

The Cell Cycle Inhibitor p16^{INK4A} Sensitizes Lymphoblastic Leukemia Cells to Apoptosis by Physiologic Glucocorticoid Levels*

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Michael J. Ausserlechner[‡], Petra Obexer[‡], G. Jan Wieggers[‡], Bernd L. Hartmann[‡],
Stephan Geley[‡], and Reinhard Kofler^{‡§¶}

From the [‡]Institute for General and Experimental Pathology, Division of Molecular Pathophysiology, University of
Innsbruck Medical School and the [§]Tyrol Cancer Research Institute, A-6020 Innsbruck, Austria

The cyclin-dependent kinase inhibitor p16^{INK4A} is frequently inactivated in childhood T-cell acute lymphoblastic leukemia. To investigate possible consequences of this genetic alteration for tumor development, we conditionally expressed p16^{INK4A} in the T-cell acute lymphoblastic leukemia line CCRF-CEM, which carries a homozygous deletion of this gene. In agreement with its reported function, p16^{INK4A} expression was associated with hypophosphorylation of the retinoblastoma protein pRB and stable cell cycle arrest in G₀/G₁, documenting that the pRB/E2F pathway is functional in these cells. Unexpectedly, p16^{INK4A} expression increased the sensitivity threshold for glucocorticoid (GC)-induced apoptosis from therapeutic to physiologic levels. As a possible explanation for this phenomenon, we found that p16^{INK4A}-arrested cells had elevated GC receptor expression associated with enhanced GC-mediated transcriptional activity and increased responsiveness of the GC-regulated cyclin D3 gene. These data are supported by our previous findings that GC receptor levels critically influence GC sensitivity and imply that p16^{INK4A} inactivation, in addition to allowing unrestricted proliferation, represents a mechanism by which lymphoid tumor cells might escape cell death triggered by endogenous GC.

The *INK4A* gene locus is located at chromosome 9p21 and encodes two different cell cycle inhibitors, namely p16^{INK4A} and p19^{ARF} (p14^{ARF}), transcribed from alternative exons of the same gene, but dissimilar in their protein structures (1, 2). Inactivation of this gene locus is observed in up to 80% of primary T-cell acute lymphoblastic leukemias (T-ALLs),¹ thereby being the most consistent genetic alteration in this disease (3–6). The high frequency in T-ALLs suggests that the tumor suppressor genes encoded by this locus might play an important role in the development of this malignancy. *Ink4a*

knockout mice develop spontaneous lymphomas with high frequency, further suggesting that p16^{INK4A} might be involved in negative regulation of proliferation in the lymphoid lineage (7). p16^{INK4A} acts by binding to and inhibiting the activity of CDK4 and CDK6 kinases (1, 8). When complexed to regulatory cyclins of the D-type, these kinases phosphorylate and inactivate the retinoblastoma protein pRB and its family members, thereby releasing transcription factors, such as E2F-1, from inhibition by pRB. This allows transcription of genes essential for the onset of S phase (9).

To further investigate a possible role of p16^{INK4A} inactivation in T-ALL tumor development, we used the childhood T-ALL model CCRF-CEM, which contains a homozygous deletion of the *INK4A* locus (10, 11), and the tetracycline-regulated gene expression system (12) to conditionally express transgenic p16^{INK4A}.

p16^{INK4A} expression was associated with a stable arrest in the G₀/G₁ phase of the cell cycle. Surprisingly, p16^{INK4A} arrest dramatically increased the sensitivity of these cells to glucocorticoid (GC), a hormone that is routinely used for its apoptosis-inducing property in the therapy of this malignancy. Since p16^{INK4A} increased the sensitivity of these leukemia cells to physiologic levels of the hormone, we speculate that p16^{INK4A} inactivation (or a functionally equivalent mechanism) might be required for lymphoid malignancies to escape an as yet unrecognized tumor surveillance for lymphoid lineage, *i.e.* GC-induced apoptosis.

MATERIALS AND METHODS

Cell Lines, Culture Conditions, and Reagents—CEM-C7H2 (13) is a highly GC-sensitive subclone of the CCRF-CEM-C7 cell line (14). The generation and analysis of the CEM-C7H2 subclone C7H2-2C8, which contains the pBrtTA plasmid coding for the reverse tetracycline-responsive transactivator (15), have been published (12). All cells were maintained in RPMI 1640 medium containing 10% fetal calf serum (Life Technologies, Paisley, United Kingdom), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (Life Technologies) at 5% CO₂ and 37 °C in saturated humidity. Dexamethasone and cortisol were stored as 10 mM stock solutions in 100% ethanol; doxycycline was kept as a 10 mM solution dissolved in phosphate-buffered saline (PBS). All reagents were from Sigma (Vienna, Austria), unless indicated otherwise. For each experiment, mid-log phase cultures (2.5–5 × 10⁵ cells/ml) were centrifuged and resuspended in fresh medium at a concentration of ~2.5 × 10⁵ cells/ml.

