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Involvement of cell cycle progression in survival signaling through CD40 in the B-lymphocyte line WEHI-231

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Abstract

The CD40 molecule transmits a signal that abrogates apoptosis induced by ligation of the antigen receptor (BCR) in both primary B cells and B-cell lines such as WEHI-231. Expression of Bcl-xL and A1, antiapoptotic members of the Bcl-2 family, is enhanced by CD40 ligation, and is suggested to mediate CD40-induced B-cell survival. CD40 ligation also promotes cell cycle progression by increasing the levels of cyclin-dependent kinases (CDKs) required for cell cycle progression, and reducing expression of the CDK inhibitor p27^{kip1}. Here we demonstrate that cell cycle inhibition by retrovirus-mediated p27^{kip1} expression does not modulate the levels of Bcl-xL or A1, but significantly reduces the survival of BCR-ligated WEHI-231 cells by CD40 ligation. This indicates that cell cycle progression is crucial for CD40-mediated survival of B cells.
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Introduction

CD40 is a member of the TNF receptor family expressed on immune cells such as B lymphocytes (B cells), macrophages and dendritic cells, and plays a crucial role in both humoral and cellular immune responses.[1,2] CD40 transmits a survival signal at least in B cells. CD40 ligation delays the apoptosis of normal B cells cultured *in vitro* with medium alone, demonstrating that CD40 transmits a signal that inhibits 'sponta-

neous apoptosis' of B cells. CD40 ligation also blocks the apoptosis induced by ligation of the B-cell antigen receptor (BCR) in both normal B cells and B-cell lines such as WEHI-231.[3–5] BCR-ligated B cells survive in the presence of CD40 ligation, thereby participating in humoral immune responses. CD40L, the ligand for CD40, is a member of the TNF family expressed on activated T cells as a membrane protein.[1] Antigen-stimulated B cells thus require interaction with CD40L-expressing T-helper cells for survival and activation. Self-reactive B cells are suggested to be unable to achieve this requirement and to undergo apoptosis, resulting in maintenance of self-tolerance.[3,6]

Two distinct pathways are involved in the induction of apoptosis.[7–9] The extrinsic pathway is activated by death receptors, which form a complex with caspases essential for apoptosis, and activate them upon interaction with the ligands. The other pathway called the intrinsic pathway or mitochondrial pathway involves mitochondrial dysfunction, which causes release of cytochrome *c* from the mitochondria. The released cytochrome *c* then activates caspase 9 by forming a complex termed apoptosome with Apaf-1 and caspase 9. Members of the Bcl-2 family regulate this pathway. Proapoptotic members of this family such as Bax and Bak induce mitochondrial dysfunction, whereas mitochondria is protected by antiapoptotic members of the Bcl-2 family such as Bcl-2 and Bcl-xL. CD40 ligation blocks B-cell apoptosis most likely by protecting mitochondria, as it abrogates mitochondrial dysfunction such as mitochondrial membrane potential transition and cytochrome *c* release induced by BCR ligation.[10,11] CD40-mediated protection of mitochondria appears to involve antiapoptotic members of the Bcl-2 family Bcl-xL and A1. Indeed, expression of these molecules is induced by CD40 ligation, and their overexpression protects B cells from BCR ligation-induced apoptosis.[12–17] These antiapoptotic members of the Bcl-2 family may thus mediate the survival of BCR-ligated B cells induced by CD40 signaling, through abrogation of mitochondrial dysfunction.

Although CD40 ligation alone induces proliferation of B cells only marginally,[1,18] CD40 signaling appears to activate the signaling pathways required for proliferation. Resting primary B cells express a high level of the cyclin-dependent kinase (CDK) inhibitor p27^{kip1}, which blocks cell cycle progression by inhibiting the CDK activity essential for cell cycle progression,[19] and CD40 signaling alone reduces p27^{kip1} almost completely in these cells.[20] CD40 ligation induces increase in the levels of CDK2 and CDK4,[15,20] as well as reduction of p27^{kip1} in BCR-ligated WEHI-231 cells. These findings indicate that CD40 signaling induces molecular events involved in cell cycle progression. This is also supported by the finding that CD40 ligation induces proliferation as well as survival of BCR-ligated WEHI-231 cells,[3] which otherwise undergo cell cycle arrest in the G1 phase prior to apoptosis.[21–24] Since overexpression of antiapoptotic

members of the Bcl-2 family, Bcl-xL and A1, blocks apoptosis but not cell cycle arrest in these cells,[15,17] survival does not restore the cell cycling of BCR-ligated WEHI-231 cells. Cell cycle progression of BCR-ligated B cells by CD40 ligation thus cannot be attributed to its antiapoptotic activity, but is induced by its cell cycle-promoting function, probably through reduction of p27[kip1] and induction of CDKs. This observation also indicates that Bcl-xL and A1, which are suggested to mediate CD40-induced survival,[12–17] do not regulate cell cycling. CD40 signaling thus appears to regulate apoptosis and cell cycling by independent signaling pathways.

To assess more closely whether CD40 signaling regulates survival independently of cell cycle progression, we blocked cell cycle progression by retrovirus expression of p27[kip1] in WEHI-231 cells, and ligated BCR and CD40 on these cells. Although p27[kip1]-induced cell cycle arrest did not modulate the expression of Bcl-xL and A1 by CD40 ligation, CD40-mediated survival was markedly reduced. Moreover, the reduced survival was restored by overexpression of Bcl-xL. These results indicate that the expression levels of endogenous antiapoptotic members of Bcl-2 family are not sufficient for blocking apoptosis by themselves, but require cell cycle progression for the survival of B cells. Our observation strongly suggests a crucial role of cell cycle progression in cell survival.

