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Increased \( p27^{\text{Kip1}} \) Cyclin-Dependent Kinase Inhibitor Gene Expression Following Anti-IgM Treatment Promotes Apoptosis of WEHI 231 B Cells

Min Wu,²* Robert E. Bellas,²* Jian Shen,²† William Yang,*, and Gail E. Sonenshein³*

Engagement of the B cell receptor of WEHI 231 immature B cells leads sequentially to a drop in c-Myc, to induction of the cyclin-dependent kinase inhibitor \( p27^{\text{Kip1}} \), and finally to apoptosis. Recently we demonstrated that the drop in c-Myc expression promotes cell death, whereas the induction of \( p27 \) has been shown to lead to growth arrest. In this paper, we demonstrate that increased \( p27 \) expression also promotes apoptosis of WEHI 231 B cells. The rescue of WEHI 231 cells by CD40 ligand engagement of its receptor prevented the increase in \( p27 \) induction. Inhibition of \( p27 \)-ablated apoptosis induced upon expression of antisense c-myc RNA. Furthermore, specific induction of \( p27 \) gene expression resulted in apoptosis of WEHI 231 cells. Lastly, inhibition of expression of c-Myc, upon induction of an antisense c-myc RNA vector, was sufficient to induce increased \( p27 \) levels and apoptosis. Thus, these findings define a signaling pathway during B cell receptor engagement in which the drop in c-Myc levels leads to an increase in \( p27 \) levels that promotes apoptosis. The Journal of Immunology, 1999, 163: 6530–6535.

Materials and Methods

Cell culture and viability analyses

WEHI 231 cells were maintained and treated with 1:1000 dilution of anti-\( \mu \)-heavy-chain Ab in the absence or presence of CD40L, as previously described (11). For immunoblot analysis, whole cell extracts were prepared as described previously (5). Except where noted, for trypan blue analysis of cell viability, cultures were incubated with 0.2% trypan blue for 10–20 min and the percentage of cells excluding dye (viable cells) was determined by examination under phase contrast microscopy at \( \times100 \). DNA fragmentation and Non-Radioactive Cell Proliferation (Promega, Madison, WI) assays were performed as described previously (5, 9).

Isolation of inducible \( p27 \) and c-myc antisense stable WEHI 231 cell transfectants

To construct an inducible \( p27 \) expression vector, a human \( p27 \) cDNA insert was cut from the vector pGlu-p27I (kindly provided Dr. Y. Xiong, Department of Biochemistry and Laboratory Medicine and Pathology, Boston University Medical School, Boston MA 02118. E-mail address: gsonensh@bu.edu


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4 Abbreviations used in this paper: BCR, B cell receptor; CAT, chloramphenicol acetyltransferase; CD40L, CD40 ligand; cdk, cyclin-dependent kinase; IPTG, isopropyl \( \beta \)-thiogalactopyranoside; ER, estrogen receptor.
University of North Carolina) with HindIII and BamHI, and was used to replace the chloramphenicol acetyltransferase (CAT) reporter gene in the pOPRSVICAT vector, generating a clone termed pOPRSVI-p27. Similarly, an expression vector for antisense c-myc RNA (pOPRSVI-as-c-myc) was prepared with the 1.8-kb HindIII and EcoRI insert, containing exon 2 and exon 3 of murine c-myc from the pRC-CMV-c-myc expression vector, which was blunt ended and subcloned in the reverse orientation into the Nod sites of pOPRSVICAT, replacing the CAT gene. Cells were electroporated as described previously (18), first with 30 μg p3′SS and stable transfectants selected with 350 μg/ml hygromycin (Boehringer Mannheim, Indianapolis, IN). Cultures were then electroporated with either 30 μg pOPRSVI-p27 or pOPRSVI-as-c-myc and were selected for stable transfectants with 350 μg/ml hygromycin plus 1.0 mg/ml G418 to isolate mixed populations of transfected cells.

