Efficiency Alleles of the *Pctr1* Modifier Locus for Plasmacytoma Susceptibility†

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The susceptibility of BALB/c mice to pristane-induced plasmacytomas is a complex genetic trait involving multiple loci, while DBA/2 and C57BL/6 strains are genetically resistant to the plasmacytomagenic effects of pristane. In this model system for human B-cell neoplasia, one of the BALB/c susceptibility and modifier loci, *Pctr1*, was mapped to a 5.7-centimorgan (cM) chromosomal region that included *Cdkn2a*, which encodes p16^{INK4a} and p19^{ARF}, and the coding sequences for the BALB/c p16^{INK4a} and p19^{ARF} alleles were found to be polymorphic with respect to their resistant *Pctr1* counterparts in DBA/2 and C57BL/6 mice (45). In the present study, alleles of *Pctr1*, *Cdkn2a*, and *D4Mit15* from a resistant strain (BALB/cDAG) carrying DBA/2 chromatin were introgressively backcrossed to the susceptible BALB/c strain. The resultant C.DAG-*Pctr1* *Cdkn2a* *D4Mit15* congenic was more resistant to plasmacytomagenesis than BALB/c, thus narrowing *Pctr1* to a 1.5-cM interval. Concomitantly, resistant C57BL/6 mice, from which both gene products of the *Cdkn2a* gene have been eliminated, developed pristane-induced plasma cell tumors over a shorter latency period than the traditionally susceptible BALB/cAn strain. Biological assays of the p16^{INK4a} and p19^{ARF} alleles from BALB/c and DBA/2 indicated that the BALB/c p16^{INK4a} allele was less active than its DBA/2 counterpart in inducing growth arrest of mouse plasmacytoma cell lines and preventing *ras*-induced transformation of NIH 3T3 cells, while the two p19^{ARF} alleles displayed similar potencies in both assays. We propose that the BALB/c susceptibility/modifier locus, *Pctr1*, is an “efficiency” allele of the p16^{INK4a} gene.

Susceptibility to cancer in human populations is a complex genetic trait. However, the field of cancer susceptibility has been defined largely by studying familial human cancers and targeted animal models that have sustained either gain- or loss-of-function mutations in genes, resulting in phenotypes which typically result in all-or-none responses. Therefore, the activation of oncogenes and the loss of tumor suppressor genes have dominated the field of cancer genetics as the effectors of tumor initiation, promotion, and progression. In contrast to all-or-none alleles, certain alleles of genes are partially functional, resulting in changes in the efficiencies of the proteins they encode. Due to their quantitative properties, natural alleles of genes that alter the efficiency of protein function have remained largely elusive in experimental studies of tumor susceptibility. However, putative “efficiency” alleles that modify tumor susceptibility might actually occur more frequently in people than all-or-none alleles and could make important contributions to cancer pathogenesis.

The *Pctr1* (22) and *Mom1* (7) loci were among the first modifiers of tumor susceptibility and resistance phenotypes identified by genome scans of restriction fragment length polymorphism and simple sequence length polymorphism markers,

respectively. The *Mom1* locus, occupied by the *Pla2g2a* gene (6, 19), was identified as a modifier of *min* (*Apc*), whose human ortholog is a major determinant of colon cancer. *Mom1* modifies *Apc*^{min} mutant mice by modulating the tumor microenvironment to control the number of adenomas that develop in their intestines (6, 7, 19). Strains whose *Mom1* locus contains a susceptibility allele have sustained a loss-of-function frameshift mutation.

Susceptibility to plasmacytomas in mice is a complex genetic trait, with at least five *Pctr* susceptibility-resistance loci determining whether mice develop hematopoietic tumors of the plasma cell lineage in response to induction with pristane, an oil which elicits a chronic inflammatory response (21, 22). The modifier loci *Pctr1*, -2, and -3 are linked to mouse chromosome 4 in noncontiguous portions of the chromosome (21, 22, 28, 45). The cyclin-dependent kinase inhibitor gene *Cdkn2a*, which encodes both p16^{INK4a} and p19^{ARF} products, falls within the *Pctr1* region of chromosome 4, and BALB/cAn mice were found to carry a rare allele of the *Cdkn2a* gene (45). Until now, none of the *Pctr* loci have been molecularly identified, as there was no genetic evidence to directly implicate the *Cdkn2a* locus or the relative roles of p16^{INK4a} and p19^{ARF} in mouse plasmacytomagenesis.

