Molecular defects in apoptotic pathways are thought to often contribute to the abnormal expansion of malignant cells and their resistance to chemotherapy. Therefore, a comprehensive knowledge of the mechanisms controlling induction of apoptosis and subsequent cellular disintegration could result in improved methods for prognosis and treatment of cancer. In this study, we have examined apoptosis-induced alterations in two proteins, nucleolin and poly(ADP-ribose) polymerase-1 (PARP-1), in U937 leukemia cells. Nucleolin is expressed at high levels in malignant cells, and it is a multifunctional and mobile protein that can shuttle among the nucleolus, nucleoplasm, cytoplasm, and plasma membrane. Here, we report our findings that UV irradiation or camptothecin treatment of U937 cells induced apoptosis and caused a significant change in the levels and localization of nucleolin within the nucleus. Additionally, nucleolin levels were dramatically decreased in extracts containing the cytoplasm and plasma membrane. These alterations could be abrogated by pre-incubation with an inhibitor of PARP-1 (3-aminobenzamide), and our data support a potential role for nucleolin in removing cleaved PARP-1 from dying cells. Furthermore, both nucleolin and cleaved PARP-1 were detected in the culture medium of cells undergoing apoptosis, associated with particles of a size consistent with apoptotic bodies. These results indicate that nucleolin plays an important role in apoptosis, and could be a useful marker for assessing apoptosis or detecting apoptotic bodies. In addition, the data provide a possible explanation for the appearance of nucleolin and PARP-1 autoantibodies in some autoimmune diseases.

Following treatment with chemotherapy agents or exposure to cellular stress such as heat shock or ionizing radiation, malignant cells can respond by undergoing apoptosis or necrosis, or may be resistant to treatment. The failure of malignant cells to undergo cell death in response to chemotherapy is a major cause of treatment failure (1), and, in many cases, chemoresistance is associated with aberrant expression of the proteins involved in the activation and regulation of apoptosis (1–3). Consequently, several therapeutic strategies based on modulating apoptotic pathways are currently in development (4, 5).

Apoptosis is characterized by well defined morphological and biochemical changes, which are generally mediated by a family of cysteine proteases, called caspases. There are at least two well characterized molecular mechanisms leading to caspase-dependent apoptosis, namely receptor-mediated and mitochondrial pathways, which have been described in some detail in recent reviews (1, 2). Both pathways lead to activation of caspase-3, which ultimately results in fragmentation of chromosomal DNA and proteolysis of selected nuclear proteins. The aim of the present study is to investigate the role of nucleolin during apoptosis of leukemia cells treated with the chemotherapy agent, camptothecin, or irradiated with UV light, and to examine the relationship between changes in nucleolin and effects on poly(ADP-ribose) polymerase-1 (PARP-1).

Human nucleolin is a 707-amino acid protein consisting of an acidic histone-like N terminus, a central domain containing four RNA binding domains, and a C terminus that is rich in arginine and glycine (RGG repeats). This multidomain structure reflects the diverse roles of nucleolin in cell growth, proliferation, and death, which have been recently highlighted in several excellent reviews (6–8). Nucleolin has been implicated in many cellular processes, including transcription, packing, and transport of ribosomal RNA, replication and recombination of DNA, cell cycle progression, and apoptosis (6–14). Although generally considered a predominantly nucleolar protein, nucleolin appears to be very mobile and can also be present in the nucleoplasm and cytoplasm and on the cell surface (9, 10, 15–19). In fact, there are several reports describing redistribution of nucleolin within the cell in response to a number of stimuli, including heat shock (9, 10), mitosis (20), T cell activation (21), treatment with a cyclin-dependent kinase inhibitor (22), and viral infection (23–25).

There is considerable interest in studying nucleolin function, not only because it is involved in so many fundamental processes, but also because of the significance of nucleolin expression in malignant cells. Levels of nucleolin are positively correlated with cellular proliferation (26) and high levels of silver-staining nucleolar proteins (of which nucleolin is the major component) predict a poor prognosis in many types of cancer (27). We have proposed nucleolin as a novel target for therapeutic intervention, based on our finding that G-rich oligonucleotides that bind to nucleolin protein can inhibit proliferation.

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† To whom correspondence should be addressed: 204B Baxter Bldg., 570 S. Preston St., Louisville, KY 40202. Tel.: 502-852-2432; Fax: 502-852-2356; E-mail: paula.bates@louisville.edu.