Microinjection analysis

A pOPRSVI-p27 construct was also prepared with reverse orientation of the p27 CDNA insert using the pOPRSVICAT as above, yielding expression of antisense p27 RNA (termed pOPRSVI-as-p27). For microinjection analysis, WEHI 231 cells were allowed to attach to tissue culture plastic in the presence of culture medium containing 0.4% FBS and supplemented with 20 mM HEPES (pH 7.3). After 30 min of incubation at 37°C, all cells in duplicate circled areas (~150–300/sample) were microinjected using a Narishige (Tokyo, Japan) micromanipulator, as previously described (19) with the indicated mammalian expression plasmid or control DNA samples, adjusted to 130 mM KCl and 10 mM sodium phosphate buffer (pH 7.3) before microinjection. Where indicated, vector DNA was microinjected in the presence of Ab or control BSA protein in the absence or presence of cognate peptide. After microinjection, cloning rings were placed over the microinjected areas and the medium was replaced with 10% FBS/DMEM. After 30 min of incubation at 37°C, cells were removed by gentle trituration, transferred to multiwell plates, and incubated at 37°C in the absence or presence of anti-IgM, as indicated. To measure cell death, one-tenth volume trypan blue solution (0.04% final) was added to the wells, and the cells were incubated for another 15 min; the percentage of trypan blue positive cells was determined by phase contrast microscopy.

Protein and RNA blot analysis

For analysis of levels of p27, c-Myc, p21, and p53 proteins, whole cell extracts were prepared as described previously (5). Immunoblotting was performed as previously described (11) using either the p27 Ab (Transduction Laboratories, Lexington, KY), affinity-purified c-Myc-specific Ab (50-23) (kindly provided by S. Hann, Vanderbilt University), p21 Ab (sc-397; Santa Cruz Biotechnology, Santa Cruz, CA), and p53 Ab (Pab 421; Oncogene Science, Cambridge, MA). Cytoplasmic RNA was isolated and analyzed as previously described (11) using either the HindIII-BamHI fragment of the pGlu-p27H or the c-myc vector (5) as probe.

Results

Increase in p27 expression is ablated during CD40L rescue from anti-IgM-induced cell death

Recently Scott and coworkers (17) noted an increase in p27 protein levels after 24 h of anti-IgM treatment of WEHI 231 cells. We first sought to determine whether CD40L-mediated rescue of WEHI 231 cell apoptosis in response to anti-IgM treatment (10, 11) ablates the increase in p27 expression. Immunoblotting for p27 expression was performed on total cellular proteins extracted from exponentially growing WEHI 231 cells treated with anti-IgM alone or costimulated in the presence of CD40L for up to 24 h. As a control to verify the effectiveness of the anti-IgM treatment, c-Myc protein levels were similarly analyzed. Treatment with anti-IgM alone for 24 h led to the expected induction in p27 protein level and drop in c-Myc (Fig. 1). In contrast, in cells cotreated with CD40L plus anti-IgM, an initial drop occurred in the level of p27 protein below baseline values, after which there was a return by 24 h to essentially the level seen in untreated cells. CD40L plus anti-IgM caused an initial increase in c-Myc levels that was maintained for 12 h, after which there was a return to essentially baseline values by 24 h, which is consistent with our previous observations (11). Treatment with CD40L alone had little effect on overall levels of either protein (data not shown). Thus, the induction of cdk inhibitor p27 levels is ablated during CD40L-induced rescue of WEHI 231 cells from receptor-mediated apoptosis. Interestingly, the changes in p27 expression varied inversely with the pattern of c-Myc expression.

Apopotis induced by antisense c-myc RNA is ablated upon reduction in p27 expression

To determine whether apoptosis resulting from the drop in c-Myc might be mediated via a p27-signaling pathway, a microinjection strategy was employed, as described previously (19). WEHI 231 cells were microinjected in duplicate with pOPRSVI-as-c-myc, a vector expressing c-myc antisense transcripts, in the absence or presence of an increasing dose of pOPRSVI-as-p27, a plasmid yielding expression of p27 transcripts in an antisense orientation. Alternatively, to control for nonspecific effects of the antisense p27 plasmid backbone, cells were microinjected with the antisense c-myc vector and the parent vector of the as-p27 construct (pOPRSVICAT). Furthermore, as additional controls, cells were microinjected with a pBluescript vector DNA or were not microinjected at all. After 20 h, cultures were analyzed for cell viability via trypan blue staining or for apoptosis using propidium iodide staining. Anti-Myc c-myc vector induced death in 33.2 ± 8.0% of cells (Fig. 2A). The presence of condensed chromatin upon staining with propidium iodide confirmed death was due to apoptosis (data not shown; see below). Upon comicroinjection of pOPRSVI-as-p27, a significant dose-dependent decrease in the extent of cell death was noted with only 10.9 ± 4.1% dead cells detected at the highest amount (p < 0.05) (Fig. 2A). In contrast, comicroinjection of a similar quantity of the pOPRSVICAT parental DNA had essentially no effect. As expected, only low levels of dead cells were seen in nonmicroinjected cells (2.5 ± 1.3%) or in cells microinjected with pBluescript DNA (4.0 ± 2.4%). Furthermore, similar protection was observed upon comicroinjection of an affinity-purified anti-p27 Ab, which could be ablated with the cognate peptide (Fig. 2B). Thus, inhibition of the induction of p27 provides significant protection against antisense c-Myc-induced apoptosis, indicating a functional role for the p27 gene in control of survival of WEHI 231 cells mediated by the drop in c-Myc.

