Protective Function of p27^{KIP1} against Apoptosis in Small Cell Lung Cancer Cells in Unfavorable Microenvironments

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A previous study of ours unexpectedly found that in contrast to frequent reductions in non-small cell lung cancer, high expression of the p27^{KIP1} cyclin-dependent kinase (CDK) inhibitor was retained in virtually all small cell lung cancers (SCLCs), suggesting the possibility of high expression of nonfunctional p27^{KIP1} in this virulent tumor. The study presented here, however, shows that p27^{KIP1} in SCLC biochemically functions as a CDK inhibitor, clearly showing induction apparently associated with G₁/G₀ arrest and efficient binding to and inhibition of the cyclin E-CDK2 complex. Interestingly, induction of p27^{KIP1} seems to confer on SCLC cells the ability to survive under culture conditions unfavorable for cell growth such as a lack of nutrients and hypoxia. Subsequent experiments manipulating p27^{KIP1} levels by using a sense p27^{KIP1} expression construct or an antisense oligonucleotide supported this notion. These observations suggest that high expression of p27^{KIP1} *in vivo* may favor the survival of SCLC by preventing apoptosis in a microenvironment unfavorable for cell proliferation. (*Am J Pathol* 2001, 158:87–96)

Lung cancer is among the leading causes of cancer death in economically developed countries. It is classified into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), which exhibit considerably distinct clinicopathological, biological, and molecular genetic characteristics.^{1,2} Prognosis of SCLC cases is generally worse than that of NSCLC patients because of the former's propensity for earlier aggressive growth and metastasis. Recent studies on cell cycle control in relation to carcinogenesis have revealed that cyclin-dependent kinase (CDK) inhibitors, which negatively regulate the ordered activation of cyclin-CDK complexes during

the cell cycle, may play a role as tumor suppressor genes.³ For example, genetic and epigenetic inactivation of the *p16* gene has been reported in various human cancers including NSCLC,^{4–7} whereas a previous report of ours suggested the potential involvement of the imprinted *p57^{KIP2}* gene in the development of lung cancers because of selective loss of the active maternal allele.⁸

p27^{KIP1}, a member of the CIP/KIP CDK inhibitor family, is expressed in large amounts in quiescent cells and declines before cellular proliferation in response to mitogenic signals.⁹ Although p27^{KIP1} may also function as a potential tumor suppressor gene, virtually no somatic mutations have been reported in human neoplasms thus far.^{10,11} We previously reported that significantly reduced p27^{KIP1} expression is frequent in NSCLC and associated with shortened patient survival. On the other hand, high p27^{KIP1} expression was unexpectedly detected in virtually all cases of SCLC, which is known to be the most virulent type of human lung cancer.¹² This observation could not be easily reconciled with this inhibitor's function as a negative regulator of the cell cycle, and raised the possibility that highly expressed p27^{KIP1} in SCLC may be in a nonfunctional state because of multiple genetic defects such as *myc* gene amplification and *Rb* inactivation. In this connection, Vlach and colleagues¹³ reported that c-myc could abrogate growth arrest by sequestering p27^{KIP1} in a form that cannot bind the cyclin E-CDK2 complex. Alternatively, the almost invariably occurring inactivation of *Rb*, which is negatively regulated by the cyclin-CDK complexes, might allow SCLC cells to proliferate in the presence of high p27^{KIP1} expression.

In the study presented here, we examined the regulation and functions of highly expressed p27^{KIP1} in SCLC as well as its biological consequences. We were able to show that p27^{KIP1} can be induced by unfavorable changes in the cellular microenvironment such as nutrient insufficiency and low oxygen pressure, that it is bio-

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chemically functional as a CDK inhibitor in apparent association with G₁/G₀ arrest, and that it confers on SCLC cells the ability to escape from apoptosis under conditions unfavorable for cell growth. Therapeutic implications of these observations are also discussed.

