The DNA of Annexin V-binding Apoptotic Cells Is Highly Fragmented

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ABSTRACT

Jurkat leukemia cells induced to undergo apoptosis by treatment with an antibody against the Fas receptor have two annexin V (AV)-binding subpopulations: (a) single-positive cells that bind AV but not propidium iodide (PI); and (b) double-positive cells that bind AV and PI. The single-positive population is thought to represent an early stage of apoptosis. We have examined the relationship between AV binding and a classical characteristic of apoptosis, DNA fragmentation. Time course studies with Jurkat cells treated for 1, 2, or 4 h with anti-Fas indicated that the proportion of AV-binding cells was increased after 2 h. A significant increase in DNA fragmentation was observed only at 4 h as measured by the mean tail moment determined with the alkaline single cell gel electrophoresis (comet) assay. This correlation suggests a temporal relationship between the two parameters, but does not provide direct evidence of what happens in individual cells. We developed a method to measure fluorescent markers of cellular structure or function with a laser scanning cytometer and then perform the comet assay on the same cells. Cells in each AV-binding subpopulation were re-examined before and after electrophoresis. Most AV+/PI− cells had no DNA damage, although a few cells showed a pattern of damage characteristic for apoptosis. Double-positive cells all had damaged DNA; approximately half had the apoptotic pattern, and the rest had a pattern typical for necrosis. Nearly all of the single-positive cells had damaged DNA with the apoptotic pattern. Both AV-positive populations contained cells with little or no detectable DNA after electrophoresis, indicating that the DNA was highly fragmented. These results indicate that AV binding is an excellent marker for apoptotic cells, but that these cells already have fragmented DNA.

INTRODUCTION

Programmed cell death (apoptosis) is an important process in normal development and in tissue homeostasis, as well as a key mechanism by which anticancer therapies exert their cytotoxic effects. Agents like anticancer drugs and ionizing radiation that damage DNA induce apoptosis through a p53-dependent pathway (1). Binding of p53 and other nuclear proteins to the sites of damage in the DNA appears to trigger the apoptotic process. Another mechanism that can induce apoptosis involves the interaction of proteins such as Fas (CD95) or tumor necrosis factor with their receptors on the surface of cells. Signaling from these so-called death receptors starts the apoptotic process (2).

Regardless of how the apoptotic process has been initiated, by intrinsic signals or extrinsic signals involving death receptors, a hallmark of apoptosis is fragmentation of the DNA. The two main steps have been identified for apoptotic DNA fragmentation (3). The first involves formation of high molecular size DNA fragments of 50–300 kb. This process is widely observed in different cells and is propagated through single- and double-strand breaks in the DNA. The second step generates small, 200–300-bp DNA fragments. These small fragments lead to DNA ladder formation classically associated with apoptosis, although it can be absent in some cell types (4).

Another characteristic of cells undergoing apoptosis is the capacity to bind the protein AV. AV binds to PS, which is normally located on the inner leaflet of the plasma membrane, but it is externalized to the outer leaflet during apoptosis. PI staining is widely used to discriminate living cells, which exclude this DNA dye, from dead cells, which are permeable to it. In populations of cells undergoing apoptosis, there are some cells that bind AV but are not stained with PI. This “single-positive” population is thought to represent cells in an early stage of apoptosis because the cells apparently exclude PI and because it appears earlier than DNA ladders can be seen (5).

The AV-stained apoptotic cells show that there is a high degree of DNA fragmentation in Jurkat cells. We have examined the relationship between AV binding and DNA fragmentation using a flow cytometer and then perform the comet assay on the same cells. Cells in each AV-binding subpopulation were re-examined before and after electrophoresis. Most AV+/PI− cells had no DNA damage, although a few cells showed a pattern of damage characteristic for apoptosis. Double-positive cells all had damaged DNA; approximately half had the apoptotic pattern, and the rest had a pattern typical for necrosis. Nearly all of the single-positive cells had damaged DNA with the apoptotic pattern. Both AV-positive populations contained cells with little or no detectable DNA after electrophoresis, indicating that the DNA was highly fragmented. These results indicate that AV binding is an excellent marker for apoptotic cells, but that these cells already have fragmented DNA.