Plasmids and cDNAs—Full-length p16^{INK4A} cDNA was subcloned from pSK-p16^{INK4A} (1) into the *EcoRI-XbaI* sites of the pUHD10-3 vector for tetracycline-regulated expression (16), generating plasmid pUHD10-3-p16^{INK4A}. pKS-tkHyg is a pBluescript II-KS vector (Stratagene, La Jolla, CA) containing the hygromycin B resistance gene under the control of a thymidine kinase promoter and a SV40 polyadenylation site (17). Transactivation reporter assays were performed using an MMTV-luciferase construct (18) cotransfected with the pRL-SV40 plasmid (Promega, Madison, WI) as a transfection control.

Stable Transfections—For stable introduction of p16^{INK4A}, C7H2-2C8 cells were transfected by electroporation as previously described (19).

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¶ To whom correspondence should be addressed: Dept. of Molecular Pathophysiology, Inst. for General and Experimental Pathology, Fritz-Pregl-Strasse 3, A-6020 Innsbruck, Austria. Tel.: 43-512-507-3102; Fax: 43-512-507-2867; E-mail: Reinhard.Kofler@uibk.ac.at.

¹ The abbreviations used are: T-ALLs, T-cell acute lymphoblastic leukemias; CDK, cyclin-dependent kinase; GC, glucocorticoid; GR, glucocorticoid receptor; PBS, phosphate-buffered saline; MMTV, murine mammary tumor virus; MOPS, 4-morpholinopropanesulfonic acid; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorter.

Briefly, $\sim 1 \times 10^7$ mid-log phase cells in 800 μ l of PBS containing 30 μ g of linearized pUHD10-3-p16^{INK4A} plasmid and 25 μ g of pKS-tkHyg plasmid were electroporated at 300 V and 500 microfarads using a Bio-Rad Gene Pulsar. The cells were cultured in two 96-well plates for 2 days and then exposed to 0.25 mg/ml hygromycin B. Resistant transformants were selected and analyzed for conditional p16^{INK4A} expression by Northern and Western blot analyses.

Northern Blot Analysis—Total RNA was extracted from 5×10^6 cells with TriReagentTM (LPS Industries, Moonachie, NJ). Approximately 10 μ g of RNA were separated by electrophoresis on a denaturing 1% agarose gel containing formaldehyde in MOPS buffer and blotted overnight onto ZetabindTM nylon membranes (Cuno, Inc., Meriden, CO) according to standard protocols. RNA was cross-linked to the membranes by UV light. Filters were then prehybridized in phosphate blocking buffer containing SDS and bovine serum albumin at 65 °C for 3 h and hybridized for 12 h to a [³²P]dATP-labeled, heat-denatured, full-length p16^{INK4A} probe. After hybridization, blots were washed in 1 \times saline/sodium phosphate/EDTA and 0.1% SDS at 65 °C and in 0.1 \times saline/sodium phosphate/EDTA and 0.1% SDS at 65 °C and exposed to Agfa Curix x-ray films with an amplifying screen at -90 °C for several hours to days. After each hybridization, the blots were stripped by boiling in 0.1% SDS and rehybridized with a full-length glyceraldehyde-3-phosphate dehydrogenase probe.

Determination of Apoptosis—For quantification of apoptosis, nuclear staining with propidium iodide in concert with forward/sideward scatter analysis was used (20). Briefly, cells were centrifuged, and the pellets were resuspended in 0.7 ml of hypotonic propidium iodide solution. The tubes were kept at 4 °C in the dark overnight. Nuclear fluorescence intensity and forward/sideward scatter were analyzed with a Becton Dickinson FACSscan. Cell debris and small particles were excluded from analysis, and nuclei in the sub-G₁ marker window were considered to represent apoptotic cells.

Immunoblotting—Cells were washed in PBS and lysed for 30 min on ice in PBS lysis buffer containing 1% Nonidet P-40 and 10 mM sodium fluoride, to which 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, 1 μ g/ml leupeptin, and 1 μ g/ml pepstatin were added just before use. Cell lysates were cleared by centrifugation. An equal amount of 2 \times SDS sample buffer containing 10% β -mercaptoethanol was added, and proteins were denatured by boiling for 2 min. Samples were separated by SDS-polyacrylamide gel electrophoresis on 7.5–15% polyacrylamide gels. Proteins were then transferred to nitrocellulose membranes by a Bio-Rad semidry transfer apparatus and stained using Ponceau red. The membranes were incubated with Tris-buffered saline blocking buffer containing 1% Tween 20 and 5% nonfat dry milk for 30 min, followed by an overnight incubation at 4 °C with primary antibody diluted in blocking buffer. Blots were probed with monoclonal or polyclonal antibodies against human cyclin D3, p16^{INK4A} (Pharmingen, Hamburg, Germany), pRB, and α -tubulin (Oncogene Research, Cambridge, MA). Membranes were washed in Tris-buffered saline and incubated with a horseradish peroxidase-conjugated anti-mouse secondary antibody (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) diluted in blocking buffer. Finally, the blots were developed by the enhanced chemiluminescence substrate ECL (Amersham Pharmacia Biotech) according to the manufacturer's instructions and exposed to Agfa Curix x-ray films from seconds to several minutes. Stripping and reprobing were performed as described by the manufacturer.