In this report we provide in vivo evidence from congenic and knockout studies that the *Cdkn2a* locus can modify susceptibility to pristane-induced plasmacytomagenesis. Moreover, cell culture-based complementation studies support the conclusion that p16^{INK4a} is functionally impaired in BALB/cAn plasma

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† We dedicate this paper to the memory of Richard P. Nordan.

cells and can be specifically corrected by the DBA/2 allele. In contrast, the biological potencies of BALB/c and DBA/2 p19^{ARF} were found to be identical. Correspondingly, p16^{INK4a}, but not p19^{ARF}, from BALB/c mice was less effective than DBA/2 p16^{INK4a} at suppressing *ras* transformation in NIH 3T3 cells.

MATERIALS AND METHODS

Mice. BALB/cDAG mice were obtained from Hillyard Festenstein (Laboratory Animal Center, Carshalton, England). They were originally created by immunizing BALB/c mice with cells bearing LY6 alloantigens from DBA/2 mice. When these mice were brought into our conventional colony, we performed a modest genome scan and identified DBA markers as passenger segments on chromosomes 3 (*D3Mit21*), 4 (*Mtv13*), 11 (*D11Mit2*), 15 (*Ly6*), and 17 (*Qa*). BALB/cDAG mice were found to be resistant to plasmacytomagenesis. C.DAG-*Mtv13* mice were created from the BALB/cDAG mice by introgressively backcrossing DBA/2 *Mtv13* and *D4Lgm1* alleles onto a BALB/c background. DBA/2 alleles on chromosomes 2, 11, 15, and 17 were actively selected against, and the subsequent C.DAG-*Mtv13* mouse carried BALB/c alleles on these chromosomes. This strain was found to carry approximately 6 centimorgans (cM) of genetic material from DBA/2 mice. When BALB/c mice were found to carry a rare allele of *Cdkn2a*, we made the C.DAG-*Cdkn2a* *D4Mit15* N20F5 mouse by selecting for DBA/2 alleles of *Cdkn2a* and *D4Mit15* and BALB/c alleles of *Mtv13*. This mouse was found to carry approximately 1.5 cM of DBA chromatin surrounding the *Cdkn2a* and *D4Mit15* loci.

The *INK4a* knockout mice, with portions of exons 2 and 3 replaced by a *neo* cassette, were bred at Albert Einstein College of Medicine to N3F3 (36). They were transferred to a conventional mouse facility at the National Institutes of Health, where they were backcrossed onto the C57BL/6N background for an additional generation. Mice heterozygous for the knockout were intercrossed, and homozygous mice (N4F4) were tested for susceptibility and resistance to plasmacytomagenesis following two 0.5-ml injections of pristane. The N4F4 mice were examined for allelic variation at three microsatellite markers per chromosome, with special emphasis on markers linked to the *Pctrl* to -4 and *Pctm* modifier loci. For each interval examined, 129 mice carried the same allele as resistant C57BL/6 and DBA/2 mice. Specifically, 129 mice were identical to C57BL/6 mice at the *Cdkn2a* locus.

Mice were examined for plasma cell tumors by examination of Wright-Giemsa-stained slides of peritoneal ascites fluid cytopins. Autopsies of congenic strains were performed as the mice developed tumors over the course of 360 days, and autopsies of knockout mice were performed at day 120 postpristane due to the significant morbidity observed in the mice. Tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (American Histology Labs, Gaithersburg, Md.).

Construction of expression plasmids. DBA/2N (wild-type) p16^{INK4a} and p19^{ARF} cDNAs were cloned into pBluescript KS. The BALB/c-derived variants A134C (H18P), G232A (V51I), and A134C+G232A of p16^{INK4a} and BALB/c p19^{ARF} variant G257A (R72H) were constructed by PCR-directed mutagenesis with *Pfu* polymerase (Stratagene) (13). The following primers generated the A134C variant: (forward) TGTGCTGACGTGCGGGCACT and (reverse) AGTGCCGCACGTCTAGGCACA. Primers (forward) GGCAACGTTTACATAGCAGCTCTTC and (reverse) GAAGAGCTGCTATGTGAACGTTGCC generated the G232A and G257A variants of p16^{INK4a} and p19^{ARF}, respectively. The wild-type and variant sequences of p16^{INK4a} and p19^{ARF} were cloned into the pcDNA3.1 vector (Invitrogen). All p16^{INK4a} and p19^{ARF} plasmids were confirmed by direct sequencing of isolated plasmids (fmol DNA Sequencing System; Promega).