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The abbreviations used are: AV, annexin V; PI, propidium iodide; PS, phosphatidylserine; LMP, low melting point; LSC, laser scanning cytometer.
voltages were adjusted to have the unlabeled Jurkat cell population fall in the first decade of fluorescence. Cells labeled with only AV-FITC or PI were used to adjust the compensation. Data acquisition and analysis were performed by the CellQuest program (Becton Dickinson).

**Single Cell Gel Electrophoresis (Comet Assay).** Agarose-coated slides were made by dipping half-frosted slides (Superfrost+, VWR, Batavia, IL) into hot (80°C) 1% agarose (SIGMA) in distilled water and drying in air. A total of 2 × 10^6 cells were suspended in 80 μl of 0.75% LMP agarose (Boehringer Mannheim, Indianapolis, IN) prepared in AB buffer and layered onto the agarose-coated slides. The agarose was solidified by placing the slides on ice for 2 min. A second layer of 0.75% LMP agarose was applied to cover the cells. The cells were lysed in ice-cold alkaline lysis buffer [1% lauryl-sarcosine, 2.5 M NaCl, 10 mm Tris, 100 mm EDTA, 10% DMSO, 1% Triton-X-100 (pH 10)] for 1 h. Then DNA was unwound for 15 min in cold running buffer (300 mM NaOH, 1 mM EDTA) and electrophoresed for 20 min. Slides were neutralized in cold 0.4 M Tris buffer (pH 7.4) for 5 min and fixed in cold ethanol for 5 min. The slides were stained with SYBR Green I (Molecular Probes, Eugene, OR) diluted 1:1000 in TE [10 mM Tris (pH 8.0) and 2 mM EDTA] for 15 min and covered with antifade solution (ProLong, Molecular Probes). Comets were quantitatively evaluated using a fluorescence microscope (Zeiss, Germany) with the Komet 3.1 image analysis system (Kinetic Imaging, Bromborough, United Kingdom). LMP agarose was prepared in AB buffer.

Tail moment is defined as a product of the distance between the head and tail mass centers and the relative amount of DNA in the tail compared with the total DNA in each comet.

**Combination Assay Using the LSC.** Cells were stained with AV and PI as described above. To each 100-μl aliquot of labeled cells, 300 μl of 1% LMP agarose were added. From this mixture, 80 μl were layered on the surface of each agarose-coated slide. After the agarose with cells solidified, a second layer of agarose was added, and coverslips were place on top. The slides were kept on ice until measurements were done using the LSC (CompuCyte Corporation, Cambridge, MA). The hardware was controlled by the WinCyte 3.1 software for Windows NT. The 488-nm argon-ion laser line was used for excitation. Green fluorescence and red fluorescence emission as well as forward scatter light was collected. The contour threshold was setup using the “added” parameter, which is a sum of the forward scatter light, green fluorescence, and red fluorescence parameters. Compensation was determined using single-labeled samples. We have used a 10× objective to reduce the time needed for scanning a given area. The size of an apoptotic comet is in the range of 100 μm, which determines maximal cell concentration that can be used. Under our experimental conditions, 3000 cells on each slide could be scanned in ~10 min. After the AV-PI measurements were completed, coverslips were removed from the slides, and they were immersed in the lysis buffer. The DNA was stained with SYBR Green I, neutralized, and fixed as described above. To perform the comet assay, the cells of interest were relocated by the LSC based on the previous measurements. The video signal from the color video camera of the LSC was ported over to the Komet 3.1 image system.