Flow Cytometric Analysis of Glucocorticoid Receptor (GR) Expression—Cultured cells were washed twice in PBS and 1% bovine serum albumin. Aliquots of 1×10^6 cells were fixed in 1% paraformaldehyde at room temperature for 30 min, washed, and permeabilized with 0.1% Triton X-100 in 0.1% citrate buffer for 5 min on ice. Cells were washed twice and incubated at room temperature for 70 min in the presence of either FITC-conjugated anti-GR antibody 5E4 (a kind gift from Dr. A. Falus) (21) or an FITC-conjugated isotype control (IgG₁, Pharmingen). Finally, cells were washed three times, incubated with propidium iodide, resuspended in PBS and 1% bovine serum albumin, and analyzed on a FACSscan.

Radioligand Binding Assay—For determination of ligand binding by the GR, whole cell ligand binding assays were performed as described previously (22). Briefly, $\sim 5 \times 10^6$ cells were incubated in triplicate with increasing amounts of [³H]triamcinolone acetonide (PerkinElmer Life Sciences) in the presence or absence of a 500-fold molar excess of unlabeled triamcinolone acetonide at 37 °C for 1 h, washed three times, and resuspended in scintillation mixture (Packard Instrument Co., Groningen, The Netherlands). The samples were counted in a scintillation counter.

Luciferase Reporter Assays—For transient MMTV reporter transfections, 5×10^6 mid-log phase cells were washed in PBS and resuspended

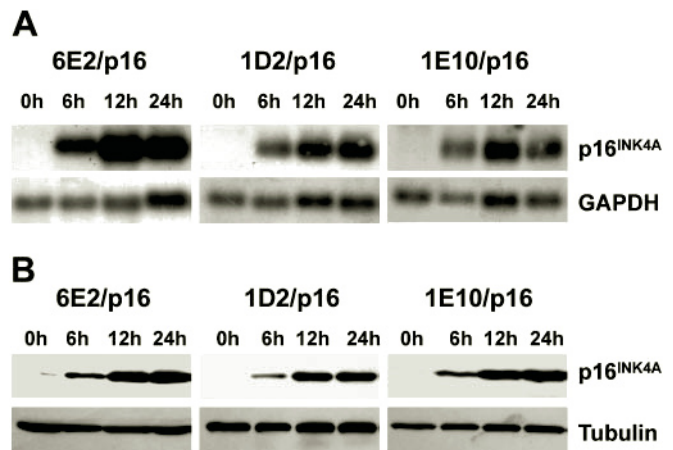


FIG. 1. Stably transfected CCRF-CEM subclones with doxycycline-regulated expression of p16^{INK4A}. C7H2-2C8 subclones 6E2/p16, 1D2/p16, and 1E10/p16 were cultured in the presence or absence of 200 ng/ml doxycycline for the times indicated. The samples were subjected to Northern blot analysis with ³²P-labeled cDNA probes to p16^{INK4A} and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (A) and to immunoblot analysis of cytoplasmic extracts with anti-p16^{INK4A} and anti- α -tubulin antibodies (B).

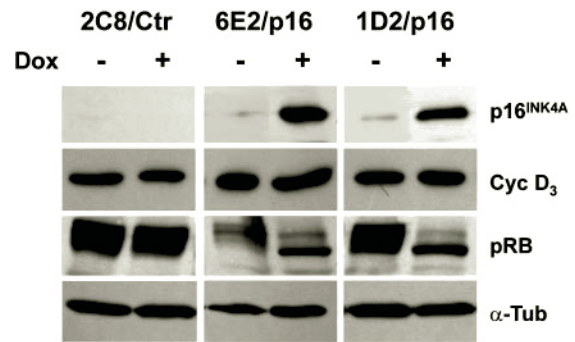


FIG. 2. p16^{INK4A} expression in stably transfected CCRF-CEM leukemia cells is associated with pRb hypophosphorylation. Parental C7H2-2C8 control (2C8/Ctr) and p16^{INK4A}-transfected 6E2/p16 and 1D2/p16 cells were cultured in the presence or absence of 200 ng/ml doxycycline (*Dox*) for 24 h. Cytoplasmic extracts were analyzed by immunoblotting using monoclonal antibodies directed against p16^{INK4A}, cyclin D3 (*Cyc D3*), pRB, and α -tubulin (α -Tub).