‡ From the Molecular Targets Group, James Graham Brown Cancer Center, Departments of Medicine and Biochemistry/Molecular Biology, University of Louisville, Louisville, Kentucky 40202

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1 The abbreviations used are: PARP-1, poly(ADP-ribose) polymerase-1; 3-ABA, 3-aminobenzamide; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; TUNEL, terminal dUTP nick-end labeling; PBSF, phosphate-buffered saline plus Tween 20; SLE, systemic lupus erythematosus.
and induce apoptosis in many cell lines derived from solid tumors (28–30) and leukemias (31).

The levels and characteristics of PARP-1 protein have also been examined in this study for two reasons. First, PARP-1 cleavage is a characteristic feature of apoptosis and the timing of apoptosis-associated events is often determined relative to the onset of PARP-1 cleavage. Second, it has been reported that nucleolin can form a complex with PARP-1 in B-cells (11) and in kidney cells (32), suggesting that PARP-1 could potentially be involved in the regulation of nucleolin function, or vice versa. PARP-1 catalyzes the addition of poly(ADP-ribose) chains to a number of nuclear proteins in response to DNA damage. It is clear that this short-lived post-translational modification plays an important, although incompletely defined, role in DNA damage response and apoptosis (33, 34). PARP-1 enzymatic activity is dependent upon binding to DNA strand breaks and is rapidly activated in response to cellular stresses, such as heat shock, gamma radiation, exposure to carcinogens, and treatment with chemotherapy agents. Synthesis of poly(ADP-ribose) uses nicotinamide adenine dinucleotide (NAD\(^+)\), and cleavage of PARP-1 during apoptosis is thought to occur to conserve NAD\(^+)\) and allow production of ATP, which is needed for execution of apoptosis. This cleavage appears to be a universal part of the apoptotic process, and appearance of the 89- and 24-kDa proteolytic fragments of PARP-1 has become one of the classical hallmarks of apoptosis (33, 34).

Here, we describe the effect of apoptosis in U937 leukemia cells on the expression of nucleolin and PARP-1 proteins. Because nucleolin can translocate between different cellular compartments, alterations in nucleolin in both nuclear (containing the nucleoli and nucleoplasm) and S-100 (containing the cytoplasm and plasma membrane) extracts have been evaluated.

**Experimental Procedures**

**Materials**—Camptothecin is an anti-neoplastic topoisomerase I inhibitor, and 3-aminoenbenzamide (3-ABA) is an inhibitor of PARP-1. Both were purchased from Sigma, dissolved in Me\(2\)SO, and diluted with phosphate-buffered saline (PBS) to give stock solutions in 0.5% Me\(2\)SO. Anti-nucleolin antibody (mouse monoclonal IgG\(_1\)), anti-PARP-1 antibody (mouse monoclonal IgG\(_\lambda\)), and normal mouse IgG were from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-poly(ADP-ribose) (rabbit polyclonal) was from Calbiochem (San Diego, CA). Protein A conjugated goat anti-mouse antibody (diluted 1:500 in blocking buffer) for 1 h at room temperature, washed three times in PBS, and placed on glass slides using a cytosensor. Samples were stained using the same procedure described for cells.

**TUNEL Staining**—Slides containing the small bodies from the medium of apoptotic cells were prepared as described above. Slides were washed with PBS and incubated in permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 min on ice, washed twice with PBS, and stained as previously described (28). Briefly, 50 \(\mu\)l of TUNEL reaction mixture (Roche) was added to each sample and slides were incubated in the dark in a humidified chamber for 60 min at 37 °C, then washed three times with PBS and observed as above.

**Preparation of Cellular Extracts and Protein from Cell Culture Medium**—At the appropriate time after treatment, cells were harvested and washed twice with cold PBS, and nuclear and S-100 extracts were prepared according to the method of Coqueret et al. (37). Briefly, 100 \(\mu\)l of ice-cold extraction buffer B (10 mM HEPES, pH 7.9, 1.5 mM MgCl\(_2\), 10 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 1 \(\mu\)g/ml leupeptin, 1 \(\mu\)g/ml aprotinin) was added to the cells. After three cycles of freeze-thaw, S-100 extracts were stored as supernatants following centrifugation at 12,000 \(\times\)g for 1 min, and pellets (nuclei) were resuspended in 40 \(\mu\)l of buffer C (20 mM HEPES, pH 7.9, 1.5 mM MgCl\(_2\), 420 mM KCl, 0.2 mM EDTA, 25% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 \(\mu\)g/ml leupeptin, 1 \(\mu\)g/ml aprotinin). Following 30 min of incubation at 4 °C, insoluble material was precipitated by centrifugation at 12,000 \(\times\)g for 5 min and nuclear extracts were collected as supernatants. Extracts were either used immediately or stored at −80 °C.