Cell culture and transfection. MOPC460 and TEPC1165 cells were cultured in RPMI 1640–2 mM L-glutamine–10% fetal calf serum–50 μ M β -mercaptoethanol with 3.6 ng of interleukin 6 (IL-6)/ml (40 U/ml) at 37°C in 5% CO₂ to a maximum density of 5×10^5 /ml. All plasmids used in transfection experiments were purified twice in cesium chloride-ethidium bromide gradients (34). The cells were pelleted and resuspended to a concentration of 10^7 /0.25 ml; they were electroporated at room temperature with a Cell-Porator (Life Technologies, Inc., Gaithersburg, Md.) at 180 V and 1,600 μ F at the low-resistance setting (3). The cells were diluted in 10 ml of medium and incubated for 48 h before fluorescence-activated cell sorter analysis. NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1% penicillin and streptomycin at 37°C in 5% CO₂. The cells were split into 100- by 15-mm tissue culture dishes (5×10^5 cells/dish) 5 h prior to transfection. The calcium phosphate transfection method (34) was used for NIH 3T3 cells with

slight modification. All transfections were done in duplicate in each experiment, and all experiments were performed at least three times. p16^{INK4a} protein levels were overexpressed at 4, 16, 24, and 48 h posttransfection (data not shown) as detected by standard Western blot analysis using rabbit polyclonal anti-p16 antibody (catalog no. SC-1207; Santa Cruz) followed by incubation with a sheep anti-rabbit immunoglobulin linked with horseradish peroxidase.

Cell cycle arrest analysis. Cells were transiently cotransfected with pEGFP (2 μ g), an enhanced membrane-targeted green fluorescent protein farnesylated (EGFPF) construct (14), and one of the p16^{INK4a} or p19^{ARF} constructs described above (18 μ g). The cells were harvested 48 h posttransfection. Dead cells were removed with Ficoll-Paque (Pharmacia Biotech), and the remaining live cells were washed twice with phosphate-buffered saline (containing 0.1% bovine serum albumin and 0.1% EDTA) and fixed in 70% ethanol for 1 h at 4°C. The fixed cells were washed once with phosphate-buffered saline containing 0.1% bovine serum albumin and 0.1% EDTA; DNA was stained with propidium iodide (50 μ g/ml) containing 250 μ g of RNase A/ml for 30 min at room temperature. Flow cytometry analysis was conducted with a Becton-Dickinson FACScan. EGFPF was used as a marker for analysis of transfected cells. The gate was set to select EGFPF-positive cells with a green fluorescent signal at least 40 times stronger than that of negative cells. The DNA content from at least 4,000 EGFPF-positive cells is presented in the DNA histograms. Modfit LT software (Verity Software House, Inc.) was used to analyze DNA content and determine the percentage of cells in the G₁, S, and G₂/M phases of the cell cycle. To analyze apoptosis in response to adriamycin treatment, 4×10^5 cells/ml were treated with adriamycin (Sigma, St. Louis, Mo.) and assayed by FACScan analysis of Annexin V-fluorescein isothiocyanate binding (Oncogene Research Products, Cambridge, Mass.) at four time points within 24 h of treatment. The percentage of specific apoptosis was calculated as $100 \times (\text{experimental apoptosis} - \text{spontaneous apoptosis}) / (100 \text{ spontaneous apoptosis})$ (33).

Ras transformation assays. NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, and fresh medium was added twice a week. For transfection, cells were plated in 35-mm-diameter dishes at a density of 10^5 /plate, and transfection was performed using the standard calcium phosphate method (44). NIH 3T3 cells were transfected using a constant amount of *v-ras* (150 ng) expression plasmid (pPA90) and an increasing amount of DBA/2 p16 or p19 or BALB/c p16 or p19. Foci were counted 7 days after transfection.

RESULTS

Congenic strains of mice with DBA/2 alleles of genes surrounding *Cdkn2a* are relatively resistant to tumor induction.