Induction of p27 expression promotes apoptosis of WEHI 231 cells

To directly investigate the potential role of p27 protein in mediating signals leading to apoptosis, the isopropyl β-D-thiogalactoside (IPTG)-inducible pOPRSVI LacSwitch two-vector system was employed (18). Cells were transfected sequentially with the p3′SS eukaryotic vector carrying the lacI gene expressing the lac repressor and then with pOPRSVI-p27 DNA encoding the

FIGURE 1. Induction of p27 protein levels is ablated upon CD40L-mediated rescue of WEHI 231 cells from anti-IgM-induced apoptosis. WEHI 231 cultures were treated with anti-IgM in the absence (−) or presence (+) of CD40L for the indicated times. Total cellular proteins were prepared, and samples (20 μg) were subjected to immunoblot analysis for p27 and c-Myc expression.

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full-length p27 protein under the control of the lac repressor. Expression of the lac repressor was confirmed by immunoblotting of cell extracts (data not shown). Immunoblotting of total proteins extracted at 24 h after 20 mM IPTG treatment confirmed a 2.0-fold induction of p27 protein in the stable pOPRSVI-p27 transfectants, which is consistent with the 1.9-fold increase seen following anti-lgM treatment (Fig. 1), but not in the control p3'SS cells (Fig. 3A).

Treatment of pOPRSVI-p27 transfectants with IPTG led to a significant increase in cell death, as judged by DNA ladder fragmentation assays (Fig. 3B) and quantified using trypan blue exclusion assays (Fig. 3C). Induction of p27 resulted in death of ~30% of cells in the mixed population of WEHI 231 pOPRSVI-p27 transfectants. As expected, IPTG had little effect on the survival of cells containing only the p3'SS vector expressing the lac repressor (Fig. 3, and data not shown). Thus, induction of p27 is sufficient to promote apoptosis of WEHI 231 cells.

Inhibition of c-Myc via ectopic expression of c-myc antisense RNA induces p27 and apoptosis

To confirm the role of the drop in c-Myc expression on p27 expression and apoptosis of WEHI 231 cells, a stable population of cells containing the IPTG-inducible antisense c-myc expression vector pOPRSVI-as-c-myc was similarly isolated. RNA and proteins were prepared 0, 14, and 24 h after addition of 20 mM IPTG. These were used in Northern blot (Fig. 4A) and immunoblot analyses (Fig. 4B) and confirmed the ability of the antisense RNA to effectively inhibit c-myc RNA and protein expression, respectively. We next evaluated the effects of IPTG stimulation on levels of p27 and cell death. An increase in p27 levels was detected within 14 h of IPTG treatment and was even more pronounced at 24 h (Fig. 4C). In contrast, no increase was noted in the levels of the p21 cdk inhibitor (Fig. 4D). Thus, the drop in c-myc expression is sufficient to lead to a selective increase in p27 protein levels. We next assessed the effects of the IPTG treatment on cell survival using DNA fragmentation to verify apoptosis and trypan blue staining to quantify the extent of cell death. The pOPRSVI-as-c-myc and control p3'SS cells were incubated in the absence or presence of IPTG for 24 h and DNA was assessed for fragmentation (Fig. 5A). Inhibition of c-myc expression in WEHI 231 cells resulted in extensive DNA laddering, whereas no effect was seen in the control cells. As judged by trypan blue staining, IPTG induction of c-myc antisense transcripts led to a time-dependent loss in cell viability (Fig. 5B). By 14 h, ~25% of cells were trypan blue positive and the numbers increased to >40% within 40 h. Individual clones were isolated by limiting dilution and two of these, pYA2 and pYA7, were subjected to a similar analysis. Even higher levels of cell death were observed after IPTG induction of c-myc antisense RNA in pYA2 and pYA7 cells (Fig. 6). Furthermore, extensive cell death was confirmed using the Non-Radioactive Cell Proliferation assay, i.e., 51% of pYA2 cells lost viability after 15 h of IPTG treatment. Thus, consistent with the microinjection analysis, decreased c-Myc expression in WEHI 231 cells leads to increased levels of p27 and to apoptosis.