in 4 ml of RPMI 1640 medium containing 10% fetal calf serum. In each experiment, 5 μ g of pMMTV-luc and 1 μ g of pRL-SV40 DNA were transfected using SuperfectTM (QIAGEN Inc., Valencia, CA) according to the manufacturer's instructions. After a 3-h incubation, 15 ml of medium were added, and cells were split and incubated in the presence or absence of 200 ng/ml doxycycline for another 18 h. Cultures were then split again, and 100 nM dexamethasone was added for another 10 h to study specific induction of the MMTV reporter. Thereafter, the cells were harvested washed once in PBS, and cell pellets were lysed in 25 μ l of reporter lysis buffer for 15 min at room temperature and centrifuged. The supernatant of each sample was analyzed using the dual-luciferase reporter system (Promega) according to the manufacturer's instructions.

RESULTS

Ectopic Expression of p16^{INK4A} in CCRF-CEM Leukemia Cells Causes G₀/G₁ Arrest—To determine whether the CDK4/6 inhibitor p16^{INK4A} can induce cell cycle arrest in CCRF-CEM cells, we generated stably transfected CCRF-CEM derivatives with tetracycline-inducible p16^{INK4A} expression. For this purpose, C7H2-2C8, a CEM-C7H2 subclone with constitutive reverse tetracycline-responsive transactivator expression (12), was cotransfected with a plasmid expressing p16^{INK4A} from a reverse tetracycline-transactivator-responsive promoter and a hygromycin resistance plasmid. Three hygromycin-resistant

clones, referred to as 6E2/p16, 1D2/p16, and 1E10/p16, showed high levels of p16^{INK4A} mRNA (Fig. 1A) and protein expression (Fig. 1B) upon addition of the tetracycline analog doxycycline, but essentially no p16^{INK4A} expression in its absence. The p16^{INK4A} protein expression level was comparable to that found in aged human fibroblasts (data not shown). Two of these subclones, 6E2/p16 and 1D2/p16, were selected for further analysis. Induction of p16^{INK4A} increased the electrophoretic mobility of pRB (Fig. 2), presumably by prevention of pRB phosphorylation. Cyclin D3, which forms a functional complex with CDK4 and CDK6, remained at unchanged levels. Twenty-four hours after addition of doxycycline, p16^{INK4A}-expressing cells were completely arrested in the G₀/G₁ phase of the cell cycle, as demonstrated by FACS cell cycle analysis (Fig. 3), suggesting that the downstream components of the p16^{INK4A}/pRB pathway are intact. p16^{INK4A}-mediated cell cycle arrest was maintained over 72 h without signs of reduced viability.

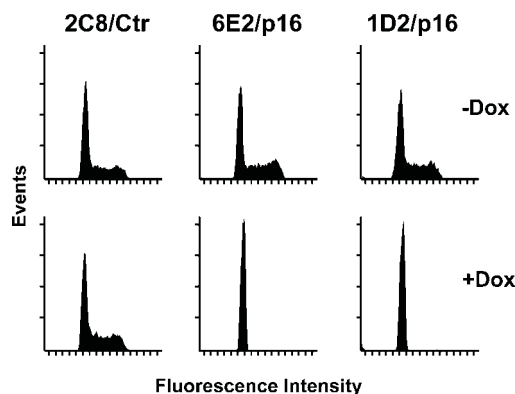


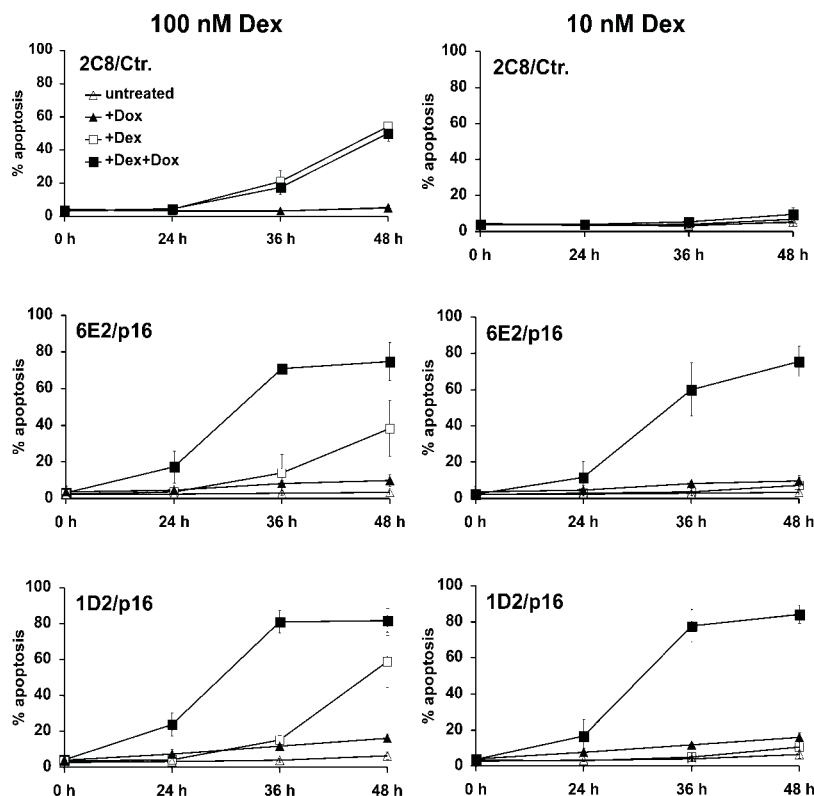
FIG. 3. p16^{INK4A} expression in stably transfected CCRF-CEM leukemia cells is followed by a stable arrest in G₀/G₁. C7H2-2C8 control (2C8/Ctr) and p16^{INK4A}-transfected 6E2/p16 and 1D2/p16 cells were cultured in the presence (+Dox) or absence (-Dox) of 200 ng/ml doxycycline for 36 h. The cells were subjected to cell cycle determination by FACS analysis of propidium iodide-stained nuclei.

