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Flow Cytometry: A Screening Tool for High Molecular Weight DNA

ABSTRACT: Decomposition is the degradation of tissues due to a combination of autolysis and putrefaction. As the postmortem interval lengthens, chromatin is degraded until no high molecular weight DNA (HMW-DNA) remains. The DNA complement per cell can be measured by flow cytometry; the integrity of the DNA within a tissue can be determined using gel electrophoresis. In this study, splenic tissue was harvested at autopsy and was allowed to decompose over several days. Serial samples were taken and submitted for gel electrophoresis and flow cytometric analysis. Gel electrophoresis showed HMW-DNA was present at postmortem days one through three but little remained by day four. Flow cytometry closely reproduced the same results with peaks near the 2n control indicating the presence of HMW-DNA. This implies that flow cytometry may substitute for gel electrophoresis as a quick, inexpensive assay for the presence of HMW-DNA.

KEYWORDS: pathology and biology, decomposition, flow cytometry, electrophoresis, HMW-DNA, DNA

In living human beings, cell death occurs predominantly by way of necrosis or apoptosis [*1*]. The latter mechanism involves activation or synthesis of endonucleases that specifically cleave nuclear chromatin into double-stranded DNA fragments [*1*]. This has been referred to as "cell suicide" and it is considered a normal physiologic process active in cell turnover and tissue embryogenesis [*1*]. Apoptosis requires a burst of activity by the cell before its death; it is not a passive process. The other common type of cell death, necrosis, is characterized by lethal cell membrane damage as a common response to irreversible cellular injury [*1*]. Chromatin degradation occurs as lysosomal enzymes are released resulting in a more random pattern of DNA cleavage. The manner of death at the cellular level is

best classified as "homicide or accident" if necrosis is involved, as the cells die due to extrinsic trauma.

When a body is dead, on the other hand, the constituent cells and tissues are most often degraded through decomposition. Decomposition is the combination of autolysis, the destruction of cells by their own lysosomal enzymes, and putrefaction, the action of bacterial enzymes on tissues [2]. Depending on the tissue, one of the two mechanisms may predomi-nate (for example, autolysis occurs quickly in the pancreas, an exocrine organ containing powerful enzymes). Following the cessation of circulation, the tissues of the body begin to die. Since energy is required to produce the endonucleases responsible for apoptosis, postmortem DNA degradation more closely resembles the random fragmentation characteris-tic of necrosis.

Flow cytometry can be used to quantitate the amount of DNA within each cell of a submitted tissue [3]. When the laser beam hits a cell whose nucleus has been stained with propidium iodide, fluorescent light is emitted that can be used to quantitate the amount of DNA relative to a standard. Since the fluorescence intensity is proportional to the amount of propidium iodide bound, nuclei that have lost DNA will stain less intensely than those with the diploid amount of DNA. A histogram can then be generated plotting the number of cells with a given DNA content on the Y-axis versus the nuclear DNA content (that is, fluorescence intensity) on the X-axis. Normally, a 2n peak representing cells in the G_0 and G_1 phases of the cell cycle predominates. Any fluorescence intensity to the left of this peak on the histogram would indicate the presence of a cell population containing less than 2n intact DNA (that is, cells with degraded DNA). This principle can be used to detect nuclear DNA degradation during decomposition. As the postmortem interval lengthens, fewer cells have intact DNA and more contain fragmented DNA [3].

Gel electrophoresis can also be used to assay the integrity of the DNA within a given tissue. Following purification, DNA molecules can be electrophoretically separated based on their molecular weight. Since high molecular weight DNA (HMW-DNA) has a low mobility, whereas low molecular weight DNA has a higher mobility, gel electrophoresis can be used to assess the presence of intact DNA versus fragmented DNA in any tissue. This technique can be employed to measure the extent of DNA degradation during decomposition.

This study compares flow cytometric analysis and gel electrophoretic evaluation of decomposing splenic tissue. In particular, the role of flow cytometry as a screening tool for the presence of HMW-DNA is being investigated.

