Transcriptional Activation of the cdc2 Gene Is Associated with Fas-induced Apoptosis of Human Hematopoietic Cells*

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Apoptosis has recently been hypothesized to be the result of aberrant cell cycle control. In this study, we have investigated the role of cell cycle-regulatory elements in Fas-induced apoptosis of hematopoietic cells. When HL-60 cells were treated with anti-Fas antibody, rapid activation of growth-associated histone H1 kinase was observed without any change in cell cycle distribution. This was accompanied by the increase in cdc2 mRNA expression and Cdc2 kinase activity. Up-regulation of cdc2 mRNA was similarly induced in BCL-2-overexpressing HL-60 subline by anti-Fas treatment independently of the appearance of apoptotic phenotypes. Fas-induced apoptosis was completely inhibited by butyrolactone I, a specific inhibitor of Cdc2 kinase. Moreover, the same phenomenon was observed during Fas-induced but not spontaneous apoptosis of postmitotic granulocytes. Finally, we have found that "Fas-responsive element" was located between nucleotides −739 and −552 of the cdc2 promoter and was responsive for transcriptional activation of the cdc2 gene during Fas-induced apoptosis. These results indicate that aberrant activation of Cdc2 is associated with Fas-induced apoptosis of hematopoietic cells, and that the mechanism of cdc2 transcription during Fas-induced apoptosis is different from that in normal cell cycle control.

Apoptosis is the physiological process of cell death that functions to control cell populations in many aspects of the biological events. It is well known that apoptosis occurs in most tissues during embryonal development (1). It plays an important role in many aspects of immune responses such as immunotolerance and elimination of virus-infected cells or tumor cells (2, 3). Recent evidence indicates that apoptosis is also a major physiological mechanism to control the homeostasis of the hematopoietic cell system. Many precursor cells succumb to apoptotic cell death during hematopoietic differentiation, because there is competition for limiting amounts of hematopoietic growth factors in the bone marrow (4). The numbers of terminally differentiated hematopoietic cells such as monocytes and granulocytes are also regulated by means of an apoptotic mechanism (5, 6). Activated monocytes and granulocytes are potentially harmful to the host and, thus, should be removed as soon as they finish their role during infection or immune response. Escape from apoptotic cell death might result in abnormal accumulation of these effector cells, leading to fatal tissue damage as observed in transgenic mice overexpressing granulocyte-macrophage colony-stimulating factor (7). Prevention of apoptosis may cause neoplastic transformation of hematopoietic cells, and in contrast, inappropriate induction of apoptosis may result in ineffective hemopoiesis as observed in myelodysplastic syndromes (8). It is obvious from these facts that clarification of the molecular mechanisms regulating the apoptotic program is important, but these mechanisms have not been fully elucidated in spite of recent extensive investigations.

Fas is a type I membrane protein with a molecular mass of 45 kDa, which belongs to the tumor necrosis factor/hormone growth factor receptor family. It mediates apoptosis after Fas ligand binding or cross-linking with an agonistic anti-Fas antibody (9, 10). The physiological significance of Fas-mediated cell death is demonstrated by the development of autoimmune lymphoproliferative disease in the mice bearing a mutation either in the fas gene or its ligand (10). This suggests that the Fas/Fas ligand system is mainly involved in activation-induced cell death of T-lymphocytes, which is necessary for clonal deletion and down-modulation of the function of immune system. Recently, it has been reported that Fas antigen is also expressed on the surface of nonlymphoid hematopoietic cells including immature myeloid cells, monocytes, and granulocytes (11). Although this strongly suggests the involvement of the Fas/Fas ligand system in the regulation of hematopoietic cell death and survival, the mechanisms by which Fas triggers apoptosis in these cells are largely unclear.

Apoptosis has recently been hypothesized to be the result of aberrant cell cycle control. First, it is frequently observed in highly proliferative cells such as embryonal cells, hematopoietic cells, and neoplastic cells (1–4). Second, some of the morphological features observed during apoptosis are similar to those of normal mitosis, including cell rounding, nuclear envelope breakdown, chromatin condensation, and nuclear lamina disassembly (12, 13). Furthermore, activation of a number of genes that mediate the transition of cells from quiescence to the proliferative state (e.g., c-myc and E2F-1) is associated with apoptosis (14–16). Finally, we have recently found that HL-60 cells in S phase of the cell cycle are more susceptible to apoptosis induced by various stimuli than those arrested in G<sub>s</sub>/G<sub>i</sub> phase (17). This evidence allows us to speculate that some cell cycle components are implicated in the process of apoptosis.