For preparation of proteins from the cell culture medium, the medium was first replaced with serum-free medium and cells were irradiated as described above. At the appropriate time, samples were centrifuged at 400 \(\times\)g for 10 min to pellet intact cells and supernatant was aspirated. The medium was filtered (syringe filters with polyvinylidene difluoride membranes, Whatman) where indicated, and proteins present in the medium were concentrated using Centricon YM-30 (Millipore, Bedford, MA) according to the instructions of the manufacturer.

**Immunoblot Analysis**—The concentration of extracted proteins was determined using the Bio-Rad DC protein assay kit. Samples (10 \(\mu\)g) were incubated in sodium dodecyl sulfate (SDS)-loading buffer (100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromphenol blue, 20% glycerol) at 65 °C for 15 min, and separated on 10% (for nucleolin detection) or 8% (for PARP-1) polyacrylamide-SDS gels, followed by electrophoretic transfer to polyvinylidene difluoride membranes (Bio-Rad). After blocking nonspecific binding sites for 1 h in 5% nonfat dried milk in PBS (0.1% Tween 20 in PBS), the membranes were incubated with primary antibody. The membranes were washed three times with cold PBS, then overnight at 4 °C with primary antibody (1:1000 anti-nucleolin or anti-PARP-1 in PBS-T). After three washes in PBS-T, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse antibody for 45 min at room temperature, washed three times in PBST, and detected using enhanced chemiluminescence (ECL kit from Amersham Biosciences). Equal gel loading and transfer of proteins were confirmed by staining membranes with India ink (28).

**Immunoprecipitation**—Nuclear extracts were prepared from untreated cells or following UV irradiation. Immunoprecipitations were performed by incubating 200 \(\mu\)g of extract with 2 \(\mu\)g of PARP-1 antibody overnight at 4 °C, followed by centrifugation and resuspension in PBS containing 5 mM MgCl\(_2\). Cells were lysed by overnight incubation at 37 °C in a buffer containing 0.1% SDS and 1.5 mM Mg\(_2\) proteinase K. Samples were extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), the aqueous layer was transferred to a new tube, and DNA was precipitated with ethanol and digested with 400 \(\mu\)g/ml RNase A. Eluted phosphorylation was performed on 1% agarose gels, stained with ethidium bromide.

**Immunofluorescence Staining of Nucleolin**—Cells were collected by centrifugation, washed twice with PBS, and placed on glass slides using a cytosensor. Samples were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature, and then permeabilized with 0.2% Triton X-100 in PBS for 10 min. After two washes with PBS, nonspecific binding of antibody was blocked by a 1-h incubation at room temperature with 5% normal goat serum in PBS. After three washes with PBS, slides were incubated in primary antibody (1:100 anti-nucleolin antibody in blocking buffer) for 1 h at room temperature and then washed three times in PBS. Slides were incubated with Alexa-488-conjugated anti-mouse (diluted 1:500 in blocking buffer) for 1 h at room temperature. Washed three times in PBS, then observed using an Axioskop BX60F fluorescence microscope and photographed using an Olympus DP10 camera. To stain the small bodies from the culture medium, cells were cultured in serum-free medium, irradiated with UV light, and then returned to the incubator. At the indicated time, cells were pelleted by centrifugation and medium was collected and placed onto glass slides using a cytosensor. Slides were stained using the same procedure described for cells.
Changes in Nucleolin during Apoptosis of Leukemia Cells

**RESULTS**

*Alterations of Nucleolin and PARP-1 Proteins after UV-induced Apoptosis*—Before investigating alterations in protein levels, we confirmed that apoptosis was occurring in treated U937 cells using a DNA fragmentation assay (36). In this assay, apoptosis is indicated by the appearance of a DNA “ladder,” which is produced by endonuclease cleavage of chromosomal DNA into nucleosomal fragments. Fig. 1 shows that apoptosis could be clearly detected within 2 h following treatment of cells by UV irradiation for 30 s or incubation with the chemotherapy agent, camptothecin at 10 μM final concentration.