p16^{INK4A}-induced G₀/G₁ Arrest Sensitizes Cells to GC-induced Apoptosis—Since GCs are central components in the therapy of childhood T-ALL, we investigated whether p16^{INK4A} expression might exert any effects on the sensitivity of these leukemia cells to GC-induced apoptosis. To this end, 6E2/p16, 1D2/p16, and parental C7H2-2C8 control cells were cultured for 24 h in the presence or absence of doxycycline and subsequently exposed to a 10 or 100 nM concentration of the therapeutic GC analog dexamethasone for another 24, 36, and 48 h. As already shown in Fig. 3, exposure to doxycycline induced cell cycle arrest in 6E2/p16 and 1D2/p16, but had no effect upon the parental C7H2-2C8 control line. When G₀/G₁-arrested cells, *i.e.* doxycycline-treated 6E2/p16 and 1D2/p16 cells, were treated with 100 nM dexamethasone, they exhibited accelerated apoptosis compared with cycling cells, starting within 24 h and reaching a plateau during the following 12 h. In contrast, doxycycline had no effect on the kinetics of cell death induced by 100 nM dexamethasone in parental C7H2-2C8 control cells (Fig. 4, left panels). Moreover, 10 nM dexamethasone (Fig. 4, right panels), which had no apoptotic effect on proliferating cells, was almost as efficient as 100 nM dexamethasone in cell death induction in G₀/G₁-arrested 6E2/p16 and 1D2/p16 cells.

p16^{INK4A} Expression Sensitizes Cortisol-resistant CEM Cells to Physiologic Concentrations of Cortisol—We next studied the sensitivity of these cells to the physiologic GC, cortisol. The parental 2C8 control line and its p16^{INK4A}-transfected subclones (in the absence of doxycycline) were highly resistant to cortisol at concentrations up to 5000 nM, which is ~100-fold higher than free cortisol in healthy humans (Fig. 5A). Surprisingly, massive apoptosis induction was observed in p16^{INK4A}-expressing cells treated with as little as 50 nM cortisol (Fig. 5A). Apoptosis induction by physiologic cortisol in G₀/G₁-arrested cells was prevented by addition of RU486, suggesting that the observed effect was mediated by the GR (Fig. 5B). Thus, p16^{INK4A} expression sensitized the cells to physiologic concentrations of cortisol.

Reintroduction of p16^{INK4A} Increases Expression of the

FIG. 4. p16^{INK4A} expression sensitizes CCRF-CEM cells to GC-induced apoptosis. Parental C7H2-2C8 control (2C8/Ctr.) cells and sublines 6E2/p16 and 1D2/p16 were maintained for 24 h in the presence (■ and ▲) or absence (□ and △) of 200 ng/ml doxycycline (Dox). Subsequently, the cell populations were split and cultured either in the presence (□ and ■) or absence (△ and ▲) of 10 nM (right panels) or 100 nM (left panels) dexamethasone (Dex) for the times indicated. The cells were then subjected to apoptosis detection by FACS analysis of propidium iodide-stained nuclei. Each panel represents the mean ± S.D. from three independent experiments.