Materials and Methods

Splenic tissue was sampled from a series of twenty nonconsecutive autopsies performed at the Medical University of South Carolina. Both natural and violent deaths were included. The decedents were men and women over eighteen years of age.

Flow cytometric analysis was performed in a previously described manner [3]. At nec-ropsy, a one centimeter-thick section of spleen was harvested and placed in a clean, nonsterile container containing eight ounces of sterile normal saline. The container was covered and the tissue was allowed to decompose over five days at room temperature (25°C). Samples of tissue were harvested at the time of autopsy, then daily for the duration of the experiment. Flow cytometric analysis was performed on 15 cases; tissue was frozen in a −70 degree C freezer for later electrophoretic DNA analysis in four cases. A one cubic centimeter cube of tissue was minced into a single-cell suspension in Dulbecco's solution. The suspension was centrifuged and the pellet was resuspended in one-hundred per cent ethanol while vortexing to form a single cell suspension. The cells were stained with propidium iodide using a Coulter DNA Prep and analyzed within two hours. The suspension was run on the Epics Elite Flow Cytometer using Elite Version 3.0 software. A fresh peripheral blood sample was used as a control. Cells were gated on peak red fluorescence versus integrated

fluorescence in order to exclude cellular aggregates and doublets. A histogram was then generated plotting number of cells on the Y-axis versus red fluorescence intensity (that is, representing nuclear DNA content) on the X-axis. A cursor was placed immediately preceding the 2n control peak to act as a point of reference. Fluorescence intensity to the left of the cursor represents cells containing partially or completely degraded DNA depending on the degree of shift to the left.

For gel electrophoresis, DNA was prepared by standard extraction methods [4]. Splenic tissue was ground in liquid nitrogen, lysed with SDS, and digested with proteinase K. The nucleic acid was recovered by phenol extraction and RNA was digested with ribonuclease. The DNA was then serially extracted with phenol, pheno-chloroform, and chloroform, then precipitated by ethanol. DNA quality and concentrations were determined by UV spectrophotometry with a Beckman DU64 scanning spectrophotometer. Five microgram aliquots of DNA were electrophoresed, accompanied by a one kilobase ladder, in an 0.8% agarose gel containing 0.07 μg/mL of ethidium bromide. A track contained DNA isolated from a Taxol-treated, anueploid HUT lymphoma cell line as an apoptotic control. The gels were photographed by UV transillumination.

On two occasions, attempts were made to separate nuclear DNA from fragmented cytoplasmic DNA. In these cases, the tissues were treated with a lysis buffer containing 10 mM Tris, 1 mM EDTA, and 0.2% Triton-X 100 to disrupt the cell membrane but not the nuclear membrane. The nuclei were then recovered by low-speed centrifugation. Both the nuclei and the supernatant were processed for DNA extraction as described above. Recovery of DNA was successful from the nuclear fractions only. Lastly, bacterial cultures were submitted from the decomposing tissue in three cases.

Results

Histograms from serial flow cytometric analyses detected a decrease in the numbers of cells with intact 2n DNA as the postmortem interval lengthened. Concurrently, the number of cells to the left of the 2n control cursor increased over postmortem days one through five. By day four, the majority of cells contained degraded DNA (Figs. 1 and 2). These histograms were more similar to those generated in previous reports by cell lines undergoing necrosis rather than apoptosis [5,6]. Flow cytometric analysis performed on our control apoptotic lymphoma cell line yielded a histogram similar to those seen on day three of decomposition.