To examine apoptosis-induced changes in nucleolin and PARP-1, cells were irradiated with UV, and protein extracts were collected at different time points after irradiation. Equal amounts of protein fractions were examined and consisted of nuclear extracts (soluble nuclear proteins) or S-100 extracts (soluble proteins from the plasma membrane, cytosol and non-nuclear organelles). Our previous studies (28) suggest that the majority of the nucleolin present in the S-100 fraction is derived from the plasma membrane. Fig. 2 (lane 1, panels A–C) shows that untreated U937 cells contained high basal levels of nucleolin in both S-100 and nuclear fractions, and of PARP-1 in nuclear extracts. PARP-1 existed predominantly as the full-length product (116 kDa) and nucleolin migrated on SDS-polyacrylamide gels as an ~110-kDa band. An additional minor band was sometimes observed in the S-100 extracts blotted for nucleolin, and the significance of this band is not known, but the mobility of the major S-100 nucleolin band corresponded to that of the nuclear fraction. Following irradiation with UV light for 30 s (equivalent to ~30 J/m²), a profound decrease in the levels of S-100 nucleolin was observed (Fig. 2A), that by 24 h this band was almost undetectable. Apoptosis also resulted in decreased levels of nuclear nucleolin (Fig. 2B). These nuclear changes were less pronounced than in the S-100 fraction, but occurred more rapidly and were already obvious by 2 h after irradiation. By 72 h after irradiation, levels of nuclear nucleolin had returned to base-line levels. Clearly, apoptosis-induced alterations in nuclear and S-100 nucleolin did not occur in parallel. It is possible that apoptosis causes some of the nuclear nucleolin to relocate outside of the nucleus or vice versa, but because the nuclear and S-100 nucleolin bands are indistinguishable, we were not able to investigate this possibility by this method.

Presumably, UV irradiation also caused activation of PARP-1, because cleavage was induced within 2 h following treatment (Fig. 2C). Although UV does not generally induce DNA strand breaks directly, it can lead to both DNA damage (thymine dimers) and the production of free radicals. The presence of either intracellular free radicals or unrepaired DNA damage could initiate the apoptotic cascade (38), and PARP-1 is likely activated in response to DNA fragmentation. In any case, PARP-1 cleavage preceded the disappearance of S-100 nucleolin by several hours. On the other hand, the inhibition of nuclear nucleolin levels appears to occur roughly in parallel with cleavage of PARP-1.

The overall intensity of PARP-1 bands was significantly increased in apoptotic cells relative to untreated cells. This could be the result of increased PARP-1 expression or possibly to an enhanced affinity of the monoclonal antibody for the 89-kDa fragment. The reappearance of PARP-1 and nucleolin at 48–72 h after irradiation was most likely caused by proliferation of cells that survived treatment.

In addition, the dose dependences of the alterations in nucleolin and PARP-1 were examined. Panels D and E of Fig. 2 show that there was a clear dose-dependent decrease in nucleolin levels in both the nuclear and S-100 extracts of cells at 8 h following irradiation with UV for 5, 10, 30, or 45 s (equivalent to ~5, 10, 30, and 45 J/m², respectively). Induction of PARP-1 cleavage apparently had a lower threshold, and complete cleavage to the 89-kDa fragment was achieved with only 5 s of irradiation (Fig. 2, panel F).

*Effect of PARP-1 Inhibitor, 3-ABA*—To investigate whether there was a direct relationship between PARP-1 and UV-induced changes in nucleolin, similar experiments were carried out in the absence or presence of a PARP inhibitor, 3-ABA. This compound blocks the enzymatic activity of PARP-1 (and also other ADP-ribosylation enzymes), probably by competing for the substrate (NAD+ binding site (34)).

**Fig. 1.** UV irradiation and camptothecin-induced fragmentation (nucleosomal ladder) of genomic DNA in U937 leukemia cells, indicative of apoptosis. Samples were derived from untreated cells (lane 6), UV-irradiated cells at 2, 4, or 24 h after irradiation for 30 s (lanes 1–5), or cells that were treated with 10 μM camptothecin for 4 or 24 h (lanes 4 and 5).