Results

Expression of p27[kip1] induces cell cycle arrest and partially inhibits CD40-mediated survival of WEHI-231 cells

To assess the role of cell cycle progression in CD40-mediated survival of B cells, we infected WEHI-231 cells with retrovirus expressing both p27[kip1] and GFP, or a control virus expressing GFP alone. After 24 h, we analyzed the expression of GFP by flow cytometry. When we analyzed WEHI-231 cells infected with undiluted virus, almost all the cells expressed

GFP (Figures 1,2a), indicating excellent infection efficiency of these cells. Most of the WEHI-231 cells expressed GFP when cells were infected with virus diluted by two- or three-folds. The fluorescent intensity of GFP-positive cells was almost

Figure 1 Schematic structures of retroviral vectors. Vectors encoding retrovirus expressing p27[kip1], p16[INK4a], p21[cip1] or Bcl-xL are schematically represented. These viruses are designed to coexpress GFP, YFP or CD8 for assessing the efficiency of infection. LTR, long-terminal repeat; asterisk, packaging signal; IRES, internal ribosomal entry site; HA, HA-tag
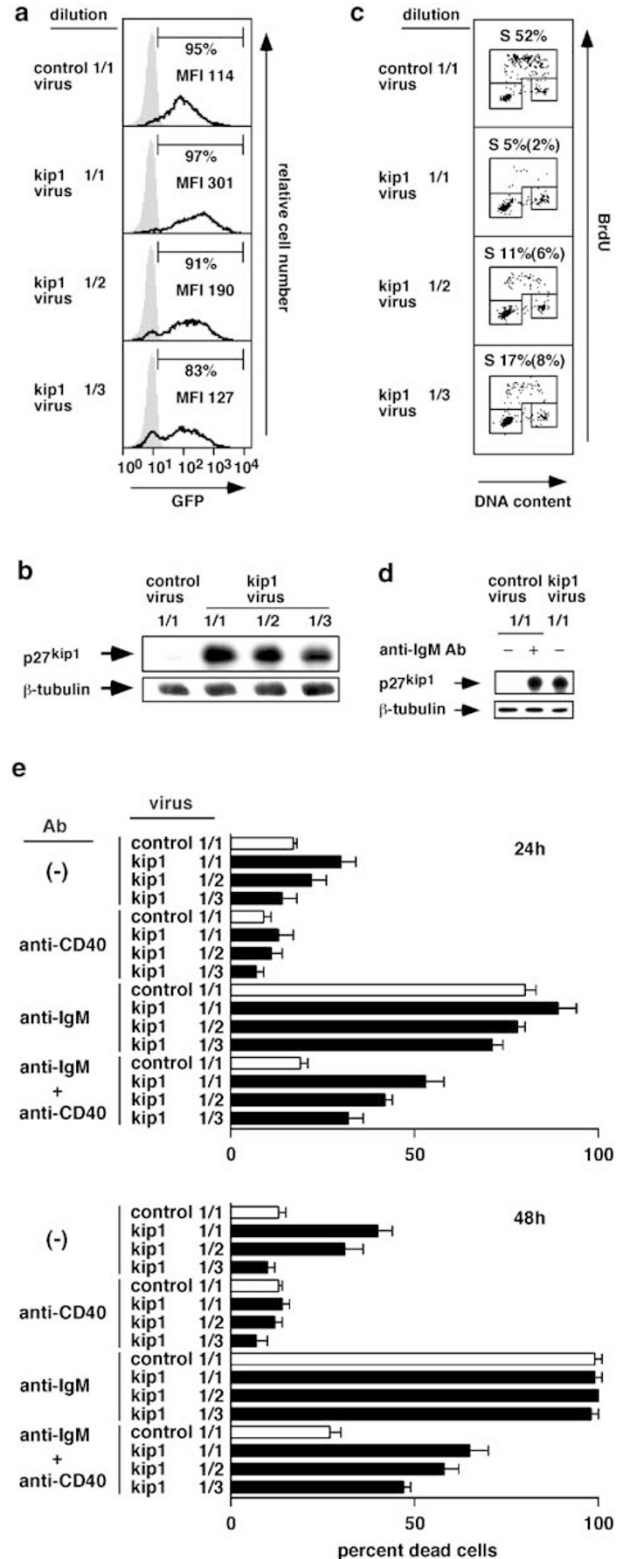
proportional to the virus titer, suggesting that expression of the protein encoded by the virus depends on the titer of virus used for infection. Indeed, expression of p27[kip1] protein was proportional to the amount of virus (Figure 2b). These results indicate that retrovirus infects WEHI-231 cells efficiently, and express p27[kip1] protein whose amount is roughly proportional to the virus titer.

We next compared the expression level of virus-mediated p27[kip1] protein to that induced by BCR ligation using anti-IgM antibody (Ab). To avoid the influence of protein degradation associated with BCR-mediated apoptosis, we treated the WEHI-231 Bcl-2 transfectants WEHI-Bcl2 with kip1 virus or anti-IgM Ab, because Bcl-2 expression confers resistance to BCR-mediated apoptosis in WEHI-231 cells.[18] The level of p27[kip1] in WEHI-Bcl2 cells infected with the kip1 virus was similar to that in anti-IgM-treated WEHI-Bcl2 cells (Figure 2d), indicating that virus-mediated expression of p27[kip1] is comparable to that induced by BCR ligation in WEHI-231 cells.

