidy, growth rate, and p53 status. The cdk4.2∗ cells demonstrated increased growth rate relative to that seen at 3 months (Fig. 1C), maintained evidence of mutant p53 by immunocytochemical staining, and lacked p21 induction after camptothecan treatment. Surprisingly, flow cytometry showed that the population shifted from being mainly octaploid at 3 months to mainly tetraploid at 9 months. Presumably, the increased growth rate observed between the cdk4.2 population after 6 additional months in culture reflects the further occurrence of mutations, epigenetic events, or both.
The existence of a CDK4-immortalized and hyperploid culture with wild-type p53 function (cdk4.1) implies that CDK4-overexpressing astrocytes do not require mutations in p53 to achieve extended proliferation and genomic instability. The extended life span of the cdk4.1 population could be formally explained by a secondary mutation in INK4a–ARF or ARF alone. However, this is unlikely, as INK4a–ARF; CDK4-overexpressing astrocytes are pseudodiploid and rapidly proliferating, whereas the cdk4.1 population is polyploid and grows more slowly. Furthermore, Southern analysis of the cdk4.1 population with the p19ARF cDNA did not demonstrate deletions or alterations in the genomic structure of the INK4a–ARF locus (data not shown). These data imply that additional mutations in p53 or INK4a–ARF are not required for immortalization of CDK4-overexpressing astrocytes. In contrast, immortalization of MEFs by myc overexpression selects for those that lose either p53 or p19ARF function (Zindy et al. 1998) and MEF cultures selected for spontaneous immortalization develop mutations in p53 or ARF (Kamijo et al. 1997). Although mutations in p53 have long been known to result in both immortalization of cells in culture and hyperploidy, the pathways leading to these effects are not completely understood. One of the effects of p53 is to increase the concentration of p21, which, in turn, inhibits Cdk4. Therefore, loss of p53 function might be expected to result in higher Cdk4 activity. It would be valuable to know whether the immortalization and ploidy shifts seen in p53-deficient cells are a result of, or dependent on, inappropriate elevations of Cdk4 activity.

Overexpression of cdk4 by astrocytes in vivo does not induce proliferation

To gauge the physiological significance of Cdk4 overproduction in astrocytes in vivo, we infected newborn Gtv-a transgenic mice with RCAS-cdk4. With this method, cells lining the injection track are coinfected with RCAS-AP to monitor cells (Holland and Varmus 1998). AP+ cells are no more numerous or widely dispersed after coinfection with a mixture of RCAS-AP and RCAS-cdk4 than after infection with RCAS-AP alone (Fig. 4B,C). In contrast, as described previously (Holland and Varmus 1998), AP+ cells are highly abundant and spread over a large expanse of the brain after coinfection with a mixture of RCAS-AP and RCAS-bFGF (Fig. 4A). Thus, by itself, excessive levels of Cdk4 do not appear to perturb the proliferative or migratory behavior of astrocytes in vivo.

The absence of gliomas in INK4a-ARF-deficient mice (Serrano et al. 1996) suggests that a lack of both p16INK4a and p19ARF is insufficient to produce grossly abnormal growth of glial cells in vivo; this is similar to our results with gene transfer of cdk4 to astrocytes in Gtv-a transgenic mice (Fig. 4). However, either INK4a-ARF loss or CDK4 overexpression can immortalize astrocytes in vitro; thus, additional limitations on proliferation may be mediated by unknown mechanisms in vivo. Of note, cultures of both INK4a-ARF−/− and CDK4-immortalized cells arrest in G1 upon reaching confluence, implying the

Figure 3. Mutations in p53 can, but do not necessarily, arise in CDK4-immortalized populations. (A) Immunoperoxidase staining of cell cultures using a monoclonal antibody to the mutant conformation of p53. Wild type, cdk4.1, cdk4.2, cdk4.2*, and INK4a−; cdk4+ are as in Fig. 1, (HKA) human keratinocyte cell line known to harbor a p53 mutation. (B) Western analysis for p53 in the indicated cultures. (C) Western analysis for p21 in cultured cells with and without camptothecin-induced DNA damage.