**Fig. 2.** Expression of nucleolin and PARP-1 proteins in U937 cells after apoptosis was induced by UV irradiation. The upper three immunoblots show nucleolin in S-100 extracts (A), nucleolin in nuclear extracts (B), and PARP-1 in nuclear extracts (C) at various times following 30 s of UV irradiation. The lower three immunoblots show nucleolin in S-100 extracts (D), nucleolin in nuclear extracts (E), and PARP-1 in nuclear extracts (F) at 8 h following UV irradiation for various times.
Changes in Nucleolin during Apoptosis of Leukemia Cells

Fig. 3. Effect of PARP-1 inhibitor 3-ABA on alterations in nucleolin and PARP-1 expression, and on cell proliferation, following UV-induced apoptosis. Immunoblots show nucleolin in S-100 extracts (A), nucleolin in nuclear extracts (B), PARP-1 in nuclear extracts (C), and MTT assay indicates cell viability (D).

Fig. 4. Immunoprecipitations (IP) using nuclear extracts from untreated or UV-irradiated U937 cells. To evaluate poly(ADP-ribose)ylation of nucleolin, immunoprecipitations were performed using a nucleolin antibody, and samples were analyzed by immunoblotting for nucleolin (A) or poly(ADP-ribose) (B). To determine whether nucleolin can interact with PARP-1, immunoprecipitations were performed using anti-PARP-1 antibody or control. Immunoblots of precipitated proteins were analyzed by immunoblotting for nucleolin (C) or anti-PARP-1 (D).
small degree of protection from apoptosis-induced changes in nucleolin and PARP-1.

As in the UV-treated cells, the effect of camptothecin on nucleolin alterations was found to be dose-dependent (Fig. 5, panels A and B), and, again, the dose required to induce cleavage of PARP-1 was lower than that required to alter nucleolin (panel F).

Redistribution of Nucleolin in Cells Undergoing Apoptosis—Our data clearly indicate reductions in the levels of nucleolin in both the nuclear and cytoplasm/plasma membrane fractions of apoptotic cells. However, the fate of the nucleolin protein that “disappears” is not clear. To investigate this further, we examined the nuclei of apoptotic cells using immunofluorescent techniques to detect nucleolin. Fig. 6 shows that, following either camptothecin treatment (10 μM) or UV irradiation (30 s), there was a dramatic redistribution of nucleolin in the apoptotic nuclei. In untreated cells, nucleolin was located throughout the nucleoplasm but was concentrated in the intensely stained nucleoli. In contrast, in treated cells, distinct alterations in nucleolin staining were observed. Some cells exhibited a “speckled” staining pattern, in which the nucleolin appeared to have left the nucleoli and redistributed throughout the nucleoplasm as small distinct foci (for example, the cells highlighted at 2 h following UV or camptothecin). In other cells, there was “perinucleolar” staining, where the nucleolin appeared to have migrated to the periphery of the nuclei (for example, the cells highlighted at 4 h following UV or camptothecin). Both types of staining patterns were observed at each time point examined, and many cells exhibited staining patterns that were intermediate between the speckled and perinuclear patterns. Counterstaining with propidium iodide (data not shown) was used to confirm that both speckled and perinuclear nucleolin staining was within the nuclei and not the cytoplasm. Using this approach, we also observed the appearance of nucleolin-containing bodies at the periphery of the nucleus and in extranuclear regions (examples are indicated in Fig. 6 by white arrows). Appearance of these bodies was much more pronounced in cells irradiated with UV than in camptothecin-treated cells.

It should be noted that, using this method, the portion of nucleolin located in the plasma membrane cannot be observed because the cell membrane is permeabilized by detergent and thus the nucleolin antibody is internalized where it can only bind to nuclear and cytoplasmic components. Additionally, consistent with the immunoblot data, the overall intensity of nuclear nucleolin staining was significantly decreased (relative to untreated cells) by UV irradiation or camptothecin treatment at all time points examined. This differential intensity may not be evident in Fig. 6 because the images of treated cells have been enhanced for brightness to allow clear visualization of the pattern of staining.