Next we assessed whether retrovirus-mediated p27[kip1] expression blocks cell cycle progression. When we analyzed the cell cycle status of WEHI-231 cells 24 h after infection with undiluted kip1 virus, cells in the S phase was almost undetectable, and the fraction of cells in the G1 phase was increased (Figure 2c), indicating that p27[kip1] expression induces almost complete cell cycle arrest in the G1 phase. WEHI-231 cells infected with diluted virus showed marginal increase in the percentage of cells in the S phase compared with those infected with undiluted virus. This increase was not due to increase in the percentage of virus-uninfected cells. Indeed, the percentage of cells in the S phase was increased, though marginally, in virus-infected cells, granted that uninfected cells undergo cell cycle as well as those infected with control virus. These results suggest that infection with kip1 virus induces cell cycle arrest at the G1 phase, in a manner dependent on virus titer.

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Figure 2 CD40 signaling rescues WEHI-231 cells from anti-IgM-induced cell death only partially in the presence of p27[kip1] expression. The supernatants containing retrovirus expressing both p27[kip1] and GFP (kip1 virus) or GFP alone (control virus) were either undiluted (1/1) or diluted by two-fold (1/2) or three-fold (1/3), and were incubated with WEHI-231 (**a**, **b**, **c** and **e**) or WEHI-Bcl2 (**d**) cells. (**a**–**d**) Retrovirus-mediated p27[kip1] expression. Virus-infected WEHI-231 cells were cultured with medium alone for 24 h. Expression of GFP was analyzed by flow cytometry (**a**). Percentages of GFP+ cells and the mean fluorescence intensities (MFI) of GFP+ cells are indicated. Expression of p27[kip1] in cell lysates was analyzed by Western blotting using anti-p27[kip1] mAb (**b**). The same blot was reprobed with anti-β-tubulin mAb to ensure equal loading. Cells were pulsed with BrdU at 37°C for 20 min, and cell cycle status was analyzed by flow cytometry using anti-BrdU Ab and PI (**c**). Percentages of cells in the S phase in kip1 virus-infected cells were calculated. Percentages of cells in the S phase in total cells and in kip1 virus-infected cells (parenthesis) are indicated. The representative data of three experiments are shown. Alternatively, virus-infected WEHI-Bcl2 cells and uninfected WEHI-Bcl2 cells were cultured with or without anti-CD40 Ab, and expression of p27[kip1] in cell lysates was analyzed by Western blotting using anti-p27[kip1] mAb (**d**). The same blot was reprobed with anti-β-tubulin mAb to ensure equal loading. (**e**) Effect of p27[kip1] expression on the rescue of BCR-ligated WEHI-231 cells from apoptosis. WEHI-231 cells were cultured with 10 μg/ml of F(ab')2 fragments of goat anti-mouse IgM Ab, 10 μg/ml anti-mouse CD40 mAb HM40-3 or both for 24 h (upper panel) or 48 h (lower panel). Viability of the cells was assessed by Trypan blue staining, and percentages of dead cells were calculated. The data represent mean±S.D. of triplicate cultures. Representative data of three experiments are shown

To ask whether CD40 signaling blocks BCR-mediated apoptosis in WEHI-231 cells arrested in the G1 phase, we ligated BCR on kip1 virus-infected WEHI-231 cells with anti-Ig Ab in the presence or absence of agonistic anti-CD40 Ab. In control virus-infected WEHI-231 cells, treatment with anti-Ig Ab killed almost all the cells, but the cells were rescued almost completely by CD40 signaling (Figure 2e). Cells infected with kip1 virus died by anti-Ig Ab as efficiently as those infected with control virus. Treatment with anti-CD40 Ab reduced the percentage of dead cells in p27[kip1]-expressing WEHI-231 cells treated with anti-Ig Ab. However, the percentages of dead cells treated with both anti-Ig and anti-CD40 Ab increased dependently on the virus titer, and more than 50% of the cells were dead in 24 h in WEHI-231 cells infected with undiluted virus. This result indicated that CD40 signaling rescues anti-Ig-treated WEHI-231 cells only partially in the presence of p27[kip1] expression. To ask whether p27[kip1] expression enhances apoptosis in WEHI-231 treated with both anti-Ig and anti-CD40 Abs, we measured the percentage of cells containing hypodiploid DNA characteristic for apoptotic cells by flow cytometry (Figure 3). In both kip1 virus-infected and control virus-infected cells, most of the cells underwent apoptosis by anti-Ig treatment. Anti-Ig-induced apoptosis was blocked by treatment with anti-CD40 Ab almost completely in control virus-infected WEHI-231 cells. In contrast, the same treatment rescued anti-Ig-induced apoptosis only marginally in kip1 virus-infected WEHI-231 cells.
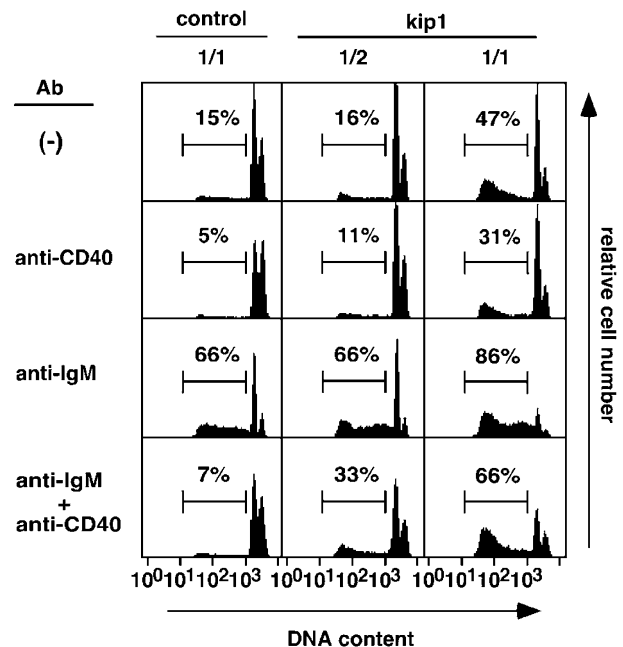