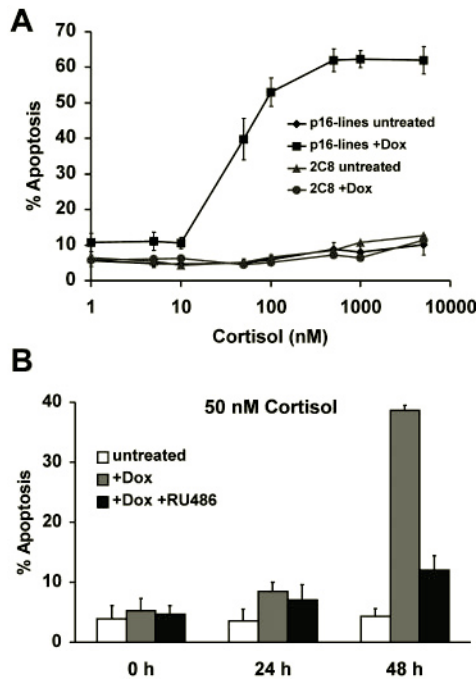


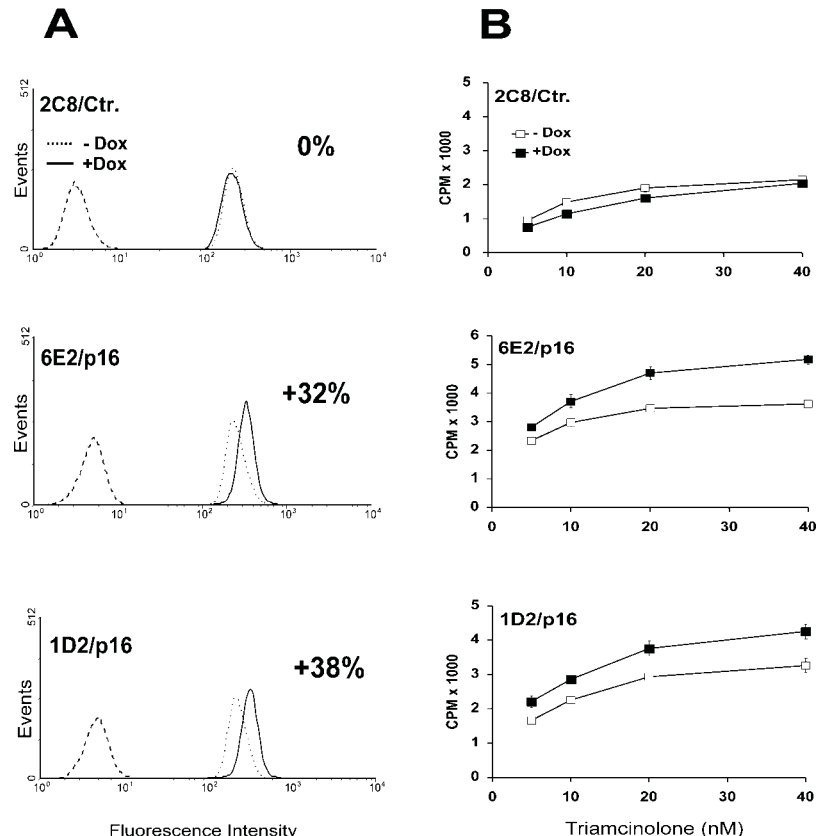
FIG. 5. p16^{INK4A}-expressing CEM cells are sensitive to physiologic concentrations of cortisol. **A**, C7H2-2C8 cells and their subclones 6E2/p16 and 1D2/p16 were cultured for 24 h in the presence or absence of 200 ng/ml doxycycline (*Dox*) and then for another 36 h in the presence of various concentrations of cortisol and finally subjected to apoptosis determination by FACS analysis of propidium iodide-stained nuclei. The results obtained with the two p16^{INK4A}-transfected cell lines were very similar and have been combined (for better readability). Shown is the mean \pm S.D. of two independent experiments. **B**, 6E2/p16 and 1D2/p16 cells were cultured for 24 h in the presence or absence of 200 ng/ml doxycycline and then for an additional 24 and 48 h in the presence of 50 nM cortisol with or without 500 nM RU486. Apoptosis was determined by FACS analysis. Shown is the mean \pm S.D. of the data derived from both cell lines and two independent experiments.

GR—To investigate possible mechanisms for the increased GC sensitivity of p16^{INK4A}-expressing cells, GR protein levels were determined in p16^{INK4A}-expressing and nonexpressing cells by flow cytometry using an FITC-labeled anti-human GR antibody. As indicated by mean fluorescence intensity, GR expression in doxycycline-treated, and hence G₀/G₁-arrested, 6E2/p16 and 1D2/p16 cells exceeded that in untreated controls by 32–38%, whereas no alteration was found in the parental control cell line, C7H2-2C8 (Fig. 6, *left panels*). Increased GR expression, measured by antibody detection, was paralleled by whole cell radioligand binding assays that showed 30–50% increases in binding of the radiolabeled GC analog triamcinolone in p16^{INK4A}-expressing cells compared with their corresponding controls. Again, ligand binding of parental C7H2-2C8 cells was not significantly influenced by addition of doxycycline (Fig. 6, *right panels*).