**RESULTS**

The LSC provides quantitative fluorescence data similar to that obtained by flow cytometry. With Jurkat cells treated with anti-Fas and stained with AV-FITC and PI, both methods identify three distinct populations (e.g., see Fig. 3A): double-negative cells (AV⁻/PI⁻), single-positive cells (AV⁺/PI⁻), and double-positive cells (AV⁺/PI⁺). Fig. 1A summarizes the results of several experiments examining the time course of appearance of AV⁺ single-positive cells after treatment with anti-Fas. Both cytometric measurements show a similar pattern, although at each time point, the LSC detects more AV⁺ cells than does the flow cytometer. Because the LSC is a microscope-based measurement and the cells remain attached to the slide, each event can be re-examined and each could be confirmed to be a cell (see Fig. 3). One hour after induction, there was only a small increase in the percentage of single-positive cells, but by the second hour, ~15% of the cells measured were positive by the LSC and 5% by flow cytometry.

![Fig. 1. Time course of induced apoptosis in Jurkat cells measured by flow cytometry, laser scanning cytometry, or the comet assay. A, mean percentage of AV⁺/PI⁻ cells from three independent experiments using the flow cytometer. •, mean percentage of AV single-positive cells from 12 (0 h), 6 (1 h), 8 (2 h), or 4 (4 h) independent experiments using the LSC. B, •, mean tail moments measured for 100 cells each from five independent experiments. Vertical bars, ± 1 SE.](https://cancerres.aacrjournals.org/)
Round cell with a sharply defined cell membrane (Fig. 3B1). After electrophoresis, the nuclei of most cells in this population remained as bright circles (Fig. 3B2). The cells in the double-positive population had indistinct borders when viewed under a bright field (Fig. 3C1). AV staining on these cells was usually uniform as shown in Fig. 3C2. Many of the cells in this population gave a typical “comet” pattern for their DNA after electrophoresis (Fig. 3C3) with a bright “head” where the nucleus was and a tail of damaged DNA. This pattern is characteristic of necrotic cells. The single-positive cells had some definition around them, but their shapes were much more irregular than those of the double-negative population (Fig. 3D1–3) and often showed localized staining with AV around the edges (Fig. 3D4–6). The DNA patterns are typical of apoptotic cells (Fig. 3D6–9), forming clouds of small pieces of DNA that migrate away from the original site, some with only traces of DNA staining (Fig. 3D8).

Based on analysis of a number of experiments, the DNA patterns of PI/AV-stained cells could be placed in four basic categories: (a) normal nuclei without DNA damage having all DNA remaining at the site of the nucleus (Fig. 3B2); (b) “Comet” with considerable DNA both in the head and in the tail, characteristic of necrotic DNA damage (Fig. 3C3); (c) “Apoptotic comets” with little DNA in the head, usually as a ring with less DNA in the center of the head, followed by...
composed of pure DNA. We will refer to these as "apoptotic remnants." Hydrophobic proteins, this may be an indication that they were not yellow than the stained DNA. Because SYBR Green I can also stain these condensed particles on SYBR Green I-stained slides was more side of Fig. 3 D9. The color of the nuclei with small amounts of fragmented DNA in a cloud (see left D9) or even without a DNA tail (Fig. 3 D9). Small condensed particles at the original locations of DNA; and (d) These patterns are segregated between the different subpopulations of these based on the relocalization of a large number of cells and the presence of DNA clouds with various fluorescent intensities indicates that they represent a continuum with progressively less DNA; and (d) Small condensed particles at the original locations of the nuclei with small amounts of fragmented DNA in a cloud (see left side of Fig. 3 D8) or even without a DNA tail (Fig. 3 D9). The color of these condensed particles on SYBR Green I-stained slides was more yellow than the stained DNA. Because SYBR Green I can also stain hydrophobic proteins, this may be an indication that they were not composed of pure DNA. We will refer to these as “apoptotic remnants.”