Figure 3 Retrovirus-induced expression of p27[kip1] inhibits CD40-mediated survival of BCR-ligated WEHI-231 cells. The supernatants containing retrovirus expressing both p27[kip1] and GFP (kip1) or GFP alone (control) were either undiluted (1/1) or diluted by two-fold (1/2), and incubated with WEHI-231 cells for 4 h. Cells were then cultured with 10 μg/ml of F(ab')2 fragments of goat anti-mouse IgM Ab, 10 μg/ml anti-mouse CD40 mAb HM40-3 or both for 24 h. Cells were fixed, stained with PI, and analyzed by flow cytometry. Percentages of cells with hypodiploid nuclei are indicated. Representative data of three experiments are shown

These results indicate that CD40 signaling rescues BCR-mediated apoptosis of WEHI-231 cells only partially in the presence of p27^{kip1} expression. Taken together, p27^{kip1} expression induces cell cycle arrest in the G1 phase and partially blocks CD40-mediated rescue from apoptosis in BCR-ligated WEHI-231 cells.

Expression of p27^{kip1} does not modulate the expression of A1 or Bcl-xL induced by CD40 signaling

CD40 signaling induces the expression of antiapoptotic members of the Bcl-2 family Bcl-xL and A1 in B cells,[12,14,15,17] and these molecules are suggested to play an important role in CD40-mediated survival. We therefore assessed whether the levels of Bcl-xL and A1 are modulated when p27^{kip1} expression reduces CD40-mediated survival of WEHI-231 cells. Western blotting revealed that expression of Bcl-xL and A1 is induced by treatment with anti-CD40 Ab, and that with both anti-CD40 and anti-Ig Abs, respectively, in control-virus infected WEHI-231 cells (Figure 4). In kip1 virus-infected cells, the expression of Bcl-xL and A1 was induced as efficiently as control virus-infected cells. This result indicated that CD40 signaling blocks BCR-mediated apoptosis only partially in p27^{kip1}-expressing WEHI-231 cells, in spite of their expression of Bcl-xL and A1 similar to control cells. For the complete rescue to BCR-ligated WEHI-231 cells, therefore, expression of Bcl-xL and A1 is not sufficient but cell cycle progression appears to be required.
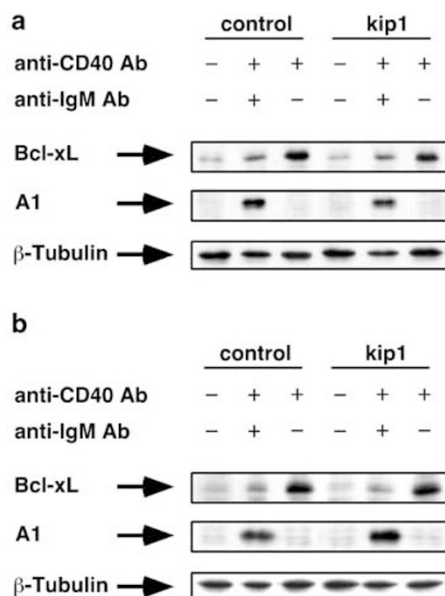

Figure 4 Expression of p27^{kip1} does not modulate the protein levels of A1 and Bcl-xL in WEHI 231 cells. WEHI-231 cells were incubated with the supernatants containing retrovirus expressing both p27^{kip1} and GFP (kip1) or GFP alone (control) for 4 h. Cells were then cultured with 10 μg/ml anti-mouse CD40 mAb HM40-3 in the presence or absence of 10 μg/ml of F(ab')$_2$ fragments of goat anti-mouse IgM Ab for 6 h (**a**) or 24 h (**b**). Cells were lysed and subjected to Western blot analysis using anti-Bcl-xL Ab (upper panel) and anti-A1 Ab (middle panel). The same blots were reprobed with anti-β-tubulin mAb to ensure equal loading (lower panel). Representative data of three experiments are shown

Overexpression of Bcl-xL restores the survival of BCR-ligated WEHI-231 cells expressing p27^{kip1}

It was previously demonstrated that expression of Bcl-xL or A1 blocks BCR ligation-induced apoptosis of WEHI-231 cells almost completely, without inducing cell cycle progression.[15,17] To resolve the apparent contradiction of this observation with our results, we obtained WEHI-231 cells overexpressing Bcl-xL (WEHI-BclxL) using a retrovirus vector. The kip1 virus efficiently infected WEHI-BclxL cells (Figure 5a), and induced cell cycle arrest in the G1 phase (Figure 5b), as is the case for parent WEHI-231 cells. When we treated kip1 virus-infected WEHI-BclxL cells with anti-Ig Ab in the presence or absence of anti-CD40 Ab, both cell death and apoptosis were completely blocked, regardless of the presence or absence of anti-CD40 Ab (Figure 5c, d), although p27^{kip1} expression was comparable to that in kip1 virus-infected WEHI-231 cells (Figure 5e). This result indicates that overexpression of Bcl-xL rescues the apoptosis of WEHI-231 cells even in the presence of p27^{kip1} expression. WEHI-BclxL cells express a much larger amount of Bcl-xL than that induced by CD40 ligation (Figure 5f). Thus, Bcl-xL is capable of completely blocking BCR-mediated apoptosis, but endogenous Bcl-xL and A1 induced by CD40 signaling in WEHI-231 cells are not sufficient for completely blocking BCR-mediated apoptosis in the absence of cell cycle progression.