In previous experiments, congenic strains of mice carrying DBA/2 alleles on a BALB/c background had limited the location of *Pctrl* to a 5.7-cM interval on mouse chromosome 4 (28). In this study, a new strain was constructed to carry an interval of 1 to 2 cM composed of DBA/2 alleles surrounding *Cdkn2a* and *D4Mit15* on a BALB/c background. This strain was found to be significantly more resistant (Fig. 1) than BALB/c. These results served to narrow the genetic interval surrounding the *Pctrl* modifier locus from a 5.7- to a 1.5-cM interval, an interval found to retain the DBA alleles of *Cdkn2a* and *D4Mit15*.

Knockout mice for the *Cdkn2a* locus (INK4a^{Δ2/3} knockouts) develop accelerated plasmacytomagenesis. INK4a^{Δ2/3} mice, which were generated by deleting exons 2 and 3 of the *Cdkn2a* locus (36), are functional knockouts for both p16^{INK4a} and p19^{ARF} genes, which share exons 2 and 3 of the *Cdkn2a* locus. To determine if the *Cdkn2a* locus influences susceptibility to plasmacytoma development, homozygous and heterozygous mutant (INK4a^{Δ2/3}) C57BL/6 mice, as well as wild-type C57BL/6 and BALB/cAn mice, were treated with pristane and examined for plasma cell tumors. The normal induction regimen for BALB/cAn involves three 0.5-ml intraperitoneal injections of pristane given at 60-day intervals, with plasmacytomas developing in 50 to 60% of the mice between 180 and 350 days after the first injection (Fig. 1 and 2). Remarkably, among 30 treated INK4a^{Δ2/3} knockout mice, 1 mouse developed a

FIG. 1. C.DAG-*Pctr1* *Cdkn2a* *D4Mit15* mice carry a 1.5-cM segment derived from DBA/2 chromatin (DBA alleles at *Cdkn2a* and *D4Mit15*; BALB/c alleles at *Jun* and *D4Mit187*) and were found to be resistant to plasmacytomagenesis relative to the susceptible BALB/cAn strain. Chr, chromosome.

plasmacytoma 60 days after the first pristane injection (Fig. 2, left inset) and three additional cases were noted by 120 days after the first injection (Fig. 2). Histopathologic sections of tissues obtained at autopsy identified an additional mouse with a plasmacytoma and seven more mice with large numbers of atypical plasma cells; two of these mice also had diffuse large-cell lymphomas. Three other pristane-treated knockout mice developed histiocytic sarcomas. No plasmacytomas were observed in untreated mice or in pristane-treated C57BL/6 mice or mice heterozygous for the *INK4a*^{Δ2/3} allele (Fig. 2). Thus, the *Cdkn2a* locus can modify both the incidence and latency of plasma cell tumor development.

As is true of Burkitt's lymphoma in humans, *myc* activation by translocation has been considered the hallmark lesion of mouse plasmacytomas, with approximately 90 to 95% of pristane-induced plasma cell tumors harboring t(12;15) translocations between *myc* and *IgH* sequences that are detectable by Southern blot and PCR analyses (18). Strikingly, only one of four plasma cell tumors that arose in the homozygous null *INK4a*^{Δ2/3} mice was found to harbor a t(12;15) translocation involving the *myc* oncogene and *IgHα* sequences. This tumor was the one that had arisen within the first 60 days post-pristane treatment. Northern blot analysis of the four tumors from *INK4a*^{Δ2/3} mice revealed that the *Myc* transcript from the tumor harboring a t(12;15) translocation was in high abundance and smaller in size, consistent with a translocation junction between exons 1 and 2. In contrast, three tumors with undetectable translocations expressed lower levels of *Myc* which were the same size as germ line transcripts (data not shown). The most straightforward interpretation of these results is that *myc* activation by translocation may not be necessary for plasmacytoma development in the *Cdkn2a* null background, although *myc* activation, when it does arise, may serve to accelerate the tumorigenic process in these mice. However, in the absence of karyotypic data, it remains possible that low-*Myc*-expressing tumors might have sustained a translocation that was undetectable by Southern blot and PCR analyses.