These patterns are segregated between the different subpopulations in the AV-PI scattergrams. Table 1 shows the proportions of each in these populations of cells treated with anti-Fas for 2 h and control cells. The double-negative population primarily consisted of cells with unfragmented DNA. In both treatment groups, >90% of the cells in this population had intact DNA. The AV single-positive population in either treatment group demonstrated highly fragmented apoptotic DNA and apoptotic remnants, with no cells having a necrotic pattern and only a few with undamaged DNA. In the double-positive population, there were both necrotic and apoptotic comets. Although treatment changes the numbers of cells in the single- and double-positive subpopulations compared with untreated cells, the type of damage in each subpopulation is the same.

We have measured the tail moments associated with each subpopulation of cells by transferring the signal from the video camera on the LSC to the image analysis system for the comet assay. Many apoptotic events in the single-positive and double-positive populations could not be scored because the total amount of DNA was too low for accurate image analysis. This includes some of the apoptotic comets that had lost most of their DNA as well as the apoptotic remnants. These were counted manually and would not have been identified in the standard alkaline comet assay. The results are shown in Fig. 4 and Table 1. The double-negative population had unfragmented nuclei, with 96.6% of the cells having tail moments <10 (Fig. 4A). After anti-Fas treatment, the proportion of cells with normal nuclei dropped to 90.8%, and those with apoptotic patterns increased from 3.0 to 8.6% (Table 1). In the double-positive population, there was a wide range of values with necrotic comets having tail moments between 10 and 40 and apoptotic comets with tail moments >40 (Fig. 4B; Table 1). This population also contained apoptotic remnants. Most of the events in the single-positive populations could not be measured, and those that could had tail moments >60 (Fig. 4C). All represented apoptotic patterns. In cells treated with anti-Fas, there was a shift to earlier stages of apoptosis, with apoptotic comets increasing from 35 to 85% and apoptotic remnants decreasing from 58 to 12% of the population. Thus, as cells are induced into apoptosis, earlier stages predominate (Table 1).

The relative proportions of measurable apoptotic comets to late apoptotic events, such as apoptotic remnants that could not be characterized by tail moment measurements in the single-positive population of cells treated with anti-Fas, were calculated. After untreated cells have been harvested and prepared for measurement (0 h), ~5% are single positive (Fig. 1), and 85% of these do not have sufficient DNA to measure comet tail moments. As the number of single positive cells increases after the addition of anti-Fas, the proportion of measurable comets increases progressively from 15 to 22%, and then to 29%, after 1 and 2 h. By the third hour of treatment, 38% of the comets represent these early stages as new cells are induced to undergo apoptosis. This proportion falls rapidly again to 12% in the next hour.

Similar studies were performed with MCF-7 breast cancer cells that had been serum-starved to induce apoptosis and with PC3 prostate cancer cells treated with ionizing radiation. The results showed that the AV single- and double-positive populations had similar DNA

![Fig. 4. Distributions of tail moments in AV^-/PI^- (A), AV^-/PI^+ (B), and AV^+/PI^- (C) Jurkat cells after treatment with anti-Fas for 2 h. Other consists of "apoptotic remnants" and those "apoptotic" comets where it was not possible to measure tail moments because there was little or no DNA remaining at the cellular locations after electrophoresis. The results are for a representative experiment from a series of six.](https://cancerres.aacrjournals.org/figs/4626-fig4)

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<th>Pattern: Anti-Fas:</th>
<th>Normal DNA (%)</th>
<th>Necrotic comet (%)</th>
<th>Apoptotic comet (%)</th>
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<td>AV^-/PI^-</td>
<td>96.6 ± 1.1</td>
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<td>56.2 ± 5.5</td>
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*Results represent averages from four experiments ± 1 SE. At least 100 cells were counted in each experiment for each AV, PI subpopulation, when possible. Data are expressed as percentages.*
patterns to those of Jurkat cells induced with anti-Fas. Single-positive cells were all apoptotic, with a high degree of fragmentation and DNA loss appearing the same as those in Fig. 3D. Double-positive cells, on the other hand, had a mixture of apoptotic and necrotic comets similar to those in Fig. 3C.