FIGURE 2. Inhibition of p27 ablates apoptosis induced by expression of antisense c-myc RNA. A, Inhibition of p27 via microinjection of a p27 antisense expression vector. Exponentially growing WEHI 231 cells were microinjected, in duplicate, with 0.5 μg/μl pOPRSVI-as-c-myc antisense c-myc expression vector (as-myc) alone or in combination with an increasing dose (0.1, 0.2, or 0.5 μg/μl) of pOPRSI-as-p27 vector expressing antisense p27 transcripts (as-p27). As a control, 0.5 μg/μl as-myc vector was microinjected with 0.5 μg/μl pOPRSVICAT DNA (as-Par), the parental vector for pOPRSVI-as-p27. If necessary, pBluescript DNA was added to bring the final DNA concentration to 1.0 μg/μl. Alternatively, cells were left nonmicroinjected (none) or microinjected with 1 μg/μl pBluescript vector DNA alone. After microinjection, cells were cultured for 20 h and then cell viability was assessed by trypan blue staining. Cell numbers per treatment ranged from 153 to 244. The statistical significance was obtained using the Student’s t test. The data are plotted as the mean percentage of cells stained positive for trypan blue ± SD.

B, Inhibition of p27 via microinjection of an Ab to p27 protein. WEHI 231 cells were microinjected with 200 ng/μl pOPRSVI-as-c-myc antisense c-myc expression vector (as-myc) or control pBluescript vector DNA (BS), as indicated. To inhibit p27 expression, 2 μg/μl anti-p27 Ab (sc-527, Santa Cruz Biotechnology) or 2 μg/μl BSA was co-microinjected in the absence or presence of cognate peptide (sc-527P). Trypan blue analysis was performed 20 h after microinjection. Cell numbers ranged from 188 to 296 per treatment. The statistical significance was obtained using the Student t test (p < 0.05), and the data are plotted as the mean percentage of cells stained positive for trypan blue ± SD.
The p53/p21 and c-Myc/p27 pathways function independently

As noted above, the drop in c-Myc, which resulted in increased p27 expression, did not cause any detectable increase in the levels of the p53-regulated p21 protein. To determine whether the inhibition of the p53 pathway affects signaling via c-Myc and p27, we used a WEHI 231 cell transfectant expressing a temperature-sensitive dominant-negative p53 protein, termed p53#11. This clone was isolated following cotransfection of WEHI 231 cells with the p53-expression plasmid pLTRp53cGVal135 and a pSV2neo plasmid, for selection, as described previously (18). In these cells, growth at 38.5°C results in expression of a dominant-negative form of p53 protein, which is unable to signal activation of p21 protein after BCR engagement (18). WEHI 231 p53#11 or control WEHI 231 cells transfected with the pSV2neo plasmid alone were treated with anti-IgM for 0, 2, 4, 8, 10, 12, or 24 h (Fig. 7). Inhibition of p53 signaling failed to ablate either the drop in c-Myc or the increase in expression of p27 protein. As expected, the p53#11 line contains extremely high levels of p53 protein, which fail to show the normal changes following BCR engagement (Fig. 7). As expected, the normal increase in p21 protein was totally ablated in the p53#11 line incubated with anti-IgM at 38.5°C (Ref. 18, and data not shown). Thus, BCR-mediated changes in c-Myc and p27 levels appear independent of p53 signaling.

Discussion

In this paper, we demonstrate that a drop in c-Myc is sufficient to increase p27 expression and that the induction of p27 gene expression promotes apoptosis of WEHI 231 immature B cells. In particular, apoptosis induced upon microinjection of an antisense c-myc expression vector was largely rescued by comicroinjection of an antisense p27 expression vector or an affinity-purified p27 Ab. Inhibition of endogenous c-Myc expression led to increased p27 levels and promoted apoptosis. Induction of p27 was sufficient to lead to death of WEHI 231 cells. These findings are consistent
induction of p27 promotes apoptosis in several cell types. For example, adenoviral-mediated induction of p27 led to increased cell death of human carcinoma, melanoma, lung fibroblast, breast cancer, and HeLa cells (21, 22). Furthermore, induction of apoptosis of skin keratinocytes upon treatment with TGF-β1 was found to be accompanied by a drop in level of c-myc and by induction of p27 expression (23). Thus, these findings suggest that enhanced cell survival may also contribute to the large mouse phenotype of the p27 knockout animals (24); however, it should be noted that cells isolated from p27−/− mice displayed increased apoptosis upon growth factor deprivation (25).