p16^{INK4A} Expression Increases the Activity of the GR—The increased expression and ligand-binding activity of the GR led to a 2–4-fold increase in transcriptional activity, as shown in transient transfection experiments with a GC-responsive MMTV-luciferase reporter construct, thereby proving that the increased levels of the GR were functional (Fig. 7A). Next, we investigated whether the sensitivity of endogenous genes to regulation by GC might also be increased in p16^{INK4A}-arrested cells. For these experiments, we studied cyclin D3, which is repressed by GC treatment in mouse lymphoma cells (23) and seems to be critical in the induction of GC-mediated cell cycle arrest in CEM cells.² Exposure to 10 nM dexamethasone had no detectable effect upon cyclin D3 expression in C7H2-2C8 control cells. It did, however, lead to its down-regulation in G₀/G₁-arrested 6E2/p16 cells (Fig. 7B). The combined data clearly showed that cells arrested in G₀/G₁ by p16^{INK4A} had elevated

² M. J. Ausserlechner, P. Obexer, S. Geley, and R. Kofler, submitted for publication.

FIG. 6. p16^{INK4A} increases expression of the GR. **A**, C7H2-2C8 control (2C8/Ctr.), 6E2/p16, and 1D2/p16 cells were cultured for 36 h in the presence (solid lines) or absence (dotted lines) of 200 ng of doxycycline (*Dox*), fixed, stained with either an FITC-conjugated anti-GR antibody (solid and dotted lines) or an FITC-labeled isotype control (dashed lines), and subjected to FACS analysis. **B**, C7H2-2C8 control cells and conditional p16^{INK4A}-expressing sublines cultured for 36 h with (■) or without (□) 200 ng/ml doxycycline were subjected to whole cell ligand binding assays with the indicated amounts of labeled triamcinolone. A representative experiment performed in triplicate is shown.



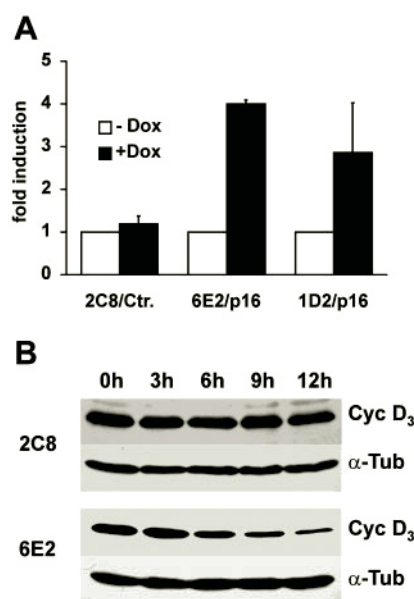


FIG. 7. p16^{INK4A} expression increases the activity of the GR. A, C7H2-2C8 control (2C8/Ctr.), 6E2/p16, and 1D2/p16 cells were transfected with a GC-responsive MMTV-firefly luciferase vector and an SV40-driven *Renilla* luciferase control vector, cultured in the presence (black bars) or absence (white bars) of 200 ng/ml doxycycline (Dox) for 18 h, and thereafter treated with 100 nM dexamethasone for another 10 h to detect GC-mediated transcriptional activity. Specific induction of the MMTV reporter is expressed as a percentage of the untreated controls (adjusted to the SV40-driven *Renilla* luciferase transfection control). Shown is the mean of two independent transient transfection experiments. B, C7H2-2C8 cells and p16^{INK4A}-expressing 6E2/p16 cells were maintained for 24 h in the presence of 200 ng/ml doxycycline and subsequently treated with 10 nM dexamethasone for the times indicated. Thereafter, lysates were analyzed by immunoblotting using specific antibodies directed against cyclin D3 (Cyc D₃) and α -tubulin (α -Tub).

levels of functional GR, which might explain, at least in part, the increased sensitivity to GC-induced cell death.

Increased GR Expression Is Not Secondary to Accumulation of Cells in G₀/G₁—To investigate whether the observed up-regulation of the GR was simply a consequence of cell accumulation in a cell cycle phase with high GR expression, the cells were double-stained with propidium iodide and anti-GR antibody, and cell cycle phase-specific GR protein levels were determined. In cycling cells, endogenous GR levels were decreased by 33% in G₀/G₁ phase compared with cells in S/G₂/M (Fig. 8A). In contrast, p16^{INK4A}-arrested G₀/G₁ cells showed a 55% increase in receptor expression compared with cycling cells in G₀/G₁ (Fig. 8B), suggesting that the increased GR expression is not the result of enriching cells in G₁, but of a specific induction in p16^{INK4A}-expressing cells.