In this study, with this background in mind, investigations were carried out to clarify the role of Cdc2 kinase, a serine/
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threonine protein kinase that is critical for G2/M transition and mitosis (18) in Fas-induced apoptosis of hematopoietic cells.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—Anti-Fas monoclonal antibody was purchased from MBL Co. Ltd. (Nagoya, Japan). This antibody was purified from ascites of BALB/c mice inoculated with hybridoma clone CH-11 that was originally established by Yonehara et al. (9). Purified mouse IgM was obtained from Pharmingen (San Diego, CA) and used as isotype-matched control antibody. Highly purified recombinant human interleukin-1 (IFN-γ) was purchased from Genzyme (Cambridge, MA). All other chemicals were ordered from Sigma unless specified.

**Cell Preparation and Culture**—Granulocytes were isolated from the healthy volunteers by the dextran sedimentation method under institutional review board-approved protocols after informed consent (5, 6). Human promyelocytic leukemia cell line HL-60, histiocytic lymphoma cell line U937 and B-lymphoblastoid cell line Daudi were obtained from the American Type Culture Collection (Rockville, MD). BCL-2-overexpressing HL-60 stable transformant (HL-60/Bcl-2) was established by introducing a mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA) containing full-length bcl-2 cDNA (provided by Dr. Yoshihide Tsujimoto, Osaka University, Osaka, Japan) as previously reported (19). HL-60 subline stably transfected with an empty vector was used as a control (HL-60/Mock).

Granulocytes were cultured at an initial concentration of 5 × 10^5 cells/ml in RPMI 1640 medium (Flow Laboratories, McLean, VA) containing 10% heat-inactivated fetal bovine serum (FBS) (Commonwealth Serum Laboratories, Melbourne, Australia) in the absence or presence of 100 ng/ml of anti-Fas monoclonal antibody.

HL-60 cells were routinely maintained in GIT medium (Wako Pure Chemicals, Osaka, Japan). For the induction of apoptosis, the cells were seeded at an initial concentration of 2 × 10^5 cells/ml in serum-free medium (10% GIT in Ham's F-12 medium) and grown in the presence of 100 ng/ml of anti-Fas monoclonal antibody.

Wright-Giemsa staining of the cytospin specimen was performed for morphological assessment of apoptotic cells. Appearance of the apoptotic body was defined as a morphological marker of apoptosis in individual cells. The percentage of apoptotic cells was determined microscopically by counting more than 200 cells on cytospin slides.

**Cell Cycle Analysis**—The cell cycle profile was determined by staining DNA with propidium iodide in preparation for a flow cytometry measurement with FACScan/CellFIT system (Becton-Dickinson, San Jose, CA).

**Analysis of DNA Fragmentation in Agarose Gels**—Approximately 1 × 10^9 cells were incubated at 50 °C in 100 μl of DNA isolation buffer (10 mM EDTA, 50 mM Tris-HCl, pH 8.0, 0.5% laurylsarcosine) containing 500 μg/ml proteinase K. Following overnight incubation, RNAase was added to a final concentration of 150 μg/ml. DNA was extracted with phenol/chloroform and then precipitated with ethanol. Ten nanograms of each sample was analyzed by 1% agarose gel electrophoresis.

**Histone H1 Kinase Assay**—Histone H1 kinase activity was measured as previously reported (21). Briefly, cells were lysed in Nonidet P-40 lysis buffer in the presence of protease inhibitors. One hundred nanograms of crude protein was incubated for 20 min at 30 °C in 40 μl of kinase buffer containing 20 μg Hepes (pH 7.5), 15 mM EGTA, 2 mM MgCl_2, 1 mM dithiothreitol, 500 nM protein kinase A inhibitory peptide, 20 μg of histone H1, and 15 μCi of [γ-32P]ATP. Samples were resolved on 12% SDS-polyacrylamide gel electrophoresis and analyzed by autoradiography after the gel was stained with Coomassie Brilliant Blue G-250. Quantitation of the results was done by scintillation counting of the incised bands after the autoradiography.