Materials and Methods

Cell Culture

RPMI 1640 powder and the selectamine kit for preparation of the isoleucine-free medium were purchased from Life Technologies, Inc. (Gaithersburg, MD) and fetal calf serum (FCS) from JRH Bioscience (Lenexa, KS). The human SCLC cell lines, ACC-LC-35, -48, -49, -51, and -67, were established in our laboratory¹⁴ and kept in a plastic flask for suspension culture (Sumilon MS-2005R; Sumitomo Bakelite, Tokyo, Japan). Twice a week the cell lines were supplied with RPMI 1640 medium supplemented with 5% FCS. Molecular genetic studies have demonstrated the inactivation of the *Rb* and *p53* genes and amplification of the *L-myc* gene in ACC-LC-48 and ACC-LC-49. For immunohistochemical, biochemical, and fluorescence-activated cell sorting (FACS) analyses, 5×10^5 cells were inoculated in 35-mm dishes for suspension culture (Falcon 1008; Becton Dickinson, Bedford, MA) with either complete or isoleucine-free RPMI 1640 medium supplemented with dialyzed 5% FCS and 0.65% methylcellulose (4000 cps; Katayama Chemical, Osaka, Japan) and incubated at 37°C for given periods. Hypoxic conditions were generated with an anaerobic sealed bag and an anaerobic culture system consisting of oxygen absorber, CO₂ generator, and oxygen indicator (FX-4; Cosmo Bio Co., Ltd., Tokyo, Japan). With this system, it was possible to keep and monitor the oxygen concentration at <0.1%.

Transplantation

Five-week-old female SCID mice were purchased from the Shizuoka Laboratory Animal Center (Hamamatsu, Japan) and maintained under specific pathogen-free conditions. Approximately 1×10^7 SCLC cells in 100 μ l of serum-free RPMI 1640 medium were injected in the subcutaneous tissue of the left abdominal wall of the mice. Tumors developed within 2 weeks. The mice were sacrificed 3 weeks after the injection and the cells from the dissected tumors were immediately minced and used for an autoradiography experiment.

Immunocytochemistry

Aggregates of SCLC cells in suspension culture were dissociated with 0.05% ethylenediaminetetraacetic acid in phosphate-buffered saline (PBS) for 5 minutes, attached to a glass slide by centrifugation with an Auto Smear CF-12D (Sakura, Tokyo, Japan), briefly air-dried, and then fixed with 4% formalin in PBS. The slides were incubated with anti-p27^{KIP1} monoclonal antibody (Signal Transduction Laboratories, Lexington, KY) at 1:1,000 di-

lution for 1 hour at room temperature, followed by incubation at room temperature for 1 hour each with biotinylated anti-mouse IgG secondary antibody and with ABC complex (Vectastain ABC kit; Vector Laboratories, Inc., Burlingame, CA). p27^{KIP1}-positive cells were visualized by incubating the slides with 3,3'-diaminobenzidine, and for light nuclear staining a hematoxylin solution was used. Cells were judged as being apoptotic when they had condensed or fragmented nuclei. At least 200 cells per slide were examined.

Autoradiography

[Methyl-³H]thymidine (Amersham, Buckinghamshire, UK) was added to the culture medium at a final concentration of 37 KBq/ml. The cells were pulse-labeled for 1 hour, washed with centrifugation once, and attached to a glass slide by means of centrifugation. After immunostaining for p27^{KIP1}, the slides were immersed in emulsion for microautoradiography (LM-1; Amersham), kept in the dark at 4°C for 7 days, and then developed according to the manufacturer's instructions.

Flow Cytometry

Flow cytometric analysis was performed with FACScan and CellFIT-DNA software (Becton Dickinson). Preparation and staining of nuclei were performed as described by Larsen.¹⁵

Western Blot Analysis

The following antibodies were used for Western blot analysis: antibodies against p27^{KIP1} (K25020) and p21^{CIP1} (C24420) from Signal Transduction Laboratories; p57^{KIP2} (SC-1040), p15 (SC-613), CDK2 (SC-163), CDK4 (SC-260), and cyclin A (SC-239) from Santa Cruz Biotechnology (Santa Cruz, CA); cyclin E (NCL-CYCLIN E) from Novocastra Laboratories (Newcastle, UK); p16 (13381A) from PharMingen (San Diego, CA); and cyclin D1 (MD-17-3) from Medical and Biological Laboratories (Nagoya, Japan). Ten micrograms of total cell lysate solubilized in Laemmli's sample buffer was electrophoresed on 10 or 12.5% sodium dodecyl sulfate-polyacrylamide gels and transferred to a Clear Blot Membrane-P filter (Atto, Tokyo, Japan). The filters were first incubated with the primary antibodies and then with horseradish peroxidase-conjugated secondary antibodies (Amersham). For visualization, an enhanced chemiluminescence system (Amersham) was used.