Expression of p16^{INK4a} or p21^{cip1} partially inhibits CD40-mediated survival of WEHI-231 cells

We asked whether other CDK inhibitors such as p16^{INK4a} and p21^{cip1} inhibit CD40-mediated survival, as is the case for p27^{kip1}. Infection with retrovirus expressing p16^{INK4a} or p21^{cip1} induced cell cycle arrest in G1 in a manner dependent on virus titer (data not shown) in WEHI-231 cells. Expression of p16^{INK4a} significantly increased the percentage of dead cells in WEHI-231 cells treated with anti-IgM and anti-CD40 antibodies (Figure 6), as is the case for expression of p27^{kip1}. Essentially, the same result was obtained when WEHI-231 cells were infected with cip1 virus (data not shown). These results indicated that CD40-mediated survival of WEHI-231 cells is partially blocked by expression of CDK inhibitors including p16^{INK4a}, p21^{cip1} and p27^{kip1}.

Discussion

We demonstrate that BCR-ligated WEHI-231 cells are rescued by CD40 ligation only partially from apoptosis when cell cycle is arrested by retrovirus-mediated p27^{kip1} expression. Since both cell cycle arrest and reduction of CD40-mediated survival of WEHI-231 are proportional to the titer of kip1-virus used for infection, it is likely that the reduction of CD40-mediated survival is due to cell cycle inhibition by p27^{kip1}, but not its toxic effect if it carries any. Exclusion of the toxic effect of p27^{kip1} is also supported by our finding that p27^{kip1} expression induces apoptosis only marginally in WEHI-231 cells, without anti-Ig treatment. Further, expression of p16^{INK4a} or p21^{cip1} also reduces CD40-mediated survival of WEHI-231 cells. Thus, CD40 appears to abrogate
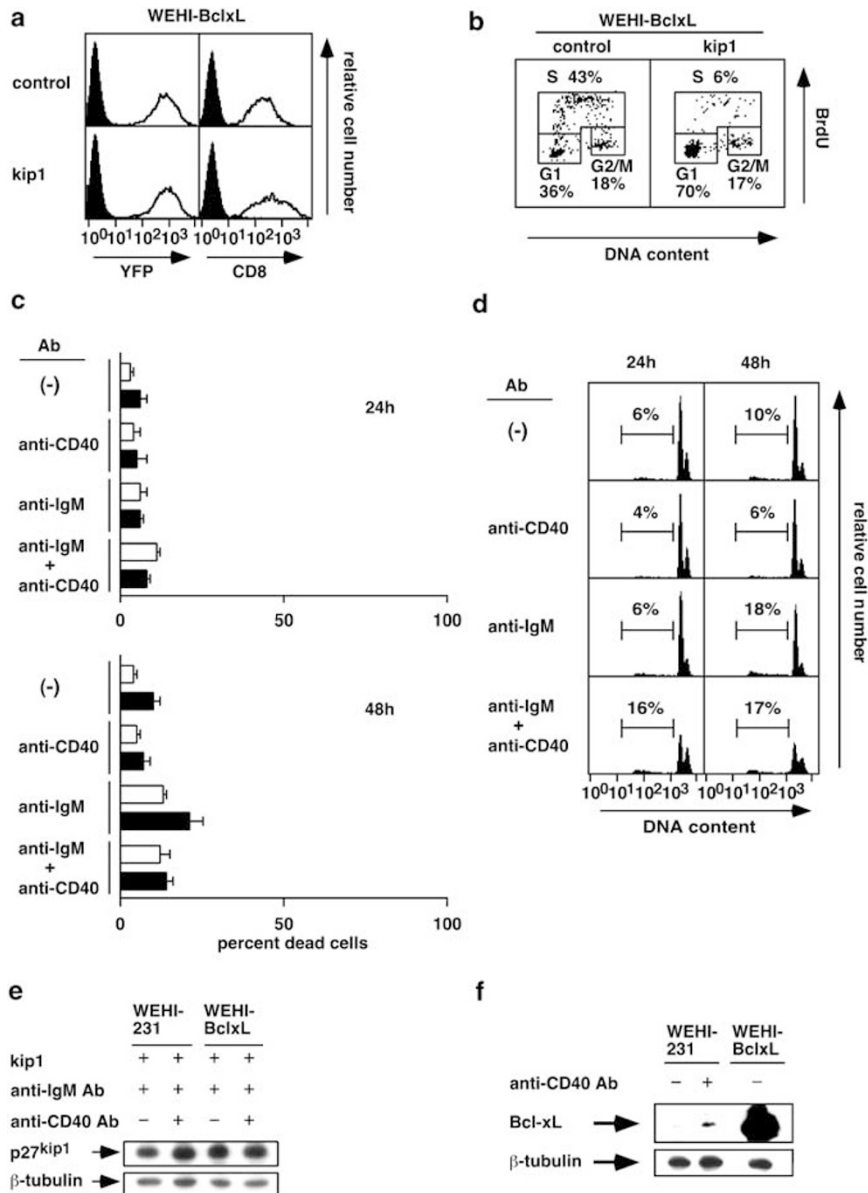
Figure 5 Overexpression of Bcl-xL blocks BCR-mediated apoptosis in p27[kip1]-expressing WEHI-231 cells. WEHI-231 cells were infected with the retrovirus expressing both Bcl-xL and YFP. The resulting WEHI-Bcl-xL cells were infected with retrovirus expressing both p27[kip1] and CD8 (kip1) or CD8 alone (control) for 4 h. (**a**, **b**) Infection with kip1 virus induces cell cycle arrest in WEHI-Bcl-xL cells. Cells were cultured in medium for 24 h. They were then analyzed for expression of YFP and CD8 by flow cytometry (**a**). Alternatively, cells were pulsed with BrdU, and cell cycle status was analyzed by flow cytometry (**b**). (**c–e**) Bcl-xL expression does not modulate p27[kip1] expression, but blocks BCR-mediated apoptosis in WEHI-231 cells. Cells were cultured with 10 μg/ml F(ab')₂ fragments of goat anti-mouse IgM Ab, 10 μg/ml anti-mouse CD40 mAb HM40-3, or both. After 24 or 48 h, percentages of dead cells (**c**) and apoptotic cells (**d**) were measured by Trypan blue staining and flow cytometry, respectively. Percentages of dead cells are shown (**c**). The data represent mean ± S.D. of triplicate cultures. Percentages of cells with hypodiploid nuclei characteristic for apoptosis are indicated (**d**). Representative data of three experiments are shown. Alternatively, cells were cultured for 24 h, and expression of p27[kip1] in cell lysates was analyzed by Western blotting using anti-p27[kip1] mAb (**e**). The same blot was reprobed with anti-β-tubulin mAb to ensure equal loading. (**f**) The level of Bcl-xL in WEHI-BclxL cells is much higher than that induced by CD40 ligation in WEHI-231 cells. Cells were cultured with or without anti-CD40 Ab for 6 h, and expression of Bcl-xL in cell lysates was analyzed by Western blotting using anti-Bcl-xL Ab. The same blot was reprobed with anti-β-tubulin mAb to ensure equal loading. Representative data of three experiments are shown