**Reporter Plasmid Construction**—The deleted cdc2 promoter fragments were generated by PCR based on the reported sequence of the 5′-flanking region of the cdc2 gene (23). cDNA fragments were subcloned into a pCR1 TA cloning vector (Invitrogen), and the recombinant clones were subjected to sequencing analysis to confirm the expected sequence. A HindIII/HpaI fragment from these clones was then subcloned into a pCDNA3 vector (Promega), for analysis of promoter activity. pCAT-control vector (Promega), which contains SV40 promoter and enhancer sequences, was used as a positive control for the chloramphenicol acetyltransferase (CAT) assay. pSV-β-gal (Promega) was co-transfected with test plasmids to monitor the transfection efficiencies of each sample. All plasmids were purified by cesium chloride gradient ultracentrifugation, linearized by appropriate restriction enzymes, and purified again by ethanol precipitation before transfection.

**Transient Transfection and CAT Assays**—Plasmids were introduced into the cells by electroporation as described (24). Exponentially growing cells (total 2 × 10^6 cells) were resuspended in 500 μl of RPMI 1640 containing 20% FBS. Electroporation was performed using a Gene Pulser apparatus (Bio-Rad) at 250 V, 960 microfarads in the presence of 0.2 μg lipofectin. The cells were placed in ice for 30 min, resuspended at 5 × 10^5 cells/ml in RPMI 1640 medium containing 10% FBS, and divided equally into two groups. Anti-Fas antibody was added into one of them at a final concentration of 100 ng/ml. After 24 h of culture, the cells were harvested for the preparation of whole cell extracts. CAT assays were carried out according to the standard procedure, and the activities were quantitatively measured by liquid scintillation counting (25).

**RESULTS**

**Histone H1 Kinase Activity in Fas-treated HL-60 Cells**—In order to examine the involvement of cell cycle-regulatory components in apoptosis of hematopoietic cells, we first measured

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1 The abbreviations used are: IFN, interferon; FBS, fetal bovine serum; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CAT, chloramphenicol acetyltransferase; CDK, cyclin-dependent kinase; RT-PCR, reverse-transcription PCR; nt, nucleotide(s).
Expression of the Genes Encoding Cyclin-dependent Kinase during Fas-induced Apoptosis—Among mammalian cyclin-dependent kinases (CDKs), Cdc2, Cdk2, Cdk5, and PITSLRE are known to effectively phosphorylate histone H1 in vitro (28). Thus, we investigated expression of these four genes during Fas-induced apoptosis of HL-60 cells in order to determine the type of kinase(s) activated by Fas. Quantitative reverse-transcription PCR (RT-PCR) analysis was used for this purpose, because relatively small amounts of RNA could be obtained from dying cells (especially in the case of granulocytes; see below), and many genes including a ubiquitously expressed control (GAPDH in this study) could be detected simultaneously in the same sample (29, 30). Total cellular RNA was isolated from Fas-treated HL-60 cells at various time points and reverse transcribed into cDNA. Equal proportions of the resultant cDNA were then subjected to quantitative PCR analysis using specific primer pairs. Cycle numbers of PCR were set for each gene to demonstrate that the amount of amplified product was directly proportional to the amount of transcripts present in the sample RNA. As shown in Fig. 3A, cdc2 and cdk2 mRNA expression was readily increased after 30 min of the treatment with anti-Fas antibody, while expression of the ubiquitous gene GAPDH remained constant. On a densitometric analysis, maximal increase of cdc2 mRNA transcript was found to be 2.5-fold over the pretreatment level, whereas that of cdk2 was only 1.5-fold (Fig. 3B). Again, this increase was not a result of the changes in cell cycle distribution (see above). In contrast, the amounts of cdk5 and PITSLRE mRNAs diminished over the time course of apoptosis with a significant decrease in expression (less than 30% of the initial amount) by 6 h (Fig. 3B). Among other cell cycle-related genes examined, cyclin A but not cyclins B, D1, and E mRNA levels was also elevated (data not shown). No significant changes in the expression of these genes were observed in HL-60 cells treated with isotype-matched control antibody (data not shown).