Immunoprecipitation and in Vitro Kinase Assays

The immunoprecipitation and *in vitro* kinase assays were performed essentially as described by Zhu and colleagues.¹⁶ Briefly, 1×10^6 cells were lysed in 0.1 ml of lysis buffer (50 mmol/L Tris, pH 7.4, 250 mmol/L NaCl, 2 mmol/L ethylenediaminetetraacetic acid, 1% Nonidet P-40, 50 mmol/L NaF, 0.1 mmol/L Na₃VO₄, and 1× Com-

Table 1. Relationship between Expression of p27^{KIP1} and DNA Synthesis (S Phase) in Five Human SCLC Cell Lines

Cell line	p27 ^{KIP1} / [³ H]thymidine			
	+/+	-/+	+/-	-/-
<i>In vitro</i>				
ACC-LC-35	0*	10.9	41.0	48.1
ACC-LC-48	0	12.7	52.1	35.2
ACC-LC-49	0	38.1	7.0	54.9
ACC-LC-51	0	6.1	31.4	62.5
ACC-LC-67	0	23.0	32.4	44.6
<i>In vivo</i>				
ACC-LC-35	0	28.4	41.1	30.2
ACC-LC-48	0	20.2	48.4	31.4

*Percentage of cells in fraction.

plete; Boehringer Mannheim, Mannheim, Germany). The cell extracts were incubated with anti-cyclin E antibody or anti-CDK2 antibody for 1 hour at 4°C. The immune complexes were then collected by incubation with protein A Sepharose CL-4B (50 μ l; Pharmacia Biotech, Uppsala, Sweden) for 1 hour at 4°C and with a rocking motion, washed five times with ice-cold lysis buffer or kinase buffer (50 mmol/L Tris-HCl at pH 7.4, 10 mmol/L MgCl₂), and used for the subsequent immunoblotting for p27^{KIP1} and histone H1 kinase assays. The extent of histone H1 phosphorylation was determined by means of sodium dodecyl sulfate-gel electrophoresis and subsequent autoradiography.

Transfection

A full-length cDNA of human p27^{KIP1} was prepared by polymerase chain reaction using the sense oligonucleotide primer 5'-AAGATGTCAAACGTGCGAG-3' and the anti-sense primer 5'-TTACGTTTGACGTCTTCTG-3'. The resulting polymerase chain reaction products were cloned into pBluescript (Stratagene, La Jolla, CA) and sequenced thoroughly to ensure their integrity. The insert was then transferred into pcDNA3 (Invitrogen, Carlsbad, CA) for sense orientation (pcDNA3-p27^{KIP1}). Transient

Figure 1. Cell cycle phase-dependent p27^{KIP1} expression in ACC-LC-48. A rapid decrease in p27^{KIP1} expression and subsequent gradual cell cycle progression to the S phase is apparent in response to release from cell cycle arrest.

Figure 2. Expression of cell cycle regulators and biochemical analysis of the activity of p27^{KIP1} in ACC-LC-48. **A:** Western blot analysis of total cell lysates. p27^{KIP1} expression significantly increases in the isoleucine-free medium. Other cell cycle regulators except for cyclin A are similarly expressed in both complete and isoleucine-free media. **B:** Western blot analysis of CDK2- or cyclin E-associated p27^{KIP1}. Increased association of p27^{KIP1} with CDK2 or cyclin E is evident when cells are cultured in the isoleucine-free medium. **C:** *In vitro* kinase assay of a cyclin-CDK complex associated with cyclin E or CDK2. The histone H1 kinase activity associated with CDK2 or cyclin E is greatly diminished to an almost undetectable level after incubation of cells in the isoleucine-free medium for 24 hours. C, complete medium; I, isoleucine-free medium.

transfection was performed using a cationic lipid reagent, DMRIE-C (Life Technologies, Inc.), according to the manufacturer's protocol. Briefly, 2 $\times$ 10⁶ cells of ACC-LC-49 were co-transfected with 3.6 μ g of the p27^{KIP1} expression vector together with either 0.4 μ g of pEGFP (Clontech, Palo Alto, CA) for detection of transfected cells by enhanced green fluorescence or pMACS 4.1 (Miltenyi Biotec, Auburn, CA) for magnetic isolation of transfected cells. After a 48-hour incubation, cells were transferred to complete or isoleucine-free RPMI 1640 medium supplemented with dialyzed 5% FCS and 0.65% methylcellulose, and incubated for a further 48 hours. The cells were then harvested, stained with 4,6-diamidino-2-phenylindole, and examined with a Nikon Fluophoto microscope (Nikon Co., Tokyo, Japan). At least 100 green fluorescence protein (GFP)-positive cells per slide were exam-