BCR-mediated apoptosis only partially when cell cycle is arrested through expression of CDK inhibitors. Expression of Bcl-xL or A1 induces the survival of BCR-ligated B cells without cell cycling.[15,17] This indicates that cell cycle progression is not required for survival induced by Bcl-xL or A1, and suggests that cell cycle arrest does not block the antiapoptotic activity of these molecules. Moreover, p27[kip1]

expression does not modulate the levels of Bcl-xL and A1. Thus, p27[kip1] expression reduces CD40-mediated survival without perturbing the expression or function of Bcl-xL or A1, indicating that CD40-mediated expression of these molecules is not sufficient for fully protecting B cells from apoptosis. In contrast, overexpression of Bcl-xL induces the survival of BCR-ligated WEHI-231 cells even in the presence of cell cycle
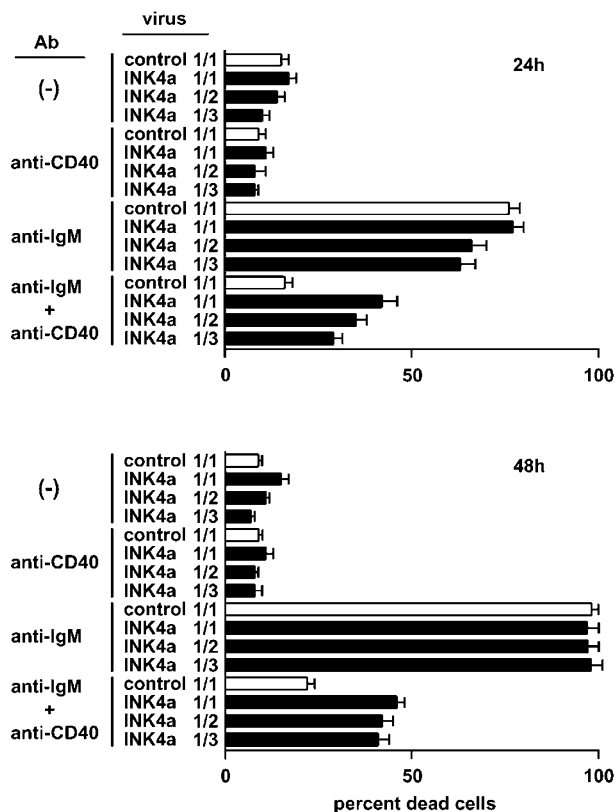
Figure 6 Expression of p16[INK4a] partially blocks CD40-mediated survival of WEHI-231 cells. The supernatants containing retrovirus expressing both p16[INK4a] and GFP (INK4a) or GFP alone (control) were either undiluted (1/1) or diluted by two-fold (1/2) or three-fold (1/3), and were incubated with WEHI-231 cells. Cells were then cultured with 10 μg/ml of F(ab')$_2$ fragments of goat anti-mouse IgM Ab, 10 μg/ml anti-mouse CD40 mAb HM40-3 or both for 24 h (upper panel) or 48 h (lower panel). Viability of the cells was assessed by Trypan blue staining, and percentages of dead cells were calculated. The data represent mean $\pm$ S.D. of triplicate cultures. Representative data of three experiments are shown

arrest, suggesting that high-level expression of antiapoptotic members of the Bcl-2 family blocks apoptosis even in the presence of cell cycle arrest. Thus, expression of these antiapoptotic members of Bcl-2 family by CD40 ligation is not sufficient, but requires coexistence of cell cycle progression for full survival of BCR-ligated B cells. Cell cycle progression appears to be involved in antiapoptotic activity of CD40 signaling.

