

## 2.1 Methods for studying apoptosis in cell populations

A number of methods have now been developed to study apoptosis in cell populations. We focus on two key apoptotic events in the cell:

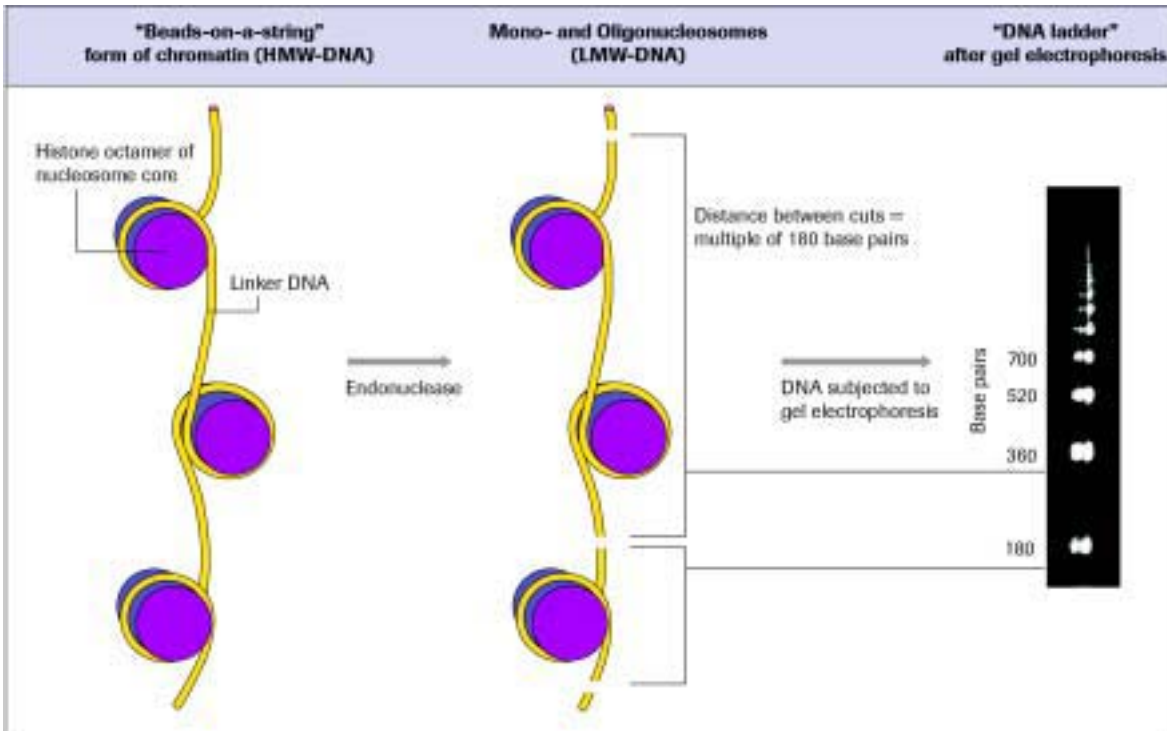
- ① Apoptosis and cell mediated cytotoxicity are characterized by cleavage of the genomic DNA into discrete fragments prior to membrane disintegration. Because DNA cleavage is a hallmark for apoptosis, assays which measure prelytic DNA fragmentation are especially attractive for the determination of apoptotic cell death. The DNA fragments may be assayed in either of two ways:
  - ▶ As “ladders” (with the 180 bp multiples as “rungs” of the ladder) derived from populations of cells, *e.g.*, with the Apoptotic DNA Ladder Kit (described on page 13 of this guide).
  - ▶ By quantification of histone complexed DNA fragments with an ELISA (described on page 15 of this guide).
- ② Further, researchers discovered that proteases were involved in the early stages of apoptosis. The appearance of these caspases sets off a cascade of events that disable a multitude of cell functions. Caspase activation can be analyzed in different ways:
  - ▶ By an *in vitro* enzyme assay. Activity of a specific caspase, for instance caspase 3, can be determined in cellular lysates by capturing of the caspase and measuring proteolytic cleavage of a suitable substrate (described on page 22 of this guide).
  - ▶ By detection of cleavage of an *in vivo* caspase substrate. For instance caspase 3 is activated during early stages (as shown in Figure 2). Its substrate PARP (Poly-ADP-Ribose-Polymerase) and the cleaved fragments can be detected with the anti PARP antibody (described on page 27 of this guide).