A Complete medium

B Ilev-free medium

- p27^{KIP1}-negative non-apoptotic cells
- p27^{KIP1}-positive non-apoptotic cells
- △— p27^{KIP1}-negative apoptotic cells
- ▲— p27^{KIP1}-positive apoptotic cells

Figure 3. No increase in apoptosis in cells expressing p27^{KIP1}. ACC-LC-48 cells were incubated in the complete (A) or isoleucine-free (B) medium for the indicated periods of time. Representative morphologies of nuclei (dark blue) and p27^{KIP1} expression (brown) on day 2 are shown on the right.

ined, and cells with condensed or fragmented nuclei were judged to be affected by apoptotic cell death. For confirmation of exogenous p27^{KIP1} expression, CD4-positive transfected cells were magnetically isolated with a MACSelect4 transfected cell selection kit (Miltenyi Biotec) and the cell lysates electrophoresed and immunoblotted.

Oligonucleotide treatment was performed to reduce the endogenous p27^{KIP1} expression in ACC-LC-48 cells. The sequences of antisense and mismatch p27^{KIP1} C-propyne-modified phosphorothioates were 5'-UGGCU-CUCCUGCGCC-3' and 5'-UCCCUUUGCGCGCC-3'

(Kurabo, Japan), respectively.¹⁷ Transfection was performed with a cytofectin reagent (Gilead Sciences, Foster City, CA) according to the manufacturer's protocol. After a 24-hour incubation, cells were transferred to either complete or isoleucine-free RPMI 1640 medium, both supplemented with dialyzed 5% FCS and 0.65% methylcellulose, and incubated for a further 72-hour period. The cells were harvested and examined for apoptotic cell death as described above. Reduced expression of p27^{KIP1} was confirmed by means of Western blot analysis using the whole cell lysate 24 hours after transfer to complete or isoleucine-free medium.

A Complete medium

B Ileu-free medium

- p27^{KIP1}-negative non-apoptotic cells
- p27^{KIP1}-positive non-apoptotic cells
- △— p27^{KIP1}-negative apoptotic cells
- ▲— p27^{KIP1}-positive apoptotic cells

Figure 4. Absence of p27^{KIP1} induction and increase in apoptosis in ACC-LC-49. ACC-LC-49 cells were incubated in the complete (A) or isoleucine-free (B) medium for the indicated periods of time. Representative morphologies of nuclei (dark blue) and p27^{KIP1} expression (brown) day 2 are shown on the right. Arrowheads indicate typical apoptotic cells with condensed or fragmented nuclei.

Results

Expression of p27^{KIP1} in SCLC Cells Accumulates at the G₀/G₁ Phase and Decreases before Progression to the S Phase in Response to Extracellular Signals

To examine the level of p27^{KIP1} expression and its relationship with the cell cycle phase, five nonsynchronized

cultured SCLC cell lines were doubly labeled with an anti-p27^{KIP1} antibody and [³H]thymidine. As shown in Table 1, 31.4 to 52.1% of the cells of four of the SCLC cell lines showed markedly increased p27^{KIP1} expression in line with the results of our previous immunohistochemical examination of SCLC specimens *in vivo*. The remaining cell line, ACC-LC-49, poorly expressed p27^{KIP1}. In all cell lines examined, it was evident that [³H]thymidine incorporation was confined exclusively to p27^{KIP1}-negative

cells. The observed strictly reciprocal relationship between p27^{KIP1} positivity and [³H]thymidine incorporation could also be confirmed in *in vivo* tumors propagated in SCID mice after subcutaneous injection of ACC-LC-35 or ACC-LC-48 cells (Table 1).