Allelic variants in the *p16*^{INK4a} locus differ in their abilities to induce G₁ arrest. The susceptibility of the *INK4a*^{Δ2/3} mice to pristane-induced plasmacytomas implied that the *Pctr1* modifier locus in BALB/c mice might be represented by the gene for *p16*^{INK4a} or *p19*^{ARF} alone or both genes together. Alleles of the *Cdkn2a* locus contain sequence differences in exon 1α and exon 2 of the *p16*^{INK4a} gene and in exon 2 of the *p19*^{ARF} gene (45). BALB/cAnPt and ABP/Le mice have rare alleles of the *p16*^{INK4a} gene (encoding proline and isoleucine at positions 18 and 51, respectively) and the *p19*^{ARF} gene (encoding histidine at amino acid 72) in contrast to DBA/2N and C57BL/6 mice, which carry the more common allele for these two genes (encoding histidine and valine at positions 18 and 51 in *p16*^{INK4a} and arginine at position 72 in *p19*^{ARF}). Therefore, the sequence divergence between the BALB/c and DBA/2 coding regions of the two genes made both genes potential candidates for the *Pctr1* modifier. To determine whether one or both genes could be implicated as the modifier, we initially compared the abilities of the BALB/c and DBA/2 coding sequences of *p16*^{INK4a} and *p19*^{ARF} to inhibit cell growth, since several studies have shown that overexpression of either *p16*^{INK4a} or *p19*^{ARF} can lead to cell cycle arrest in G₁ (17, 31).

NIH 3T3 and two plasmacytoma cell lines derived from BALB/cAn mice were transiently transfected with isogenic BALB/c and DBA/2 allelic variants of these genes. Overexpression of the DBA/2 *p16*^{INK4a} variant led to G₁ arrest for both plasmacytoma cell lines, MOPC460 and TEPC1165 (Fig. 3), with almost twice as many cells in G₁ compared to cells transfected with the empty vector. In contrast, when the BALB/c *p16*^{INK4a} allele was overexpressed in the same cell lines, there was only a marginal increase (2 to 6%) in the proportion of cells in G₁. Thus, the proliferative phenotype of the plasmacytoma cell lines could be rescued by overexpression of the more common (or wild-type) allele, while the BALB/c variant of *p16* was much less efficient in inducing G₁ arrest.

To investigate whether one or both of the codon changes in the BALB/c *p16*^{INK4a} allele was functionally relevant to the

FIG. 2. *Cdkn2a* ($INK4a^{\Delta 2/3}$; $p16^{INK4a}$ and $p19^{ARF}$) knockout (ko) mice on the C57BL/6 genetically resistant background develop plasmacytomas (PCTs) with a shorter latency period than that seen in BALB/cAnPt mice. Mice were given two 0.5-ml intraperitoneal injections of pristane at days 0 and 60. Homozygous knockout mice were autopsied at day 120 due to their significant morbidity. Insets are photomicrographs of Wright-Giemsa-stained slides of peritoneal exudate cells from knockout (left) and normal (right) mice.

genetic inhibitory activity, constructs were engineered which contained only the exon 1 α or exon 2 BALB/c variants. When expressed in the plasmacytomas, the exon 1 α mutant allele was as deficient as the BALB/c variant allele (Fig. 3) while the exon 2 mutant construct was almost as active as the DBA/2 allele. The greater impairment of the exon 1 α mutant correlates with its variant amino acid (proline versus histidine at residue 18) being less conservative than the exon 2 variant amino acid (isoleucine to valine at residue 51).

Overexpression of BALB/c or DBA/2 $p19^{ARF}$ variants leads to p53-dependent growth arrest. To determine whether the genetic polymorphism in exon 2 of the *Cdkn2a* locus affected $p19^{ARF}$ function, isogenic constructs containing the variants were transiently transfected into the two plasmacytoma cell lines and also into NIH 3T3 cells. Both $p19^{ARF}$ alleles were equally efficient at inducing G₁ arrest in NIH 3T3 and TEPC1165 cells (Fig. 4), a finding in accord with the highly conservative nature of the amino acid substitution (histidine versus arginine at residue 72). Almost twice as many NIH 3T3 and TEPC1165 cells were in G₁ following transfection with

either construct compared with cells transfected with the empty vector. These results argue that the exon 2 sequence divergence in the BALB/c $p19^{ARF}$ gene does not render its product less active biologically than the DBA/2 $p19^{ARF}$.