Fas-induced up-regulation of cdc2 mRNA was further confirmed by Northern blotting in HL-60 cells. As shown in Fig. 4, an approximately 3-fold increase in the amounts of cdc2 mRNA transcripts was observed after 30 min of the treatment with anti-Fas antibody, whereas no such increase was obtained with isotype-matched control. This is fully consistent with the result of RT-PCR analysis. Furthermore, this increase in cdc2 expression was not specific for HL-60 cells, i.e. the same pattern of induction was observed in another Fas-sensitive leukemic cell line U937 but not in Fas-negative cell line Daudi (data not shown). Taking into account the recent observation that a newly synthesized Cdc2 protein possesses a major protein kinase activity (31, 32), it is reasonable to speculate that up-regulation of cdc2 is mainly responsible for increased histone H1 kinase activity after Fas treatment of HL-60 cells, although the involvement of other kinases cannot be ruled out.

Up-regulation of cdc2 mRNA by Anti-Fas Antibody in BCL-2-overexpressing HL-60 Subline—Expression of cdc2 mRNA was also examined in BCL-2-overexpressing HL-60 subclone (HL-60/Bcl-2) to determine whether Fas-induced up-regulation of cdc2 can occur independently of the appearance of apoptotic phenotypes. HL-60/Bcl-2 cells were resistant to Fas, i.e. neither morphological changes characteristic of apoptosis nor oligonucleosomal DNA fragmentation was observed after 16 h of anti-Fas treatment, while empty vector-containing control (HL-60/Mock) underwent apoptosis to an extent similar to that of parent cells (Fig. 5A). Total cellular RNA was isolated from both cell lines at various times after Fas treatment and subjected to quantitative RT-PCR analysis for cdc2 expression. As shown in Fig. 5B, cdc2 mRNA expression was increased with a peak at 30 min in HL-60/Bcl-2 cells as observed in both parent

Histone H1 kinase activity at various times after Fas treatment of HL-60 cells. Histone H1 kinase activity is well correlated with the proportion of the cells in active cell cycle (26) and reflects the amounts of active cyclin-dependent kinases, especially Cdc2 (27). When HL-60 cells were treated with 100 ng/ml of anti-Fas antibody, approximately 25% of the cells displayed morphological changes characteristic of apoptotic cell death such as cell shrinkage and membrane blebbing after 16 h of the culture, while control culture contained less than 5% apoptotic cells (data not shown). As shown in Fig. 1A, DNA electrophoresis revealed that oligonucleosomal length DNA fragmentation, a hallmark of apoptosis, was present in these cells. On a DNA histogram obtained with propidium iodide staining, typical subdiploid fractions were observed after 16 h of the culture with anti-Fas antibody (Fig. 1B).

Histone H1 kinase activity was readily increased in Fas-treated HL-60 cells after 2 h of the treatment, although pretreatment cells already had a high activity (Fig. 2A, left panel). This increase apparently preceded the induction of apoptosis, since no DNA fragmentation was observed at this time point (Fig. 1A). There was no increase in histone H1 kinase activity in HL-60 cells cultured with isotype-matched control antibody (Fig. 2A, right panel). To confirm that H1 kinase activation is actually mediated through Fas, we carried out the same experiments with HL-60 cells preincubated with IFN-γ. IFN-γ pretreatment was known to up-regulate surface Fas expression, thereby enhancing cellular susceptibility to anti-Fas-mediated apoptotic cell death (9). In our experiments, the percentage of apoptotic cells increased from 25 to 85% by the pretreatment of HL-60 cells with 200 IU/ml of IFN-γ for 24 h. In accordance with the increase in Fas-induced apoptotic cell death, histone H1 kinase activity was significantly enhanced in HL-60 cells by pretreatment with IFN-γ (Fig. 2B). This is due to the direct effect of IFN-γ, since IFN-γ alone neither induced apoptosis nor activated histone H1 kinase in HL-60 cells (data not shown).