Identification of Nucleolin in Extracellular Apoptosis-induced Bodies—The overall disappearance of 110-kDa nucleolin is very pronounced (Fig. 2) and could possibly be the result of proteolytic digestion of nucleolin (either fragmentation or complete degradation) or loss of nucleolin from the cell by secretion or extrusion. In the previous experiments, we did not detect the appearance of nucleolin cleavage products with either a monoclonal antibody (Figs. 2 and 3), or a nucleolin polyclonal antibody (Santa Cruz, clone C-18; data not shown). In light of our observation of nucleolin-containing bodies (Fig. 6), we next investigated the possibility that nucleolin was shed into the cell culture medium by examining proteins derived from the me-
Changes in Nucleolin during Apoptosis of Leukemia Cells

Fig. 7. Levels of nucleolin detected in the culture medium of untreated and apoptotic U937 cells. Cells were cultured in serum-free medium and then treated with UV light for 30 s, with or without 3-ABA pre-incubation. Immunoblots show the presence of nucleolin in the medium of cells at different times after UV irradiation (A), and the size of the nucleolin-containing particles was determined by pre-filtering the medium using filters with pores of various sizes (B). The apoptosis-induced extracellular bodies could also be detected by TUNEL staining (C) or immunofluorescent staining for nucleolin (D). The inset to panel D shows that some of these particles contain both nucleolin (anti-nucleolin staining, green) and DNA (propidium iodide staining, red).

Fig. 8. Immunoblots showing levels of PARP-1 in the S-100 fraction (A) and the culture medium (B) of untreated and apoptotic U937 cells. Some of the culture medium samples were pre-filtered, as indicated.

There are a number of reasons why studying nucleolin function and regulation is of interest. First, nucleolin is a fascinating protein because of its multifunctionality and its ability to translocate within the cell, yet the reasons for its ubiquitous behavior have not been fully elucidated (6–8). Second, nucleo-
Changes in Nucleolin during Apoptosis of Leukemia Cells

The apoptosis-induced changes in nucleolin levels that we have described appear to depend in some way upon PARP-1, inasmuch as an inhibitor of PARP-1 (3-ABA) can repress them. Further studies will be required to determine whether this is related to poly(ADP-ribosyl)ation of nucleolin by PARP-1, or the binding of nucleolin to the 89-kDa fragment of PARP-1 (or perhaps both). In accord with our observations, previous reports have also described redistribution of 89-kDa PARP-1 in response to apoptosis (53–55). Indeed, PARP-1 can be localized in the nucleolus under some circumstances (53–56), and there is some evidence to suggest that the nucleolus is central to the process of apoptosis and the earliest site of caspase-mediated proteolysis (57, 58).

Another novel result of our study is the observation that nucleolin appears to be shed from apoptotic cells in the form of small bodies. Kerr et al. (40) first described apoptotic bodies in 1972, and since then several others types of apoptosis-associated particles, including nucleolar-derived structures, have been reported (59, 60). Apoptotic bodies are thought to be derived from collapsing nuclei, which are transported to the plasma membrane and released into the extracellular space, where they are normally cleared by phagocytosis (61). Although apoptotic bodies have not been extensively characterized, they are known to contain several components including DNA or RNA, plasma membrane components, and nucleolar matrix proteins (49, 60).

The presence of nucleolin in apoptotic bodies has a number of clinical implications. In healthy individuals, apoptotic bodies are engulfed by macrophages or neighboring cells, and cleared from the circulation. However, under conditions of excessive apoptosis, apoptotic bodies may be released into the circulation (61) and could potentially be detected in plasma or serum. This excessive apoptosis can be caused by a number of conditions including inflammation, autoimmune disease, ischemic injury, and cancer. There is evidence that the presence of apoptotic cells or bodies can be an important diagnostic or prognostic marker for several types of cancer (45, 62–64) and detection of circulating apoptotic material (e.g., nucleosomes or nucleic acids) in serum has been proposed as a non-invasive method to detect the presence of malignancy or to evaluate therapeutic response in cancer patients receiving chemotherapy or radiation (65, 66). Nucleolin may be a particularly useful marker for apoptosis in these applications because tumor-derived apoptotic bodies are expected to be rich in nucleolin. The ability of apoptotic bodies to deliver their contents to the cells that engulf them is also an interesting property, which has been linked to the horizontal transfer of oncogenes (67, 68) and exploited as a drug delivery mechanism (69).

Our data also have implications for some autoimmune diseases, which are thought to develop, at least in part, because of defects in apoptotic responses (2, 61). Autoimmune diseases are characterized by the development of autoreactive T-cells and B-cells against self-antigens, and there is mounting evidence that this response could be triggered by exposure of the immune system to excessive amounts of intracellular materials from apoptotic cells (70–73). The appearance of nucleolin autoantibodies is one of the hallmarks of the autoimmune disease systemic lupus erythematosus (SLE), and these are in fact some of the earliest autoantibodies to develop as the disease progresses (74, 75). Our observation that apoptotic bodies containing nucleolin are rapidly shed from cells undergoing apoptosis suggests a possible explanation for the early appearance of nucleolin autoantibodies in SLE. Patients with SLE also have a high frequency of PARP-1 autoantibodies (76).