If you're just starting out in the field, however, it may be difficult to decide how best to assay apoptosis in your system. Thus, in the following sections, we will describe details of each of these apoptosis assays.

### 2.1.1 Assays that measure DNA fragmentation

The biochemical hallmark of apoptosis is the fragmentation of the genomic DNA, an irreversible event that commits the cell to die. In many systems, this DNA fragmentation has been shown to result from activation of an endogenous  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -dependent nuclear endonuclease. This enzyme selectively cleaves DNA at sites located between nucleosomal units (linker DNA) generating mono- and oligonucleosomal DNA fragments (Figure 4). These DNA fragments reveal, upon agarose gel electrophoresis, a distinctive ladder pattern consisting of multiples of an approximately 180 bp subunit<sup>8</sup>.

Radioactive as well as non-radioactive methods to detect and quantify DNA fragmentation in cell populations have been developed. In general, these methods are based on the detection and/or quantification of either low molecular weight (LMW) DNA which is increased in apoptotic cells or high molecular weight (HMW) DNA which is reduced in apoptotic cells (Figure 5). The underlying principle of these methods is that DNA, which has undergone extensive double-stranded fragmentation (LMW DNA) may easily be separated from very large, chromosomal length DNA (HMW DNA), *e.g.*, by centrifugation and filtration.



▲ Figure 4: The biochemistry of DNA fragmentation and the appearance of the "DNA ladder".

For the quantification of DNA fragmentation, most methods involve a step in which the DNA of the cells has to be labeled: Prior to the addition of the cell death-inducing agent or of the effector cells, the (target) cells are incubated either with the [ $^3\text{H}$ ]-thymidine ( $^3\text{H}$ -dT) isotope or the nucleotide analog 5-bromo-2'-deoxyuridine (BrdU). During DNA synthesis (DNA replication) these modified nucleotides are incorporated into the genomic DNA. Subsequently, those labeled cells are incubated with cell death-inducing agents or effector cells and the labeled DNA is either fragmented or retained in the cell nucleus. Finally each type of DNA (HMW and LMW) is quantitated. Because the labeling of the cellular DNA has to be done prior to the induction of cell death, this labeling is also called "prelabeling".

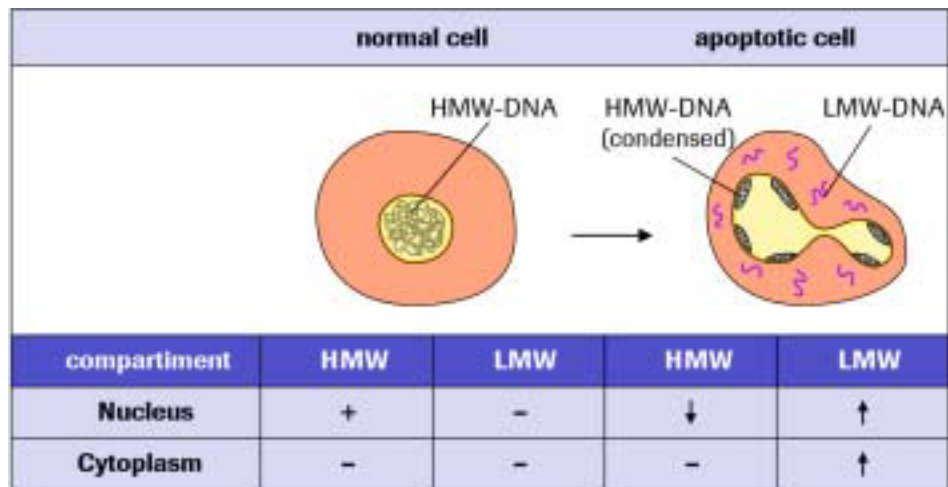
The prelabeling of one cell population (e.g., the target cells) allows the behavior of the labeled cells to be traced specifically when different cell populations are mixed.