The increase in H1 kinase activity was not a result of the changes in cell cycle distribution, since cell cycle profile determined by flow cytometric analysis was unchanged within 6 h of the culture when H1 kinase was fully activated (Fig. 1B). Therefore, this finding is rather consistent with the hypothesis that Fas-induced enhancement of histone H1 kinase activity might be due to aberrant activation of cyclin-dependent kinases.
and HL-60/Mock control cells. This suggests that Fas-induced up-regulation of cdc2 is not a simple consequence of the induction of apoptosis, and it occurs prior to the critical step of apoptosis that can be negatively regulated by Bcl-2. Histone H1 Kinase Activity during Fas-induced Apoptosis of Granulocytes—Recently, it has been reported that Fas antigen is constitutively expressed on peripheral blood granulocytes (33). Thus, we performed the same experiments using granulocytes in order to confirm the findings obtained with HL-60 cells. Granulocytes are believed to have the shortest half-life among hematopoietic cells and rapidly undergo apoptosis in vitro (34). Morphologically identifiable apoptotic cells began to accumulate after 4 h of culture in the medium containing 10% FBS and reached more than 50% of the entire population after 16 h (Fig. 6A). In the presence of anti-Fas antibody, the induction of apoptosis in granulocytes was significantly accelerated, i.e. approximately 20% of the cells showed the morphological features of apoptosis at 2 h, and more than 80% of the cells became apoptotic after 16 h (p < 0.01 versus anti-Fas− culture).

Whole cell lysates were prepared from these cells and subjected to histone H1 kinase assays. As shown in Fig. 6B, an approximately 5-fold increase in histone H1 kinase activity was observed following anti-Fas treatment in granulocytes. This increase was transient with a peak after 2 h of the treatment. Phosphorylation of histone H1 was then decreased gradually and became less than control levels at 16 h when more than 80% of the cells underwent apoptotic death. In contrast, there was no increase in H1 kinase activity during spontaneous apoptosis (data not shown).

Next, we investigated expression of cdc2 mRNA in Fas-treated granulocytes by quantitative RT-PCR analysis. As previously reported (23), little if any cdc2 mRNA transcript was present in granulocytes just after the isolation (Fig. 6C). No induction of cdc2 mRNA was observed after the culture either in the absence or presence of appropriate cytokines including granulocyte-colony stimulating factor (Fig. 6C, left). This is consistent with the notion that peripheral blood granulocytes are postmitotic cells and never reenter the cell cycle even after the stimulation (23,35). However, cdc2 mRNA was apparently induced by anti-Fas antibody with a peak at 1 h, suggesting that Cdc2 is at least in part responsible for the increased histone H1 kinase activity in dying granulocytes (Fig. 6C, right). Other cdk genes were not detected by this approach (data not shown). These results are fully consistent with the results obtained with HL-60 cells.

Cdc2 Kinase Activity in Fas-treated HL-60 Cells and Granulocytes—To further corroborate that Cdc2 kinase is activated during Fas-induced apoptosis, we directly measured Cdc2 kinase activity in Fas-treated hematopoietic cells. Specific Cdc2 kinase activity was measured with a peptide substrate derived from SV40 large T antigen, which is known to be phosphorylated by Cdc2 (22). As shown in Fig. 7, Cdc2 activity was rapidly induced in both HL-60 cells and granulocytes after the treatment with anti-Fas antibody, while pretreatment activity was already high in HL-60 cells. In granulocytes, Fas-induced activation of Cdc2 kinase was transient with a peak at 2 h, and it declined gradually after 4 h. In contrast, increased Cdc2 activ-

![Fig. 2. Histone H1 kinase activity in Fas-treated HL-60 cells. A, HL-60 cells were cultured with either anti-Fas antibody or purified mouse IgM (control antibody) for 16 h. Whole cell lysates were prepared at the indicated time points, and subjected to histone H1 kinase assay. Samples were analyzed by 12% SDS-polyacrylamide gel electrophoresis and autoradiography. The autoradiogram is shown in the upper panel. Coomassie Brilliant Blue staining of the gel is shown in the lower panel to indicate the equal amount of histone H1 in each sample. B, HL-60 cells were preincubated with 200 IU/ml of IFN-γ for 24 h, washed, and resuspended in the medium containing anti-Fas antibody. Histone H1 kinase activity was determined as shown in A. Data shown are representative of three independent experiments.](image)
city was sustained in HL-60 cells after 2 h of the treatment. This kinetics of Cdk2 kinase activation was markedly similar to that of the induction of histone H1 kinase activity by Fas in each cell type.