DISCUSSION

Deletion of the *INK4* gene locus is the most frequent genetic alteration in T-ALLs (3, 5, 24), suggesting a critical role of p16^{INK4A} and/or p19^{ARF} in the development of these tumors. Using the T-ALL model CCRF-CEM, we show that p16^{INK4A} expression alone is sufficient to mediate G₀/G₁ arrest and dramatically sensitizes these cells for apoptosis induced by GC, a hormone routinely used in the therapy of malignant lymphoproliferative disorders. The former was in good agreement with numerous studies on various malignancies that suggested a tumor suppressor role of p16^{INK4A} due to its known inhibitory effect upon cell cycle progression (1, 7, 25). The latter observation was, however, unexpected and raises questions regarding its underlying mechanism, possible significance, and implications.

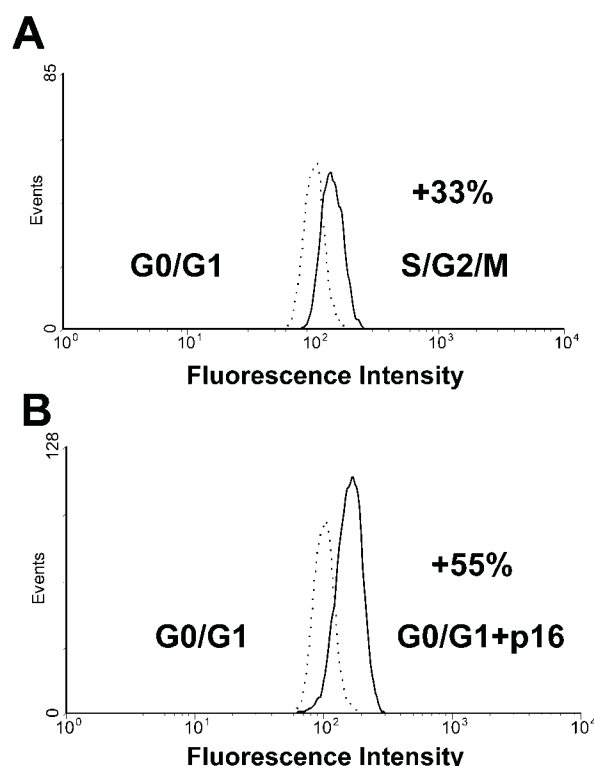


FIG. 8. Increased GR expression is not secondary to accumulation of cells in G₀/G₁. 1D2/p16 cells were cultured for 36 h in the presence or absence of 200 ng/ml doxycycline, fixed, stained with an FITC-conjugated anti-GR antibody and propidium iodide, and subjected to FACS analysis. Shown are the GR fluorescence intensity of cycling cells in G₀/G₁ phase (dotted line) versus S/G₂/M phase (solid line) (A) and the GR staining of proliferating cells in G₀/G₁ phase (dotted line) versus p16^{INK4A}-arrested G₀/G₁ cells (solid line) (B).

As to possible mechanisms for the increased GC sensitivity, we observed up-regulation of functional GR, as evidenced by flow cytometry, radio receptor assays, transient transfection studies with a GC-responsive reporter construct, and down-regulation of an endogenous GC-responsive gene. We (26) and others (27) have shown previously that GR expression levels are critical parameters for sensitivity to GC-induced apoptosis. Thus, although GR up-regulation may not be the sole cause of the dramatic increase in GC sensitivity, it may well contribute to this phenomenon. Interestingly, the observed increase in GR expression was not simply due to cell accumulation in the G₁ phase of the cell cycle since proliferating cells going through G₁ had reduced GR levels. Rather, it appeared specific for the p16-induced cell cycle arrest.

Another issue is whether the observed increase in GC sensitivity bears any significance for tumor development. Given that lymphocytes at certain stages of differentiation are sensitive to apoptosis induction by endogenous GC (28), malignant transformation of such cells might require inactivation of this lymphocyte-specific form of tumor surveillance. Whether p16^{INK4A} inactivation contributes to this escape mechanism and, if so, how this might happen is unclear. In circulating cells, p16^{INK4A} is not expressed and therefore cannot influence GC sensitivity. Stimulated lymphocytes have only a limited life span, and leukemogenesis has to invoke a program to extend the life span of proliferating T-cells, e.g. by suppressing apoptotic pathways. In addition, leukemia cells have to evolve ways to overcome replicative senescence, and inactivation of p16^{INK4A} might be one of those means. The dramatically increased sensitivity to GC associated with induction of p16^{INK4A} suggests a novel role for GC in the regulation of lymphocyte homeostasis, i.e. the

induction of cell death in p16^{INK4A}-expressing lymphocytes. Loss of p16^{INK4A} might contribute to leukemogenesis by reducing GC sensitivity and causing inability to undergo replicative senescence. In addition, the dramatic increase in GC sensitivity in p16^{INK4A}-expressing cells may have implications for the therapy of malignant lymphoproliferative disorders. Thus, combination of GC with substances that mimic p16^{INK4A} function, such as CDK4/6-inhibiting peptides or specific kinase inhibitors, might improve T-ALL therapy.

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