Figure 4. Glial-specific CDK4 gene transfer does not result in proliferation in vivo. An equal mixture of cells producing RCAS-AP and RCAS-bFGF (A), RCAS-AP alone (B), or RCAS-AP and RCAS-cdk4 (C) was injected into the right frontal lobe of Gtv-a mice. The mice were sacrificed and brains (40-μm sections) analyzed for AP activity at 10 weeks of age and counterstained.
existence of an intact G1-arrest pathway in these cells. Identification of factors and pathways causing growth arrest of either INK4a-ARF−/− or CDK4-immortalized astrocytes in culture may help elucidate the mechanism for arrest of these cells in vivo.

Recently, we have reported the utility of transgenes expressing tv-a in the glial lineage in intact animals (Holland and Varmus 1998; Holland et al. 1998). Here we demonstrate that primary cell lines from such tv-a transgenic animals can be used to perform high efficiency gene transfer to defined cells within a mixture of cell types. Selection for specific, infectable cell types is possible if the population is initially infected with RCAS-puro and subsequently grown in puromycin. The ability to manipulate populations of primary astrocytes genetically in culture has allowed us to study the effects of individual alterations in the G1 arrest pathways, something not possible in established cell lines in which the endogenous G1 arrest pathway has already been altered.

In Holland et al. (1998) we describe the contributions that excess Cdk4 or loss of the INK4a-ARF gene products make to gliomagenesis. We find that a constitutively active, mutant EGFR is insufficient to induce gliomas in mice, but mutant EGFR can induce glioma formation either in INK4a-ARF−/− mice or, less often, in combination with excess Cdk4. Furthermore, mutant EGFR does not induce gliomas in p53-deficient mice unless Cdk4 is overexpressed. Our results demonstrate that INK4a-ARF loss and Cdk4 overexpression not only immortalize astrocytes in culture, as shown here, but are also important components of gliomagenesis in mice. The fact that p53 mutations arise in some CDK4-immortalized astrocyte cultures implies that p53 loss can provide a growth advantage to these cells. The results in Holland et al. (1998) suggest that CDK4 overexpression and p53 loss also cooperate in gliomagenesis. These apparent interactions between components of the cell cycle arrest pathways in mice and in cultured mouse astrocytes resemble the genetic abnormalities found in human gliomas. Most notably, results in the two species illustrate the importance of disrupting the p16INK4a-Cdk4-Rb pathway, the nonequivalence of mutations in the pathways that govern G1-S transition, and the apparent synergy between CDK4 overexpression and p53 loss. Taken as a whole, these observations indicate that behavior of genetically defined primary astrocyte cultures reflects many aspects of gliomagenesis both in mice and man.

Materials and methods

Transgenes and viral vectors

Construction of the Gtv-a transgene and RCAS-AP and RCAS-bFGF have been described (Holland and Varmus 1998). The Gtv-a mouse line was originally generated from an FVB/N crossed with a C57B6 × BALB/c F2. The Gtv-a founder was then bred to an FVB/N to generate F2 progeny that have subsequently been interbred to maintain the transgenic line. RCAS-puro was obtained from Steve Hughes (National Cancer Institute). RCAS-cdk4 was constructed by Cial digestion of RCAS-puro to remove the Escherichia coli puromycin resistance gene and replacement with a BstBI–Clal fragment from pcdk4.1 (gift from Robert Sikorski), which contains the complete human CDK4 cDNA (Matsushime et al. 1992). RCAS–GFP was a gift of Connie Cepko (Harvard University, Cambridge, MA).

Cell culture

Primary brain cell cultures from newborn transgenic mice were obtained by mechanical dissociation of the whole brain, followed by digestion with 0.25% trypsin for 15 min at 37°C. Large debris was allowed to settle, and single cells were plated and grown in DMEM with 10% FCS (GIBCO BRL). DF-1 cells (gift from D. Foster; Schaefer-Klein et al. 1998) were grown in DMEM with 5% FCS, 5% calf serum, 1% chicken serum, and 10% tryptose phosphate broth (GIBCO BRL).