Effect of CDK Inhibitor on Fas-induced Apoptosis of HL-60 Cells—Next, we investigated the effect of inhibition of Cdc2 activity on Fas-induced apoptosis in order to determine whether the induction of Cdc2 activity was really required for apoptosis. Butyrolactone I was used for this purpose. This agent was isolated from mycelia of Aspergillus terreus, and shown to be a specific, potent inhibitor of CDK activity both in vitro and in vivo (36, 37). HL-60 cells were preincubated with various doses of butyrolactone I for 24 h, and DNA fragmentation was assessed after a further 16 h of incubation with anti-Fas antibody. As shown in Fig. 8, butyrolactone I could inhibit DNA fragmentation in a dose-dependent manner, and it could almost completely abrogate the effect of Fas at 20 μM. Simultaneous analysis of the cell cycle profile revealed that butyrolactone I-treated cells were arrested in G1 and G2 phases of the cell cycle due to the inhibition of Cdk2 and Cdc2, respectively (Fig. 8). No subdiploid peak was present in the cells pretreated with butyrolactone I, while control cells showed subdiploid fractions indicative of DNA fragmentation. This confirms that CDK activity is required for Fas-induced apoptosis.

Transcriptional Activation of the cdc2 Gene in Fas-treated Hematopoietic Cells—Finally, we tried to determine the mechanisms whereby Fas regulates transcriptional activation of the cdc2 gene. Based on the reported sequence of the 5′-flanking region of the cdc2 gene (23), we have constructed four reporter plasmids as described in Fig. 9. These reporter constructs were transiently transfected into Fas-positive (HL-60) and Fas-negative (Daudi) hematopoietic cell lines by electroporation, and CAT activity was assayed after a 24-h culture with or without anti-Fas antibody. Relative CAT activity was expressed as fold increase against the value obtained with transfection of pCAT-control vector into corresponding cells. As previously reported (23, 38), the sequence between nt −383 and +84 showed a strong promoter activity in both Daudi and HL-60 cells, i.e. the reporter plasmid containing this segment (the −383 construct) revealed more than 2-fold increase in CAT activity over pCAT-control vector, which possesses strong SV40 promoter with enhancer (Fig. 10). In agreement with the recent report (38), the promoter activity was markedly

![Image](52x353 to 294x729)
cdc2 Activation in Fas-induced Apoptosis

Recent studies have shown that Cdc2 is involved in apoptotic cell death, since increased expression of cdc2 mRNA was also detected during Fas treatment of BCL-2-overexpressing HL-60 cells. This does not seem to be a simple consequence of apoptotic cell death, since increased expression of cdc2 mRNA was also detected during Fas treatment of BCL-2-overexpressing HL-60 cells. This does not seem to be a simple consequence of apoptosis, since the increase in cdc2 expression is associated with Fas-induced apoptosis of proliferating HL-60 cells. Thus, it is reasonable to speculate that Cdc2 plays a role in the regulation of apoptosis.

Recently, several independent lines of evidence have fostered the notion of a link between cell cycle and apoptosis. Apoptosis shares a number of morphological features with mitosis, including lamina disassembly and chromatin condensation, that are known to be regulated by Cdc2 kinase (12, 13). Thus, it is reasonable to speculate that Cdc2 plays a role in the regulation of apoptosis. This is consistent with the universal view of apoptosis as a cell cycle transition.

The recent work of the authors has shown that Cdc2 is involved in the regulation of apoptosis. In particular, they have demonstrated that Cdc2 is activated during Fas-induced apoptosis, and that this activation is dependent on the presence of Fas receptor. Furthermore, they have shown that Cdc2 activation is necessary for the induction of apoptosis, since inhibition of Cdc2 activity prevents apoptosis.