DISCUSSION

The process of apoptosis has received a great deal of attention in experimental oncology. Defects in this pathway early during carcinogenesis can ultimately lead to cancer formation (7) and later to resistance to cancer treatment (1, 8). Several pathways have been described for apoptosis in different systems, but they all converge with activation of the protease caspase 3 and subsequently of endonuclease activity that results in fragmentation of nuclear DNA (4, 9–13). Experimentally, this DNA fragmentation has been demonstrated in model systems as a “ladder” following electrophoretic separation of DNA extracts prepared from the cell population. This method lacks sensitivity because the proportion of apoptotic cells in the population must be large to be detected. A method has been developed to detect DNA damage in single cells called the “comet” or single cell gel electrophoresis assay that can detect effects in subpopulations of cells (14).

The binding of AV by cells undergoing apoptosis has been proposed to be an early event based on correlative studies on cell populations (5): (a) increases in the single AV+ cell population occur much earlier than increases in the double-positive cell population; (b) the proportion of AV-binding cells is higher at all times after induction than that of cells with morphological changes in the nucleus; (c) AV binding appeared earlier than cell shrinkage as determined by flow cytometry. Until recently, studies of this type have been correlative by necessity because assays such as AV binding, which require viable cells, and morphological assays could not be performed on the same cell (15). We have developed a method using the ability of the microscope-based LSC to relocate cells that combines the AV-binding assay with assessment of DNA fragmentation by the comet assay in the same cells.

To validate our approach, we selected a well-studied apoptotic system, Jurkat leukemia cells induced to undergo apoptosis by an antibody against Fas. In contrast to other model systems in which drug- or radiation-induced DNA damage initiates the apoptotic program, anti-Fas interacts with a cell surface death receptor to initiate the process. To ensure that we were examining the early events of apoptosis, we have used treatment times of no more than 4 h.

Our initial studies comparing the results obtained by flow cytometry and laser scanning cytometry for PI-AV-FITC-stained cells demonstrated that there was no significant difference between the two methods in the time course of appearance of single- and double-positive cell populations, confirming the results of Bedner et al. (11). However, the proportions of AV+ cells measured by laser scanning cytometry were higher than those determined by flow cytometry. There are two possible explanations for this difference. The first is that the embedding of the cells in agarose immediately after staining preserves fragile apoptotic cells that may be lost in flow cytometry because of pressure changes through the flow aperture. The second possibility is that the cells might remain intact during flow analysis, but changes during the apoptotic process in the side scatter parameter used to set regions for flow analysis might remove these cells from the region being analyzed (16). In either case, each positive event detected with the LSC could be confirmed visually as a cell. Another advantage of embedding the cells in agarose is that all of the cells can be examined, avoiding the potential problem associated with other microscopic assays in which late apoptotic cells may be lost because they cannot attach to glass surfaces (11).

After lysis, electrophoresis, and ethanol fixation, it was possible to reliably relocate nuclei at the sites where cells were first detected for the PI and AV measurements. There was no PI or FITC fluorescence remaining at these locations. When objects were relocated, it was nearly always possible to classify them as intact nuclei, necrotic comets, apoptotic comets, or apoptotic remnants. At the location of single- or double-positive cells, damaged DNA with either an apoptotic or necrotic pattern was evident or small-condensed particles were found. The size of these irregularly shaped particles was ~2–4 μm in diameter, and their color was more yellow than that of normal DNA stained with SYBR Green I. They were also visible on bright field images in contrast to apoptotic or necrotic DNA. These findings suggest that apoptotic remnants are composed of heavily condensed material, probably cross-linked proteins. It has been shown that transglutaminases are activated during the last stages of apoptosis, resulting in cross-linked cellular proteins that prevent disintegration of the apoptotic cells (17).