The relationship between p27^{KIP1} expression and cell cycle phase was examined by using ACC-LC-48 as a representative SCLC cell line. Because our preliminary experiment showed that ACC-LC-48 could not be arrested at the G₀/G₁ phase, even in the RPMI 1640 medium without FCS, perhaps because of autocrine stimulation as is sometimes seen in SCLC (data not shown), we used an isoleucine-free RPMI 1640 medium as an alternative previously reported by Ley and Tobey.¹⁸ Approximately 85% of the cells became p27^{KIP1}-positive and arrested at the G₀/G₁ phase when they were incubated for 72 hours in the isoleucine-free medium (Figure 1). Release from cell cycle arrest by replacing the isoleucine-free medium with a complete one resulted in a rapid fall in p27^{KIP1} expression and ~65% of the cells became p27^{KIP1}-negative 16 hours later. After reduction in p27^{KIP1} expression, cells at the G₀/G₁ phase began to progress to the S phase around 16 hours after replacement with the complete medium and cells at the S phase gradually increased thereafter. These results indicate that p27^{KIP1} in SCLC cells accumulates at the G₀/G₁ phase and decreases before progression to the S phase.

Highly Expressed p27^{KIP1} in SCLC Is Biochemically Functional

We next examined whether p27^{KIP1} in SCLC is functional in conjunction with an inhibition of cyclin-CDK complexes. It was noted that p27^{KIP1} was greatly elevated after incubation in the isoleucine-free medium for 24 hours (Figure 2A), whereas a significant reduction of cyclin A was also observed. In contrast, CDK2, CDK4, cyclin D1, cyclin E, p15, p16, p21^{CIP1}, and p57^{KIP2} were expressed at similar levels in both complete and isoleucine-free media. The quantity of CDK2- or cyclin E-associated p27^{KIP1}, as well as the histone H1 kinase activity of cyclin-CDK complexes containing CDK2 or cyclin E, were also examined. It was found that CDK2- or cyclin E-associated p27^{KIP1} increased as a result of incubation in the isoleucine-free medium (Figure 2B). Concurrently with this increase, the histone H1 kinase activity associated with CDK2 or cyclin E was greatly diminished to an almost undetectable level (Figure 2C). These results indicate that p27^{KIP1} in SCLC retains its binding capabilities as well as inhibitory activities, suggesting that it functions normally in cell cycle regulation. The reduction in cyclin A might be a consequence of inefficient cell cycle progression caused by deficiency of CDK2-cyclin E activity. Our study also implicates p27^{KIP1} as a molecule involved in cell cycle arrest because of a lack of nutrient factors, thus identifying the mechanism which had remained unclear since the initial observation by Ley and Tobey.¹⁸

Figure 5. Reciprocal relationship between p27^{KIP1} expression and apoptosis in ACC-LC-48 and ACC-LC-49 under hypoxic conditions. **A:** Increased expression of p27^{KIP1} after a 24-hour incubation in either complete medium (C), a hypoxic environment (H) or isoleucine-free medium (I). **B:** No increased apoptosis in ACC-LC-48 cells exhibiting increased p27^{KIP1} expression induced by hypoxic conditions. **C:** Increase in apoptosis in ACC-LC-49 cells showing lack of p27^{KIP1} induction under the same conditions.

Induction of p27^{KIP1} Expression Correlates with Protection of SCLC Cells from Apoptosis under Culture Conditions Unfavorable for Cell Proliferation

SCLC characteristically exhibits numerous apoptotic appearances both *in vivo* and *in vitro*, suggesting that its overall growth represents a balance between proliferation and cell death. In the study reported here, we also asked whether p27^{KIP1} might have any relation to apoptosis in SCLC using ACC-LC-48 as an example of typical SCLCs with a high expression of p27^{KIP1}. ACC-LC-48 showed a gradual increase of only a fraction of p27^{KIP1}-positive cells when cultured in the complete medium, possibly because of progressively unfavorable changes in the microenvironment such as depletion of essential nutrients (Figure 3A). Incubation in the isoleucine-free medium resulted in a rapid increase in the p27^{KIP1}-positive fraction associated with cell cycle arrest (Figure 3B). Under both culture conditions, the proportion of apoptotic cells remained small.

Figure 6. Increased survival after transfection of p27^{KIP1} of ACC-LC-49 cells in the isoleucine-free medium. **A:** Expression of exogenous p27^{KIP1} in the transfected cells. VC-C and VC-I represent data obtained with an empty vector and p27^{KIP1}-C and p27^{KIP1}-I, data obtained with sense p27^{KIP1} in complete and isoleucine-free media, respectively. **B:** Increased survival of p27^{KIP1}-transfected cells in isoleucine-free medium. **Bars** indicate mean $\pm$ SD of the proportion of surviving cells in GFP-positive transfected cells in three independent transfections. **Open bars** represent nonapoptotic fractions in the complete, and **solid bars** those in the isoleucine-free medium. p27^{KIP1}, sense p27^{KIP1} vector; VC, empty vector. **C:** Representative pictures showing apoptosis in nontransfected cells but not in a GFP-positive p27^{KIP1}-transfected cell in isoleucine-free medium.