Unexpectedly, MOPC460 plasmacytoma cells did not undergo G₁ arrest when either allele of the $p19^{ARF}$ gene was overexpressed. $p19^{ARF}$ is known to induce cell cycle arrest and apoptosis by blocking the ability of *Mdm2* to target p53 for degradation (2, 27, 39, 46, 47), suggesting that the failure of $p19^{ARF}$ variants to induce G₁ arrest in MOPC460 could be due to alterations in MDM2 or p53 signaling. p53 mRNA and protein levels were found to be equivalent in all cell lines except for MOPC460, which had high levels of p53 expression (data not shown). cDNA and genomic sequencing of the p53 genes in a total of five plasmacytoma cell lines revealed that four (TEPC1165, X24, TEPC2027, and MOPC265) of the five lines contained only wild-type p53 while that of MOPC460 harbored a 21-bp in-frame deletion involving the splice acceptor site of exon 5. To determine if the MOPC460 p53 gene was functionally compromised, we treated both TEPC1165 and

FIG. 3. Cell cycle analysis of DBA/2 and BALB/cAn variants of p16^{INK4a}. Overexpression of DBA/2N, but not BALB/cAnPt, p16^{INK4a} by transient transfection can lead to G₁ arrest in plasmacytoma cell lines. The inefficiency of p16^{INK4a} function in BALB/cAn is largely attributable to the codon change seen in exon 1 α . DNA content was measured by propidium iodide (PI) staining. All transfections were done in duplicate in each experiment, and all experiments were performed at least three times. The means and standard errors across experiments for the number of TEPC1165 cells in G₁ are as follows: empty vector, 37 $\pm$ 3.2; DBA p16, 69 $\pm$ 3.5; BALB exon 2, 58 $\pm$ 2.2; BALB exon 1, 37 $\pm$ 0.3; BALB/c p16, 37 $\pm$ 0.7. The means and standard errors across experiments for the number of MOPC460 cells in G₁ are as follows: empty vector, 20 $\pm$ 1.5; DBA p16, 40 $\pm$ 4; BALB exon 2, 30 $\pm$ 1; BALB exon 1, 22 $\pm$ 1.5; BALB/c p16, 21 $\pm$ 2.

MOPC460 cells with adriamycin, an agent that induces cell cycle arrest and apoptosis (38). At 24 h after the start of adriamycin treatment (0.5 μ g/ml), almost 50% of TEPC1165 cells were arrested in G₂/M and exhibited apoptosis. In contrast, approximately 90% of the MOPC460 cells were arrested in G₂/M, and modest levels of apoptosis were observed (Fig. 5). Taken together, the results suggest that the p53 pathway is intact in most plasmacytoma cell lines but that it is altered in MOPC460 by the in-frame p53 deletion specific to this cell line.

Inhibition of *ras* transformation by p16^{INK4a} or p19^{ARF}. To further test the relative biological activities of the BALB/c and DBA/2 p16^{INK4a} and p19^{ARF} proteins, we compared their abilities to inhibit focal transformation induced by an oncogenic *ras*. In these experiments, NIH 3T3 cells, in which the endogenous *Cdkn2a* locus is mutationally inactivated (30, 31), were cotransfected with a constant amount of *v-ras* and increasing amounts of DBA/2 p16 or p19 or BALB/c p16 or p19 (44). Under these conditions, p16^{INK4a} and p19^{ARF} from either mouse strain inhibited the formation of *ras*-transformed foci in a dose-dependent manner (Fig. 6). However, the BALB/c p16^{INK4a}, at any of three different doses, was less efficient than

its DBA/2 counterpart, while the BALB/c p19^{ARF} variant was as active as DBA/2 p19^{ARF} in suppressing *ras* transformation. These results therefore parallel those obtained with the plasmacytoma cell lines in showing that the BALB/c p16^{INK4a} is less active than that of DBA/2, while p19^{ARF} proteins from both strains appear to possess similar degrees of activity. Furthermore, in contrast to the low activity of BALB/c p16^{INK4a} in the G₁ arrest assay of the plasmacytomas, its dose-dependent ability to inhibit *ras*-induced transformation clearly indicates that BALB/c p16^{INK4a} retains biological activity.