When B-cell lines such as WEHI-231 undergo BCR ligation-induced apoptosis, cell cycle is arrested prior to apoptosis.[21–24] Since restoration of cell cycle progression is involved in CD40-mediated abrogation of apoptosis in BCR-ligated WEHI-231 cells, cell cycle arrest induced by BCR ligation may be involved in BCR-mediated apoptosis in these cells. Involvement of cell cycle arrest in BCR-mediated apoptosis was previously suggested by Sonnenshine and colleagues. They reported that WEHI-231 transfectants constitutively expressing c-Myc, essential for cell proliferation, fail to undergo apoptosis upon BCR ligation.[25] They further reported that treatment with anti-sense oligonucleotides for cip1 and kip1 reduces BCR-mediated apoptosis.[26] Based on these

findings, they suggested that reduction of c-Myc causes cell cycle arrest by increasing the levels of CDK inhibitors, thereby inducing apoptosis in BCR-ligated WEHI-231 cells. However, later studies using inducible activation of c-Myc demonstrated that c-Myc rather enhances apoptosis,[27] and suggested that Sonnenshine's observation may be due to apoptosis-resistant variants obtained during establishment of stable transfectants. They also failed to rule out the possibility that antisense oligonucleotides to kip1 and cip1 induce the survival of WEHI-231 cells by their nonspecific effects as oligonucleotides, such as activation of TLR-9, transmitting a survival signal,[28] and of dsRNA-activated kinase.[29] Recently, Banerji et al.[30] demonstrated that expression of p27[kip1] induces both cell cycle arrest and apoptosis in WEHI-231 cells. This observation indicates that p27[kip1] can induce apoptosis, but does not show whether BCR-mediated apoptosis requires p27[kip1] and/or cell cycle arrest. Thus, whether cell cycle arrest induced by BCR ligation is involved in BCR-mediated apoptosis is to be elucidated using appropriate tools.

Cytokines such as IL-3 and IL-7 induce survival as well as proliferation.[31,32] Although these cytokines activate signaling pathways distinct from that through CD40, these cytokines induce the expression of antiapoptotic members of the Bcl-2 family, as is the case for CD40 ligation.[12,14,15,17] IL-3 induces expression of Bcl-xL,[32] and its overexpression reverses the apoptosis induced by IL-3 deprivation in BaF3 cells without restoring cell cycling.[33] IL-7 induces the expression of Bcl-2,[31] and its constitutive expression restores defects in the generation of various hematopoietic cells in IL-7-deficient mice.[34,35] These observations suggest that expression of antiapoptotic members of the Bcl-2 family is involved in survival induced by cytokines. However, these cytokines reduce the p27[kip1] level and increase the levels of CDKs,[36,37] as is the case for CD40 signaling. Therefore, it could be possible that cytokine-induced full survival requires cell cycle progression through regulation of CDKs and their inhibitors.

It is already established that cell cycle progression regulates survival when cell cycle is arrested by checkpoint activation.[38,39] For instance, ionizing irradiation induces DNA damage, which activates DNA damage checkpoint, thereby causing cell cycle arrest.[40] The arrest allows the cells to repair DNA damage before proceeding cell cycle progression. Abrogation of checkpoint causes immature cell cycle entry, resulting in mitotic catastrophe and cell death.[41] Thus, cell cycle progression promotes cell death when cell cycle checkpoints are activated. However, cell cycle progression enhances survival but not apoptosis when CD40 signaling regulates B-cell apoptosis. Moreover, cell cycle checkpoints do not appear to be activated when B cells undergo spontaneous or BCR-mediated apoptosis. Cell cycle progression by CD40 signaling thus regulates B-cell apoptosis independently of checkpoint activation. Checkpoint-independent regulation of apoptosis by cell cycle progression occurs in other cell types. For instance, expression of the CDK inhibitors p16[INK4a] and p21[cip1] enhances survival in the myoblast C2C12 upon differentiation.[42] In contrast, expression of CDK inhibitors induces apoptosis in various cell types such as HeLa.[43] In serum-deprived HUVEC, caspase-dependent degradation of CDK inhibitors promotes cell cycling and retards apoptosis.[44] In these examples, check-

point does not appear to be activated, but cell cycle inhibitors regulate apoptosis. Interestingly, cell cycle progression appears to enhance apoptosis in C2C12 cells and serum-deprived HUVEC, but is involved in B-cell survival by a signal through CD40, and is antiapoptotic in cells such as HeLa. Cell cycle progression thus regulates apoptosis in various cell types even in the absence of cell cycle checkpoint activation, but how it regulates appears to depend on the cellular context, probably including cell types and differentiation status. Since both cell cycle control and apoptosis are crucial events in cells, elucidation of crosstalk between them is likely to be crucial for understanding the regulations of both cell proliferation and apoptosis, and also actions of survival signals such as that through CD40 and cytokines.

Materials and Methods

Cell culture

The mouse B-lymphoma line WEHI-231 and its Bcl-2 transfectant WEHI-Bcl2[18] were cultured in RPMI 1640 medium supplemented with 10% FCS, 50 μM 2-mercaptoethanol and 2 mM L-glutamine in the presence or absence of 10 μg/ml F(ab')$_2$ fragments of goat anti-mouse IgM Ab (ICN Pharmaceuticals, Aurora, OH, USA) and 10 μg/ml anti-mouse CD40 monoclonal antibody (mAb) HM40-3 (kindly provided by Dr. Yagita, Juntendo University, Tokyo, Japan).