The observation that apoptosis of WEHI 231 cells is induced upon inhibition of c-Myc expression confirms and extends previous work from several groups. We have shown that ectopic expression of c-myc protects WEHI 231 cells from anti-IgM-mediated apoptosis and that inhibition of c-Myc function upon expression of Mad1 leads to apoptosis (5). Scott and coworkers (26) and Krieg and coworkers (27) have found that receptor-mediated apoptosis of WEHI 231 cells can be ablated upon treatment with CpG-containing oligonucleotides, which induce expression of NF-κB/Rel and lead to maintenance of c-Myc. TGF-β1-mediated apoptosis of WEHI 231 or CH33 B cells, which occurs after a drop in c-myc expression, can similarly be rescued by ectopic c-Myc expression (28). In contrast, however, Hagiya et

with our previous work, which shows that microinjection of a p27 Ab or antisense expression vector partially ablates apoptosis of WEHI 231 cells upon anti-IgM treatment (18). BCR engagement of WEHI 231 cells has been shown to initiate a complex signal cascade resulting in apoptosis. Our work in this study shows that one component of the signaling pathway leading to cell death is the induction of p27 cdk inhibitor levels that results from the drop in c-Myc expression.

Previously we demonstrated that anti-IgM treatment of WEHI 231 cells results in two signaling pathways that contribute approximately equally in promoting cell death. One pathway is mediated via an increase in expression of p53 and p21 (18), and the other involves a decline in the constitutive NF-κB/Rel activity that leads directly to the drop in c-Myc level (5, 9, 19). Taken together with the results presented in this paper, we propose that this latter pathway leading to cell death can now be extended as follows: anti-IgM → decreased NF-κB/Rel → decreased c-Myc → increased p27 → G1 arrest → apoptosis. Furthermore, the results suggest that signaling via regulation of p27 levels represents another mechanism for the observed effects of the Rel family of transcription factors in promoting cell proliferation and cell survival (20).

Although not universal, several studies have recently shown that induction of p27 promotes apoptosis in several cell types. For example, adenoviral-mediated induction of p27 led to increased cell death of human carcinoma, melanoma, lung fibroblast, breast cancer, and HeLa cells (21, 22). Furthermore, induction of apoptosis of skin keratinocytes upon treatment with TGF-β1 was found to be accompanied by a drop in level of c-myc and by induction of p27 expression (23). Thus, these findings suggest that enhanced cell survival may also contribute to the large mouse phenotype of the p27 knockout animals (24); however, it should be noted that cells isolated from p27−/− mice displayed increased apoptosis upon growth factor deprivation (25).

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al. (29) have recently presented evidence for overexpression of a c-Myc/estrogen receptor (ER) fusion protein promoting apoptosis upon anti-IgM treatment. It is unlikely that a simply physiologically relevant increase in c-Myc expression can result in apoptosis because cotreatment with CD40L and anti-IG or TGF-β1 induces a very large increase in expression of c-Myc but protects WEHI 231 cells from apoptosis (11, 28). However, levels of induction with ER vectors are often extremely high, and unfortunately no measurements of the functional c-Myc protein expressed before or after anti-IGM treatment were reported by Hagiyma et al. (29). Differences in WEHI 231 cell lines used in these studies are also apparent because in Hagiyma et al.’s (29) experiments, essentially no cell death was detected even after 24 h of incubation with F(ab′)2 fragments, whereas extensive cell death is normally detected within 24 h of treatment with either F(ab′)2 (11) or anti-IGM (4, 5, 11). Lastly, one cautionary note needs to be raised about the use of the Myc/ER vector because several groups have now reported evidence that some of the apparent functions of the Myc/ER are due to the ER moiety alone (12, 30). Thus, experiments to verify the absence of any effect on apoptosis in WEHI 231 cells of a control vector expressing ER alone are needed.

To date, several posttranscriptional sites of regulation of p27 activity have been observed, e.g., at the levels of protein stability and protein sequestration (33). Furthermore, increased p27 activity have been observed, e.g., at the levels of protein stability and protein sequestration (33). Interestingly, transcription of many genes containing initiator elements can be repressed by c-Myc (37). Thus, the findings presented in this paper suggest the intriguing possibility that p27 is a novel c-Myc target gene within a signaling pathway induced by BCR engagement, and that derepression of its expression leads to growth arrest and apoptosis. Work is in progress to test this hypothesis.

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