*Note: Because cell-mediated cytotoxicity (CMT) proceeds, at least in part, by apoptotic mechanisms, the DNA fragmentation assay may also be used as a CMT assay.*

In a study of cell-mediated cytotoxicity the target cell population is labeled before the effector cells (e.g., CTL) are added. Subsequently, due to pore formation in the target cell plasma membrane, the fragmented LMW DNA is released from the cytoplasm of the target cell into the culture supernatant (Table 2). The cytotoxic potential of the effector cells is measured by quantification of the label released from the damaged target cells.

Because this metabolic prelabeling of the genomic DNA requires DNA synthesis, only cells proliferating *in vitro* (e.g., cell lines) may be labeled in this way; cells which do not proliferate *in vitro* (e.g., primary cell cultures, tumor cells *ex vivo*) do not replicate their DNA and therefore, do not incorporate labeled nucleotides (see also Section A 3.2.1. "Cellular DNA Fragmentation ELISA" page 64).

To detect fragmented DNA in cells which do not replicate *in vitro*, the DNA has to be isolated and analyzed by agarose gel electrophoresis (“DNA ladder assay”, Figure 6, see also Figure 4). Roche Applied Science offers a kit, the Apoptotic DNA Ladder Kit, that simplifies this assay.



▲ Figure 5: Compartmentalization of HMW and LMW DNA in normal and apoptotic cells. (↓ = decreasing, ↑ = increasing)

| Compartment | Apoptosis |         | Cell mediated cytotoxicity |         |
|-------------|-----------|---------|----------------------------|---------|
|             | HMW DNA   | LMW DNA | HMW DNA                    | LMW DNA |
| Nucleus     | +         | +       | +                          | +       |
| Cytoplasm   | -         | +       | -                          | +       |
| Supernatant | -         | -       | -                          | +       |

▲ Table 2: Distribution of HMW and LMW DNA in cells undergoing apoptosis and target cells during cell mediated cytotoxicity.

*Note: In the early phases of apoptosis, no DNA is released into the supernatant (prelytic DNA fragmentation). However, in vitro, the apoptotic cells will lyse (“secondary necrosis”). Therefore, LMW DNA is found in the supernatant late in apoptosis.*

An alternative method which circumvents the isolation and electrophoretic analysis of DNA is the immunological detection of LMW DNA (histone-complexed DNA fragments) by an immunoassay (Cell Death Detection ELISA<sup>PLUS</sup>, see page 15).

This nonradioactive immunoassay, offered by Roche Applied Science quantifies that hallmark of apoptosis. The Cell Death Detection ELISA<sup>PLUS</sup> has been designed for relative quantification of DNA fragmentation in cells which do not proliferate *in vitro* (since the kit requires no prelabeling of the cells). This kit measures the enrichment of histone-complexed DNA fragments (mono- and oligonucleosomes) in the cytoplasm of apoptotic cells.

Each of the methods to detect and measure apoptosis has its advantages and limitations. Because the cellular mechanisms that result in apoptosis are complex, most published methods cannot by themselves detect apoptosis unambiguously.

To ensure that the mode of cell death in the individual cell system or experiment is apoptotic, one also has to consider other criteria like the cellular morphology. Morphologic criteria for apoptotic cell death include, for example, chromatin condensation with aggregation along the nuclear envelope and plasma membrane blebbing followed by separation into small, apoptotic bodies. When internucleosomal DNA fragmentation is accompanied by these morphological features it provides an additional useful criterion to define cell death as apoptotic.