Plasmids

The retroviral vector pMx-IRES-CD8 and pMx-IRES-YFP were constructed by replacing the GFP cDNA in the pMx-IRES-GFP (kindly provided by Drs. Kitamura and Miyajima, University of Tokyo, Tokyo, Japan)[45] by a NotI–SalI fragment encoding mouse CD8[46] and a NotI–SalI fragment encoding YFP, respectively. The EcoRI–XhoI fragment containing kip1 cDNA was isolated from pBS-Kip1[20] and inserted into the EcoRI-XhoI site of pMx-IRES-GFP and pMx-IRES-CD8, resulting in pMx-Kip1-IRES-GFP and pMx-Kip1-IRES-CD8, respectively (Figure 1). The BamHI fragment containing INK4a cDNA was isolated from pCMV-FLp16 (kindly provided by Dr. Harlow, MGH Cancer Center, Charlestown, USA),[47] inserted into the BamHI site of pUC19, and then recovered as an EcoRI–SalI fragment. pMx-INK4a-IRES-GFP was constructed by inserting this EcoRI–SalI fragment into the EcoRI–XhoI site of pMx-IRES-GFP. The cip1 cDNA was obtained by RT-PCR using WEHI-231 cells with a set of primers: 5′-AGAATTCATGTCCAATCCTGGTGAT-3′ and 5′-ACTCGAGT-CAGGGTTTTCTCTTGCA-3′. The EcoRI–XhoI fragment containing cip1 cDNA was inserted into the EcoRI–XhoI site of pMx-IRES-GFP, resulting in the retrovirus vector pMxCip1-IRES-GFP. A pair of synthetic oligonucleotides (5′-AATTCAACTCGAGAAGCGGCCGGCG-3′ and 5′-TCGACGCGGCCGCTTCTCGAGTTG-3′) was annealed and ligated with the EcoRI-XhoI-opened pMx-IRES-YFP. The resulting plasmid was digested with EcoRI and inserted with the DNA fragment containing the HA tag generated by annealing a set of synthetic oligonucleotides (5′-AATTAAGAAAATGTATCCCTATGACGTGCCCGACTATGCGG-3′ and 5′-AATTCCGCATAGTCGGGCACGTCATAGGGATACATTTTCTT-3′). The resulting plasmid was designated as pMx-HA-IRES-YFP. The Bcl-xL cDNA[48] was obtained by RT-PCR from WEHI-231 cells using a set of primers: 5′-AACTCGAGAATGTCTCAGAGCAACCGG-3′ and 5′-AAGCGGCCGCTCACTTCCGACTGAAGAG-3′. The XhoI–NotI fragment containing Bcl-xL cDNA was inserted into the XhoI–NotI site of pMx-HA-

IRES-YFP, resulting in the retrovirus vector pMxBcl-xL-IRES-YFP encoding the retrovirus expressing both Bcl-xL and YFP.

Retrovirus infection

The retrovirus packaging cell line Plat-E (kindly provided by Dr. Kitamura)[49] was maintained in Dulbecco's Modified Eagle Medium supplemented with 10% FCS and 2 mM L-glutamine. Plat-E cells (1×10^6) were seeded into 60 mm dishes. After 24 h, cells were transfected retroviral vectors using Fugene6 (Roche Diagnostics Corp., Indianapolis, USA). Cells were cultured for 48 h, and 1.5 ml of the culture supernatant was incubated with 5×10^5 WEHI-231 for 4 h. After 24 h, expression of GFP and YFP was analyzed by flow cytometry using a FACSCalibur (BD Biosciences, San Jose, USA). Alternatively, cells were stained with biotinylated anti-CD8 mAb TIB-105 and phycoerythrin (PE)-conjugated streptavidine (DAKO, Glostrup, Denmark), and analyzed by flow cytometry.

Western blot analysis

Cells were washed with PBS, and lysed in SDS-PAGE sample buffer. Proteins were subjected to SDS-PAGE on a 10 or 15% acrylamide gel under reducing conditions, and transferred to PVDF membranes (Amersham Biosciences, NJ, USA). Membranes were incubated with mouse anti-p27[kip1] mAb (BD Biosciences) or mouse anti-β-tubulin mAb (Seikagaku Co., Tokyo, Japan), followed by incubation with peroxidase-conjugated sheep anti-mouse Ig Ab (Amersham Biosciences). Alternatively, membranes were reacted by goat anti-A1 Ab (Santa Cruz Biotechnology, CA, USA) or rabbit anti-Bcl-xL Ab (Santa Cruz Biotechnology), followed by incubation with peroxidase-conjugated donkey anti-goat IgG Ab (Santa Cruz Biotechnology) or peroxidase-conjugated goat anti-rabbit Ig Ab (New England Biolabs Inc., MA, USA), respectively. Proteins were then visualized by ECL system (Amersham Biosciences).

Cell cycle analysis

Cells were pulsed with 20 μM of 5-bromo-2′-deoxyuridine (BrdU) at 37°C for the last 20 min of culture, and then fixed with 70% ethanol overnight at -20°C. Cells were then stained with fluorescein isothiocyanate (FITC)-labeled anti-BrdU Ab (BD Biosciences) and 5 μg/ml of propidium iodide (PI), and analyzed by flow cytometry using a FACSCalibur (BD Biosciences).

Assay of apoptotic cells

Cells were fixed in 70% ethanol overnight at -20°C, washed with PBS, and incubated in 1 ml PBS containing 50 μg/ml RNase A for 30 min at 37°C. PI was added to a final concentration of 50 μg/ml, and cells were analyzed by flow cytometry using a FACSCalibur (BD Biosciences).

Acknowledgements

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