ACC-LC-49, which showed unusually weak p27^{KIP1} expression during continuous growth (Table 1), was also examined. p27^{KIP1} positivity did not increase significantly under either complete or isoleucine-free conditions (Figure 4). However, there was a marked difference with regard to the occurrence of apoptotic cell death. Typical apoptotic cells showing condensed or fragmented nuclei rapidly appeared in the isoleucine-free medium (Figure 4B) and >80% of the cells had also been heavily stained with the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling method by day 3 (data not shown). In contrast, there were very few apoptotic cells in the complete medium (Figure 4A). Putting these results together suggests that induction of p27^{KIP1} may play a role in the protection of SCLC cells from apoptosis under culture conditions unfavorable for cell growth.

SCLCs, which feature poor vascularity relative to their rich cellularity, are thought to be hypoxic *in vivo* and tumor cell hypoxia is considered as one of the causes of tumor treatment resistance.^{19,20} We therefore examined whether another unfavorable microenvironment, hypoxia, can induce p27^{KIP1} expression in SCLC cell lines. Western blot analysis showed that p27^{KIP1} expression was clearly increased in ACC-LC-48 within 24 hours when cells were placed in a hypoxic environment, resulting in the expression of p27^{KIP1} at a level similar to that observed under incom-

plete nutrient conditions (Figure 5). ACC-LC-48 cells with increased p27^{KIP1} expression were found to be resistant to apoptosis under hypoxic conditions. In contrast, an increase in apoptotic cells in a hypoxic environment was evident in ACC-LC-49, which did not show a marked induction of p27^{KIP1}.

These results offer further support for the suggestion that induction of p27^{KIP1} may play a role in the protection of SCLC cells from apoptosis under culture conditions unfavorable for cell growth such as insufficient nutrients and lack of oxygen.

Manipulation of p27^{KIP1} Expression Alters Susceptibility to Apoptosis of SCLC Cells

To further examine the relationship between anti-apoptotic activity and induction of p27^{KIP1} in SCLC, a human p27^{KIP1} cDNA was introduced together with a GFP (green fluorescence protein) expression vector into ACC-LC-49 with a constitutive low expression of p27^{KIP1} even in the isoleucine-free medium. Two days after transfection, the cells were transferred to either the complete or the isoleucine-free medium, and further incubated for 2 days. As shown in Figure 6, the introduction of p27^{KIP1} resulted in a significant increase in surviving GFP-posi-

Figure 7. Significant reduction of nonapoptotic surviving ACC-LC-48 cells in the isoleucine-free medium as a result of the introduction of an antisense p27^{KIP1} oligonucleotide. **A:** Reduced expression of p27^{KIP1} in cells transfected with antisense (AS) but not in those transfected with mismatch (MM) oligonucleotides. Numbers denote concentrations (nmol/L) used for various oligonucleotide treatments. **B:** Dose-dependent reduction resulting from the antisense (AS) p27^{KIP1} oligonucleotide treatment of nonapoptotic surviving cells in isoleucine-free medium. This result was not seen when the mismatch (MM) oligonucleotide was used. **Bars** indicate mean $\pm$ SD for three independent transfections. C, complete medium; I, isoleucine-free medium. **C:** Representative pictures of cells transfected with either 10 nmol/L antisense (AS) or mismatch (MM) oligonucleotide in isoleucine-free medium.

tive cells in the isoleucine-free medium, whereas there was no such augmentation when they were cultured in the complete medium.

A complementary experiment was performed using ACC-LC-48 cells that contain highly inducible p27^{KIP1}. Either an antisense or mismatch oligonucleotide was transfected to ACC-LC-48. After a 1-day incubation of the transfected cells in the complete medium, they were transferred into either the complete or isoleucine-free medium. A significant decrease in the number of non-apoptotic surviving cells was observed in the isoleucine-free medium but not in the complete medium, in association with the reduction in p27^{KIP1} expression resulting from the antisense p27^{KIP1} oligonucleotide treatment (Figure 7). Introduction of the mismatch oligonucleotide did not produce such effects.