We did not observe comets with intermediate levels of DNA damage in the double-negative or single-positive populations in these experiments. Most double-negative cells had undamaged DNA with tail moments <10, whereas the single-positive population was composed primarily of heavily damaged cells with apoptotic fragmentation patterns having tail moments >40. In some apoptotic cells, tail moment was not possible to calculate because little or no DNA remained in the head. These observations are in good agreement with field inversion gel electrophoresis data, where an abrupt (within 15 min) DNA fragmentation was found in apoptotic cells (18). We observed intermediate DNA damage (tail moments between 10 and 40) only in the double-positive population, and these cells had comet shapes typical of necrotic DNA damage (19). Thus, it appears that in apoptotic cells, the transition from undamaged DNA to a high degree of DNA fragmentation is very rapid. After the initial fragmentation that gives rise to measurable comets, the DNA is further fragmented, giving rise to comets in which all of the DNA has migrated from the nuclear area during electrophoresis. Finally, there is what appears to be a gradual loss of DNA from the comets leaving only the apoptotic remnants. This last stage may take between 6 and 12 h in Jurkat cells induced by anti-Fas (5).

When measured with the LSC, there was a background of apoptotic cells in the control samples (0 h). These cells that underwent spontaneous apoptosis were all in the late stages of apoptosis. When cells were induced to undergo apoptosis with anti-Fas, the numbers of cells in the early stages increased gradually through the first 3 h and then decreased. This suggests that an initial wave of apoptosis occurs during the first 3 h. Whether or not further waves occur in this population and their relationships to proliferative status of the cells at the time of treatment remain to be determined. Our results confirm that AV binding is an excellent marker for apoptotic cells (5, 20, 21). Virtually all of the cells in the single-positive population had apoptotic comets or apoptotic remnants. In contrast, the double-positive population was composed of cells giving rise to comets having necrotic or apoptotic patterns in about equal numbers. This means that counting only the single-positive cells will underestimate the actual numbers of apoptotic cells.

We have further shown that AV binding does not precede DNA fragmentation because all of the single-positive cells, which are purported to be earlier in the apoptotic process than double-positive cells, have highly fragmented DNA. In contrast to our data, it has been suggested that AV binding is an earlier step in the execution phase than DNA fragmentation (21, 22), based on use of the TUNEL assay to measure DNA fragmentation. This method is not sensitive enough.
to detect high molecular size DNA fragmentation that occurs in the early phases of apoptosis, but only detects the final internucleosomal DNA degradation (23, 24). The comet assay, on the other hand, also detects the 50-kb DNA fragments in apoptotic cells (14), so we are detecting the earlier high molecular size fragmentation in our assays using the comet assay. Furthermore, we are looking at early times after the addition of anti-Fas, and if PS exposure was an earlier event than DNA fragmentation, we would expect to find cells containing undamaged DNA in the single- or double-positive populations at these times, which is not the case. Our findings that AV-binding cells have a high degree of DNA fragmentation and loss are also supported by the results of Martin et al. (5) showing a correlation between the proportions of peripheral blood neutrophils binding AV after anti-Fas or drug treatment and the numbers of nuclei with sub-G1 DNA content as determined by flow cytometry. Our studies with MCF-7 and PC3 demonstrate that the relationship between AV binding and DNA fragmentation is not restricted to cells of hematopoietic origin.

In conclusion, our method using the LSC to relocate cells after subpopulations have been identified and to measure DNA damage by the comet assay provides a powerful tool for studies on apoptosis. This technique can be used to examine other markers of apoptosis, such as binding of mitochondrial specific dyes, to determine their relationship to DNA damage in apoptotic cells. It will also allow analysis of biological relationships between virtually any cell surface or cytoplasmic marker and DNA damage in individual cells.

REFERENCES

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