Infection with RCAS vectors

The supernatant from DF-1 cells infected with and producing RCAS vectors was filtered through a 0.45-μm filter and plated directly onto primary brain cell cultures from Gtv-a mice. INK4a-ARF−/−; Gtv-a cultures were generated from the F2 progeny of Gtv-a mated with mice having targeted deletions of INK4a-ARF (gift of Ron DePinho, Harvard Medical School, Boston, MA). These primary brain cultures were infected with filtered medium from RCAS-puro-producing cells and then selected in 4 μg/ml puromycin. To infect cells in Gtv-a transgenic mice, DF-1 cells infected with RCAS vectors were harvested by trypsin digestion and pelleted by centrifugation, the cell pellets were resuspended in −50 μl of medium, and placed on ice. Using a 10-μl gas-tight Hamilton syringe, a single intracranial injection of 1 μl (containing 106 cells) was made in the right frontal region, just anterior to the striatum, with the tip of the needle just touching the skull base.

Brain sectioning and staining

Animals were sacrificed at 10 weeks of age, the brains fixed in 4% formaldehyde, 0.4% glutaraldehyde, 1× PBS for 36 hr, and dehydrated in 20% sucrose, 2% glycerol, and 1× PBS. Frozen sections (40 μm) were obtained using a sledge microtome (Zeiss) and stained in solution for alkaline phosphatase activity using 5-bromo-4-chloro-indolyl-phosphate and 4-nitro-blue-tetrazolium-chloride (Boehringer), after treatment at 65°C (pH 9.5) for 30 min to remove endogenous alkaline phosphatase activity. The sections were then mounted on glass slides and counterstained with hematoxylin and eosin.

Flow cytometry

Cells were either grown to confluence and maintained for 24 hr or treated with 0.12 µg/ml nocodazole (Sigma) for 16 hr. Cells (5 × 106) were harvested by trypsin digestion, centrifuged, resuspended in 500 μl of propidium iodide solution (Electa), incubated for 20 min at 37°C, and analyzed on a Beckman FaxScan using ModFit LT software (Verity).

Immunofluorescence and immunohistochemistry

Cell cultures were grown on glass coverslips precoated with 0.1 mg/ml poly-c-ornithine (Sigma). For staining with anti-GFAP antibodies, cells were fixed in 4% paraformaldehyde (pH 7.4 in PBS) for 15 min, permeabilized in 95% ethanol/5% acetic acid for 10 min, and incubated with 1:300 diluted rabbit anti-human GFAP antibody (Chemicon) for 1 hr. After incubation with fluorescein- or rhodamine-conjugated goat anti-rabbit (GAR; Cappel-Organon Teknika) for 45 min, cells were washed extensively in PBS and mounted in Vectashield (Vector Laboratories). For staining with anti-nestin antibody, cells were fixed and permeabilized as described above and incubated overnight at 4°C with an anti-nestin polyclonal rabbit antibody (gift from Ron McKay; Tohyama et al. 1992) (1:1000; in 1% fetal bovine serum + 0.5% bovine serum albumin). After incubation with fluorescein- or rhodamine-conjugated GAR for 45 min at room temperature, cells were washed extensively in PBS and mounted in Vectashield. The immunofluorescence micrographs presented are representative of two to three experiments and were taken on a Zeiss Axiophot fluorescence microscope (40× N-ko- fluar objectives).

For detecting mutant p53, cells were initially fixed with 100% methanol and incubated with Tris-buffered saline (pH 8.0), 0.1% Tween 20, (TBST) 5% dried milk, and 1% goat serum (TBST). A mouse monoclonal antibody recognizing mutant p53 in nondenaturing conditions (Ab-3, Oncogene Science) was incubated at a 1:200 concentration in TBST for 1 hr. After incubation with fluorescein- or rhodamine-conjugated goat anti-rabbit (GAR; Cappel-Organon Teknika) for 45 min, cells were washed extensively in PBS and mounted in Vectashield. The immunofluorescence micrographs presented are representative of two to three experiments and were taken on a Zeiss Axiophot fluorescence microscope (40× N-ko-fluar objectives).

For detecting cell proliferation, cultures were stained with fluorescein- or rhodamine-conjugated GAR for 45 min at room temperature, cells were washed extensively in PBS and mounted in Vectashield. The immunofluorescence micrographs presented are representative of two to three experiments and were taken on a Zeiss Axiophot fluorescence microscope (40× N-ko-fluar objectives).

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Metaphase spread

Cells were treated with 0.02 mg/ml Colcemid (Sigma) for 6 hr and harvested by trypsin digestion and centrifugation. The cells were resus-
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