These findings support the idea that cell cycle and apoptosis are linked, and provide a potential mechanism for the regulation of apoptosis by the cell cycle. The identification of the specific substrates for Cdc2 during apoptosis is necessary to clarify this point. These experiments are currently under way in our laboratory. In addition, the requirement of Cdc2 in Fas-induced apoptosis was confirmed by the complete inhibition of cdc2 mRNA expression in BCL-2-overexpressing HL-60 cells. This suggests that Fas-responsive elements may be present in the region between nt −370 and −552. Transcriptional activation of the cdc2 gene during Fas-induced apoptosis might be mediated through this region and, thus, is regulated by a different mechanism from that of normal cell cycle transition.

DISCUSSION

Recently, several independent lines of evidence have fostered the notion of a link between cell cycle and apoptosis. Apoptosis shares a number of morphological features with mitosis, including lamina disassembly and chromatin condensation, that are known to be regulated by Cdc2 kinase (12, 13). Thus, it is reasonable to speculate that Cdc2 plays a role in the regulation of apoptosis. This is consistent with the universal view of apoptosis as a cell cycle transition.
that neoplastic lesions that generate uncontrolled cell proliferation can also act potent triggers of apoptosis, since Cdc2 is constitutively activated in a majority of tumors including hematologic malignancies (40, 41). These data also provide a good explanation for our recent observation that HL-60 cells in S phase of the cell cycle are more susceptible to apoptosis induced by various stimuli than those arrested in G0/G1 phase (17). This can be explained by the fact that Cdc2 kinase activity is high in S phase HL-60 cells, while it is down-regulated in G0/G1 phase of the cell cycle (23). A similar finding was also reported in HeLa cells, i.e. apoptosis was selectively induced in S phase arrested cells by staurosporine, caffeine, 6-dimethylaminopurine, and okadaic acid concomitantly with activation of Cdc2 kinase (42). Belizario et al. (43) also reported that treatment with agents that arrest cells in G1 phase offered protection from apoptosis. Taken together, these findings indicate that Cdc2 is considered to be one of the most important mediators of apoptosis in highly proliferative cells like immature hematopoietic cells or neoplastic cells.

Moreover, we have found that the same phenomenon was observed during Fas-induced apoptosis of peripheral blood granulocytes independently of the cell cycle entry. Recently, Iwai et al. (33) reported that anti-Fas could enhance apoptosis of granulocytes, but little is known about its underlying mechanisms. It is somewhat surprising that Cdc2 kinase was also activated and cdc2 mRNA was up-regulated in granulocytes, because they are postmitotic cells and cdc2 mRNA expression was not usually observed even after the culture with appropriate stimulants such as granulocyte colony-stimulating factor or lipopolysaccharides (23). Intriguingly, the reagents that normally induce activation of granulocytes are unable to induce cdc2 mRNA expression; rather, they protect them from spontaneous apoptosis (44). Induction of cdc2 mRNA expression was not usually observed even after the culture with appropriate stimulants such as granulocyte colony-stimulating factor or lipopolysaccharides (23). Intriguingly, the reagents that normally induce activation of granulocytes are unable to induce cdc2 mRNA expression; rather, they protect them from spontaneous apoptosis (44). Induction of cdc2 mRNA seems specific for Fas treatment in granulocytes, suggesting that it plays a critical role in Fas-induced apoptosis. On the basis of this finding, it has been proposed that inappropriate activation of the cell cycle-regulatory elements in postmitotic cells might result in entry into an abortive cell cycle, leading them to apoptotic cell death. A series of recent reports support this hypothesis. First, castration-induced regression of the rat ventral prostate is associated with the induction of cyclin A mRNA and other markers of cell proliferation including proliferative cell nuclear antigen and bromodeoxyuridine uptake (45). Second, apoptosis is frequently associated with activation of genes.
that mediate the transition of cells from quiescence to the proliferative state (46). For example, cyclin D1 is reported to be selectively induced in postmitotic neurons that undergo apoptosis upon withdrawal of nerve growth factor (30). Finally, overexpression of growth-promoting genes such as c-myc (14, 47), adenovirus EIA (48, 49), and E2F-1 (15, 16) in serum-deprived or p53-overexpressed quiescent cells resulted in apoptotic cell death. These data indicate that at least some forms of apoptosis might be the result of abnormal or conflicting growth signals. aberrant activation of Cdc2 in postmitotic granulocytes may be included in this category.