On postmortem day one, gel electrophoresis revealed that the majority of cellular DNA was intact. At this time the DNA was larger than the twelve kilobase rung of the molecular weight ladder. This is consistent with data that estimates the size of uncut DNA at 20 kilobase pairs in size [7]. Over postmortem days two and three, however, as DNA fragmentation progressed, the DNA in the tracks began to "smear" indicating the appearance of degraded, low molecular weight DNA as well as HMW-DNA (>2 kilobase pairs). By postmortem day four, most of the DNA had been degraded to low-molecular weight fragments less than 506 base pairs in length (Fig. 3). As expected, the pattern of DNA decomposition was nonspecific compared to the "stepladder" pattern noted in the apoptotic control. Interestingly, a slight degree of banding was noted in the low-molecular weight fragment region of the "smears" derived from decomposing splenic tissue. Perhaps this was the result of specific cleavage by bacterial enzymes or semispecific cleavage by lysosomal enzymes. Alternatively, the low molecular weight bands represented bacterial DNA.

Gel electrophoresis confirmed the DNA fragmentation detected by flow cytometry. Splenic tissue taken from the same cases, sampled at the same postmortem intervals, yielded data consistent with fragmentation by both flow cytometry and gel electrophoresis. By postmortem day four, little or no HMW-DNA remained. If the histogram had a 2n "peak"
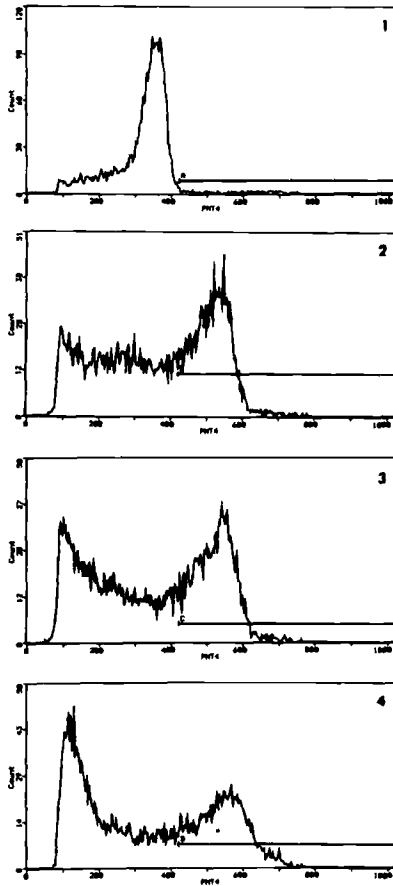
FIG. 1—*Flow cytometric analysis of splenic tissue from subject X. The histograms show progressive DNA fragmentation over postmortem days 1–4* (top to bottom). *The cursor marks the 2n control peak.*

higher than the left-shifted fragment "peak," HMW-DNA was detected by gel electrophoresis. This held true irrespective of the width of the peaks or the areas beneath them.

By postmortem day four, the splenic tissue was autolytic and foul-smelling. Anaerobic and aerobic cultures yielded Enterobacter, Enterococcus, Clostridium, Klebsiella, and Eschericia coli. These finding are similar to those we have seen in cultures taken from bodies in a state of mild-to-moderate decomposition.

Discussion

The isolation of HMW-DNA (>2 kilobase pairs) is essential for many forensic applications of DNA, including restriction fragment length polymorphism (RFLP) analysis [7]. Unfortunately, gel electrophoresis, unparalleled at identifying and isolating HMW-DNA, is an expensive, labor-intensive, time-consuming procedure. Further, while most practicing pathologists and many medical examiners have access to a flow cytometer, gel electrophoresis is commonplace only in academic centers and referral laboratories. Our data suggests that flow cytometric analysis may be performed on decomposing or autolytic tissues to screen for the presence of HMW-DNA. If the 2n "peak" is higher than the fragment "peak"
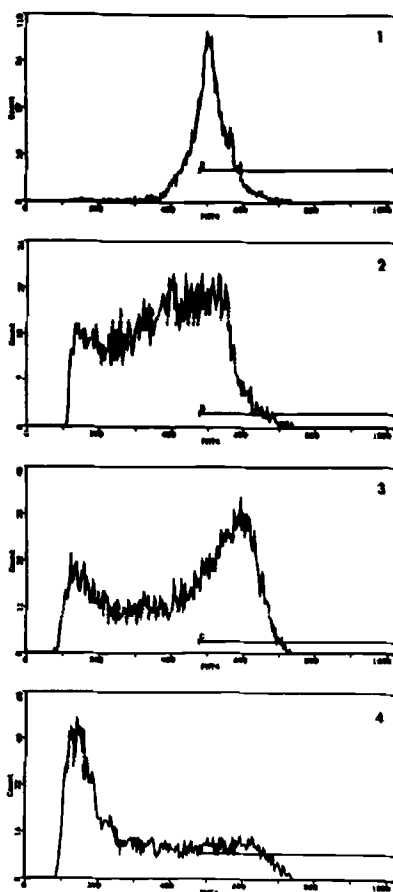
FIG. 2—*Flow cytometric analysis of splenic tissue from subject Y. The histograms show progressive DNA fragmentation over postmortem days 1–4* (top to bottom). *The cursor marks the 2n control peak.*