In conclusion, our studies have identified nucleolin as an important component of the apoptotic pathway in leukemia cells. Although we have not yet elucidated its precise role in this complex process, our results indicate that nucleolin may be involved in the processing of a proteolytic fragment of PARP-1.

lin is associated with many of the processes that are dysfunctional in neoplastic cells (proliferation, cell cycle control, apoptosis) and elevated levels of nucleolin expression are generally related to malignancy (26, 27). Third, we have identified nucleolin as the probable molecular target of a class of non-antiser G-rich oligonucleotides that inhibit the proliferation of tumor and leukemia cell lines, and therefore have significant promise as novel therapeutic agents (28–31).

In this report, we have described alterations in the levels and localization of nucleolin protein that occur in leukemia cells induced to undergo apoptosis by UV-irradiation or treatment with the topoisomerase I inhibitor, camptothecin. We found that induction of apoptosis was accompanied by a rapid reduction in the levels of nuclear nucleolin, followed several hours later by the disappearance of nucleolin from the S-100 fraction containing plasma membrane nucleolin. A distinct redistribution of nucleolin within the nucleus and the appearance of extracellular apoptotic bodies containing nucleolin and PARP-1 were also observed. By examining levels and localization of nucleolin in relation to other apoptosis-induced changes, we have shown that alterations in nucleolin occur very early in the apoptotic process. For example, at 1 h following UV irradiation of cells (for 30 s), nucleosomal fragmentation of DNA is barely detectable on ethidium-stained agarose gels, and only ~50% of PARP-1 has been cleaved. At the same time point, levels of nuclear nucleolin are clearly reduced, a distinct alteration in the staining pattern of nuclear nucleolin can be observed, and low levels of nucleolin-containing apoptotic bodies can be detected (Figs. 4 and 6, and data not shown). The fact that alterations in nucleolin are such an early event may indicate that this protein plays an active role in the initiation or progression of apoptosis, although further experiments would be required to confirm this.

Previously, there have been several studies that have examined the role of nucleolin in cell death. Martelli et al. (47–49) have used light and electron microscopy to examine changes in nucleolar proteins in HL60 leukemia cells treated with camptothecin (to induce apoptosis) or ethanol (to induce necrosis). These authors reported a redistribution (associated with fragmentation of nuclei) of nucleolin in apoptotic cells but not necrotic cells, and found that nucleolin was not degraded during apoptosis, in agreement with our data. In contrast to our results and those of Martelli and colleagues, several other reports suggest that nucleolin is proteolyzed in response to apoptosis. Brockstedt et al. (14) used two-dimensional electrophoresis to identify nucleolin as a protein that was cleaved in response to anti-IgM antibody-mediated apoptosis in a Burkitt’s lymphoma cell line. Morimoto et al. (50) described the proteolysis of a 110-kDa silver-stained nucleolar protein (presumed to be nucleolin) to give a 80-kDa fragment when human salivary gland or oral carcinoma cells were treated with okadaic acid. Finally, Pasternack et al. (51) reported that nucleolin was a substrate (in vitro) for cleavage by granzyme A, an apoptosis-associated protease secreted by cytotoxic T lymphocytes. The seemingly contradictory findings regarding apoptosis-induced cleavage of nucleolin could potentially be caused by cell death occurring via different mechanisms. Although the precise apoptotic pathways for each treatment are far from clear, it seems reasonable to expect that DNA damage (such as UV irradiation or camptothecin treatment) could activate a different pathway from anti-IgM, which binds to B-cell surface receptors (14), or okadaic acid, which is known to up-regulate Fas receptor (52).

The apoptosis-induced changes in nucleolin levels that we have described appear to depend in some way upon PARP-1, inasmuch as an inhibitor of PARP-1 (3-ABA) can repress them.
Furthermore, the distinct nuclear redistribution of nucleolin, its disappearance from the plasma membrane, and its presence in apoptotic bodies may be useful markers to detect apoptosis in experimental and whole animal systems.

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Apoptosis in Leukemia Cells Is Accompanied by Alterations in the Levels and Localization of Nucleolin

Yingchang Mi, Shelia D. Thomas, Xiaohua Xu, Lavona K. Casson, Donald M. Miller and Paula J. Bates

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