Although our present finding has implicated Cdc2 kinase in the pathway of Fas-mediated apoptosis in hematopoietic cells, Cdc2 activation during apoptosis is not specific for Fas. Premature activation of Cdc2 was first demonstrated in apoptosis of YAC-1 lymphoma cells induced by fragmentin-2, a cytotoxic T-cell granule protease (50). Subsequently, it has been shown that Cdc2 kinase activity was required for human immunodeficiency virus-1 Tat protein-induced apoptosis in T-lymphocytes (51). Similar findings were later obtained in apoptosis of HeLa cells triggered by tumor necrosis factor or staurosporine (42). In addition, Cdc2 is not the only kinase that is activated during apoptosis. Lahti et al. reported that proteolytic activation of PITSLRE kinase, a member of the CKD family, was observed in Fas-induced apoptosis of human T-cell lines (52). They have also demonstrated that ectopic expression of PITSLRE could induce telophase arrest with ensuing apoptosis. Activation of Cdk2 was also reported in some forms of apoptosis (42). In this study, we have also observed that a minor increase in cdk2 mRNA expression was accompanied by Fas-induced apoptosis in HL-60 cells but not in granulocytes. However, the extent of the increase in cdk2 mRNA was less striking than that of cdc2 in our system. In contrast to the report by Lahti et al. (52), no elevation of PITSLRE expression was observed in our experiments. This discrepancy might be due to the difference in cell types used in each study or due to the fact that there are multiple mechanisms and signaling pathways in apoptosis depending on cell types and inducers. Further studies are required to elucidate this point.

Finally, we have investigated the mechanism of transcriptional activation of the cdc2 gene during Fas-induced apoptosis. Expression of cdc2 mRNA is regulated in a cell cycle-dependent manner, i.e. it is suppressed in G0/G1 phase and induced at the G1/S boundary (53). Recent investigations revealed that cellular transcription factor E2F might confer cell cycle regulation of cdc2 mRNA expression. Tommassi et al. (54) reported that suppression of cdc2 mRNA in G0/G1 phase was mediated by the binding of p130-E2F-4 complex at the position of nucleotide −20 relative to the transcription start site. The binding of pRB-E2F-1 complex at the position between nt −124 and −117 of the cdc2 promoter and its dual role in the regulation of cdc2 transcription has been reported (32, 38, 55, 56). Activation of the cdc2 gene was indeed induced by overexpression of E2F in cDNA in quiescent 293 cells with adenovirus vector (57). In the present study, we have shown that 5′-untranslated sequence of the cdc2 gene up to nt −383 had a strong promoter activity in both Fas-positive and negative cells in accordance with our previous report (52). This activity was not affected by anti-Fas treatment even in Fas-responsive HL-60 cells. In contrast, anti-Fas antibody significantly enhanced promoter activity of the sequence up to nt −730 as well as −942, whereas that of the sequence up to nt −552 was unaffected. This suggests the presence of a Fas-responsive element between nt −730 and −552. Among known transcription factors, the putative binding site for YY1 is present in this region (the position from nt −590 to −583). YY1 is a zinc finger-containing transcription factor that can either activate or repress transcription, depending on its promoter context and orientation (58). The YY1-binding site also displays the characteristics of an initiator element in that it can direct specific transcription in the absence of binding sites for other factors (59). With this background, we can speculate that Fas activates cdc2 transcription through a YY1-binding site at the position between nt −590 and −583 of the cdc2 promoter. However, in a preliminary experiment, introduction of a nonbinding mutation at this region (5′-aaaaagt3′ to 5′-caaaccgtt3′) did not affect the increase in the promoter activity of the sequence up to nt −730 by anti-Fas antibody, suggesting that YY1 is not responsible for Fas-induced activation of cdc2 transcription (data not shown).

We are now trying to determine the Fas-responsive element by the more precise analyses. Nevertheless, these results have demonstrated that the mechanism of cdc2 transcription during Fas-induced apoptosis is somewhat different from that in normal cell cycle control.

In summary, these data suggest that, in addition to the well characterized role in cell cycle control and cellular differentiation, Cdc2 may contribute to the maintenance of tissue homeostasis by the regulation of cell mortality, although its physiological relevance is currently unknown. Insights into the molecular mechanism of this "new" function of Cdc2 may offer a better understanding of the physiology of many important biological phenomena.

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