on the histogram, HMW-DNA is likely to be present and the specimen may be suitable for further processing (for example, analysis by RFLP). If only small fragments are present, as seen in day four of splenic decomposition, other methods, such as the polymerase chain reaction (PCR), may need to be employed to amplify any remaining DNA [7].

Different tissues may yield HMW-DNA that can be detected by flow cytometry over a greater postmortem interval. The recent literature suggests that cerebral cortical tissue may yield useful HMW-DNA up to 85 days postmortem [8]. Although this tissue may also be amenable to flow cytometric screening the high lipid content has resulted in difficulty with flow cytometric study in our laboratory. Analysis of cerebellar tissue is currently under investigation.

Although our in-vitro conditions seem to approximate the natural process of decomposition at room temperature, it remains of interest to compare our results with those that would be obtained from decomposed bodies with a known time of death. A correlation of success of PCR and/or RFLP analysis with flow cytometric analysis should also be investigated, however this is beyond the scope of this project.
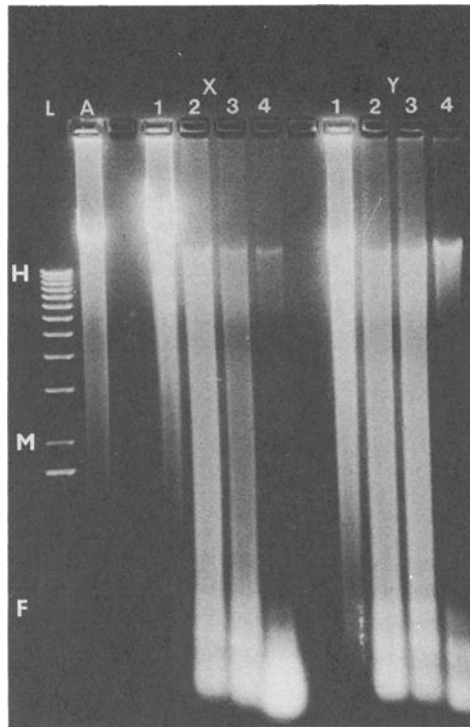
FIG. 3—*Electrophoretic analysis of DNA from decomposing splenic tissue from subjects X and Y. HMW-DNA is at the top of the gel, fragments migrate to the bottom. DNA is progressively degraded into fragments over postmortem days 1–4 in both subjects X and Y. A "step-ladder" is formed by the apoptotic control DNA (A). A 12 kilobase molecular weight ladder (L) is on the far left; the 12 kilobase (H), 2 kilobase (M), and 506 base pair (F) rungs are identified.*

Conclusions

The aim of this investigation has been to evaluate the ability of flow cytometry to determine the extent of postmortem DNA degradation by measuring the HMW-DNA content of cells relative to the standard electrophoretic method. Since the degree of DNA degradation detected by gel electrophoresis could be estimated from the histograms generated by flow cytometric analysis, it was concluded that flow cytometry can be used in place of gel electrophoresis to quickly and inexpensively detect the presence of HMW-DNA in decomposing tissue.

Acknowledgment

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