DISCUSSION

The current data provide evidence that efficiency alleles of the p16^{INK4a} gene are likely to serve as modifiers of plasma cell tumor susceptibility among inbred strains of mice. In the present congenic mouse studies, the *Pctrl* interval was narrowed from 5.7 to 1.5 cM by introgressively backcrossing resistant DBA/2 alleles from the *Pctrl* interval, which includes *Cdkn2a*, onto a susceptible BALB/c background. Compared with wild-type BALB/cAn mice, the congenic displayed a delayed onset of plasmacytomagenesis and decreased tumor in-

FIG. 4. Cell cycle analysis of BALB/cAnPt and DBA/2N p19^{ARF} allelomorphs indicated normal function of each allele. Ectopic expression of all p19^{ARF} variants led to G₁ growth arrest in NIH 3T3 fibroblasts and TEPC1165 plasmacytoma cells but not in the p53 compromised MOPC460 plasmacytoma cells. PI, propidium iodide.

cidence. Although the DBA/2 *Cdkn2a* locus was present in the congenic strain, it could not be determined whether the *Pctr1* modifier was the *Cdkn2a* locus or another tightly linked DBA/2 gene within the congenic interval. In the knockout studies, the impact of the *Cdkn2a* locus on plasmacytoma susceptibility is underscored by the short latency period and increased incidence of clinically apparent tumors by 120 days after pristane treatment, even though the knockout of the *Cdkn2a* locus was on the plasmacytoma-resistant C57BL/6 (N4) background strain. In models of accelerated plasmacytomagenesis, by the INK4a^{Δ2/3} knockout or by the inoculation of retroviruses harboring combinations of *myc* and *ras* or *abl* oncogenes (20, 43), it is plausible to hypothesize that the shorter latency period results from fewer required genetic changes. Consistent with this possibility, rearrangement of the *myc* gene does not occur in the retroviral systems (43) and was identified in only one out of four plasma cell tumors that arose in the INK4a^{Δ2/3} knock-

out mice by assays that can identify rearrangement of *myc* in more than 90% of these tumors in BALB/c mice.

Fibrosarcomas and lymphomas have been the two most common spontaneous tumor types described for the INK4a^{Δ2/3} knockout of the *Cdkn2a* locus and for p19^{ARF} knockout mice (15, 36). These tumors developed more slowly than those in the present study and were either spontaneous or induced with two-stage carcinogenesis protocols involving the reagents DMBA and UVB commonly used to induce skin tumors. In this report, the plasma cell tumors were induced with pristane, which promotes chronic inflammation and increased production of IL-6 (29, 37). The short latency period for plasmacytoma formation in the INK4a^{Δ2/3} knockout mice probably results from inactivation of both p16^{INK4a} and p19^{ARF}, which disrupt the Rb and p53 pathways, respectively. In our present studies, BALB/c mice exhibit partially impaired p16^{INK4a} function but appear to have an intact p19^{ARF}-mdm2-p53 pathway

FIG. 5. TEPC1165 (wild-type p53) and MOPC460 (21-bp deletion of p53) plasmacytoma cells differed in induction of specific apoptosis induced by adriamycin treatment (0.1 to 0.5 $\mu\text{g/ml}$). MOPC460 cells exhibited attenuated apoptotic responses following treatment with adriamycin.

in normal cells and in most plasmacytomas. The longer tumor latency period in BALB/c mice probably results from the partially functional $p16^{\text{INK4a}}$ gene and completely functional $p19^{\text{ARF}}$ gene. The development of the tumors in the knockout mice resulted from the homozygous disruption of the *Cdkn2a* locus, since neither the C57BL/6 mice nor the $\text{INK4a}^{\Delta 2/3}$ heterozygotes developed plasma cell tumors. The tumor incidence studies in the *Cdkn2a* knockouts support the view that one or both of the genes included in the *Cdkn2a* locus govern susceptibility to pristane-induced plasmacytomagenesis.

Since the *Cdkn2a* locus includes two genes that inhibit cell growth, several biological studies were carried out to determine whether the BALB/c modifier locus was a result of $p16^{\text{INK4a}}$ and/or $p19^{\text{ARF}}$ alleles. In two different assays, the BALB/c $p16^{\text{INK4a}}$ protein variant was found to be less active than DBA/2 $p16^{\text{INK4a}}$, while the $p19^{\text{ARF}}$ variants of the two strains behaved similarly. Overexpression of the BALB/c allele was not sufficient to induce G_1 arrest in mouse plasma cell tumor lines. Furthermore, overexpression of the wild-type (DBA/2 and C57BL/6) allele of $p16^{\text{INK4a}}$ was able to complement the defect inherent in the BALB/c-derived plasma cell tumor lines. Inefficient BALB/c $p16^{\text{INK4a}}$ may allow phosphorylation of Rb and its release from promoter-bound E2F transcription factor, which leads to the transactivation of several genes involved in S-phase progression (24, 42); this results in cells progressing through the cell cycle and allows for sustained rounds of proliferation. Our present biological studies support the notion that BALB/c $p16^{\text{INK4a}}$ is less effective in inducing G_1 arrest and that it could in fact contribute to continued rounds of B-cell proliferation and the possible accumulation of further genetic changes, leading to neoplasia.