Together, these observations suggest that p27^{KIP1} may play a role in the protection of SCLC cells from apoptosis under unfavorable conditions for cell growth. It should be noted that our preliminary experiments showed NSCLC cell lines to be rather refractory to the induction of apoptosis after the introduction of antisense p27^{KIP1} even in the isoleucine-free medium. This suggests that p27^{KIP1}-dependent resistance to apoptosis may, to certain extent, be specific to SCLC cells (unpublished observation).

Discussion

Our previous immunohistochemical examination of p27^{KIP1} expression in human lung cancers unexpectedly showed a marked increase in virtually all SCLC cases. The study reported here clearly demonstrates that p27^{KIP1} expression in SCLC cell lines is normally regulated by the extracellular microenvironment and is not constitutively expressed in a tissue culture or in transplanted tumors. We were also able to show that the cyclin E- or CDK2-associated histone H1-kinase activity is markedly inhibited in SCLC cells under culture conditions unfavorable for cell growth, presumably because of a significant increase of p27^{KIP1} in the cyclin-CDK complexes. Although SCLC is known to frequently carry *c-myc* amplification and overexpression,¹ it was previously reported that in an experimental system using Rat1 cells, *c-myc* could induce abrogation of growth arrest by sequestering p27^{KIP1} from the cyclin E-CDK2 complex.¹³ The present findings, however, suggest that p27^{KIP1} is functional as a cell cycle regulator not only in normal lung epithelial cells¹² but also in SCLC cells. It is nevertheless possible that high p27^{KIP1} expression in SCLC may stoichiometrically titrate out the sequestering action of *c-myc* and that such changes in the delicate balance might con-

sequently induce growth arrest despite myc overexpression.

One of the characteristic histological features of SCLC tumors is the presence of patches of massive cell death, suggesting their susceptibility to apoptosis.² SCLC shows very rich cellularity together with relatively poor vascularity, which is possibly an architectural prerequisite for insufficient blood supply and low oxygen pressure.^{19,20} Our study suggests that p27^{KIP1} can confer on SCLC cells resistance to apoptosis because of an unfavorable microenvironment. It is possible that expression of p27^{KIP1} in SCLC cells *in vivo* may be a reflection of unfavorable growth conditions such as insufficient nutrients and low tissue oxygen pressure as shown *in vitro* in our study. Thus, p27^{KIP1} might function as a remaining gatekeeper in SCLC cells, which show neuroendocrine differentiation, harbor multiple genetic defects including invariable *Rb* inactivation,²¹ and very frequent *p53* mutations.²² In this regard, it has been suggested that p27^{KIP1} may play a part in protecting cells and tissues from inflammatory injury by acting as a safeguard against excessive cell proliferation and apoptosis.²³ A similar protective function of p27^{KIP1} was also implicated in growth factor-deprived fibroblasts from p27^{KIP1} null mice.²⁴ In addition, it may be of interest that a previous study of ours suggested a possible link between high expression of p27^{KIP1} and neuroendocrine differentiation of SCLC cells, because prominent apoptosis was reported to be elicited in neuronal cells of *lnk4d*- and p27^{KIP1}-double-knockout mice,²⁵ which can be viewed as reminiscent of p27^{KIP1}-deprived SCLC cells carrying *Rb* mutations in terms of cell cycle control.

SCLC is generally very sensitive to chemotherapeutic agents and irradiation, but recurrence is quite frequent, indicating inadequate eradication of tumor cells by currently available therapeutic modalities.²⁶ The existence of quiescent cells such as hypoxic cells is considered to be one of the multifactorial causes of tumor treatment resistance. For this reason, the results presented here warrant further studies aiming at a thorough elimination of the residual SCLC cells by forced reduction of the p27^{KIP1} expression level. Potential strategies for such novel therapeutics may well include p27^{KIP1} antisense oligonucleotides and viral vectors as well as agents that selectively enhance degradation of p27^{KIP1}. It is also worth noting that an association between induction of p27^{KIP1} and drug resistance was shown in a murine mammary tumor cell line and that p27^{KIP1} antisense oligonucleotides in combination with conventional anticancer drugs enhanced apoptosis.^{17,27} Such a combinatorial treatment may prove to be useful as an adjunct therapy for the treatment of SCLC.

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