In another bioassay, the abilities of the alleles to inhibit focal transformation of NIH 3T3 cells by activated *ras* were examined. Activation of the Ras pathway has been implicated in mouse plasmacytomas, since the combination of constitutively active *ras* and *myc* can collaborate to induce plasmacytomas

(20) whose growth is dependent upon IL-6 (5). IL-6 can activate the Ras pathway (23, 26). In the in vitro *ras* transformation assay, we found, in four separate assays, that BALB/c $p16^{\text{INK4a}}$ suppressed *ras*-induced transformation in a dose-dependent manner, although this allele was less efficient than the DBA allele. These biological results confirm the relevance of our previous biochemical assays for BALB/c versus DBA/2 $p16^{\text{INK4a}}$ proteins. Taken together, these data argue that the *Pctr1* modifier is encoded by the $p16^{\text{INK4a}}$ gene and that the BALB/c $p16^{\text{INK4a}}$ allele is functional, but less active, than the more common allele found in DBA/2 and C57BL/6 mice. Using the cell cycle and *ras* suppression bioassays, the BALB/c and DBA/2 $p19^{\text{ARF}}$ proteins were found to be equally active. Therefore, we have no evidence that the $p19^{\text{ARF}}$ gene is part of the *Pctr1* modifier, although it remains possible that other assays might uncover biological differences between them.

In contrast to the *Pctr1* plasmacytoma modifier described here, mouse strains which carry the recessive susceptibility allele of the *Mom1* modifier have sustained a frameshift loss-of-function mutation in its encoded PLA2G2A protein (19). In the plasmacytoma system, the recessive susceptible allele (a single-base substitution) encodes a mutant full-length $p16^{\text{INK4a}}$ protein that is less efficient biologically and biochemically. Our data are in keeping with information derived from studies of a growing number of disease gene alleles in which point mutations account for differences in disease susceptibility (1, 4, 10, 11, 35, 41).

The molecular identification of modifier loci leading to a genetic predisposition for mouse plasmacytomas provides a powerful model for predicting genes and pathways involved in the etiologies of a variety of human B-cell neoplasias. Recently, a multiple-myeloma patient was described who was a member of a melanoma-prone family and had a germ line mutation in the *Cdkn2a* locus consisting of a duplication of the first eight codons of the $p16^{\text{INK4a}}$ gene, a lesion previously identified in other melanoma-prone families (8). Subsequent

FIG. 6. Inhibition of *ras*-mediated focus formation. (A and B) Cotransfection of BALB/c and DBA/2 variants of p16^{INK4a} in pcDNA3.1 with *v-ras* in NIH 3T3 cells. The percentages of residual foci formed relative to the foci detected on the plates transfected with *ras* alone are shown. (C and D) Cotransfection of BALB/c and DBA/2 variants of p19^{ARF} in pcDNA3.1 with *v-ras* in NIH 3T3 cells. The transfection assays were repeated three times with similar results.

analysis of bone marrow plasma cells from the patient revealed loss of the wild-type CDKN2A allele. The results reported here should stimulate efforts to identify similar linkage in humans afflicted with plasmacytomas, Burkitt's lymphomas, and multiple myelomas. In many human and mouse tumors, it is already known that there is somatic selection against functional CDKN2A by either homozygous deletion or hypermethylation (9, 12, 16, 25, 32, 40). Relatively few tumors outside of melanoma and certain digestive cancers have shown missense mutations in the p16^{INK4a} gene (32). Our results suggest that single-base changes in important growth-regulatory genes, particularly when they are present as several defective efficiency alleles, may be frequent predisposing events in tumorigenesis.

The majority of human cancers are not the clear result of predisposition determined by a single genetic defect. Instead, they are likely to represent the outcome of complex interactions between multiple genetic alleles and environmental factors. Mouse plasma cell tumors provide a model system for studying neoplastic processes which result from the complex interplay among several predisposing genetic factors and environmental stresses.

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