Trends in Molecular Medicine

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Review

Cell-Free DNA and Apoptosis: How Dead Cells Inform About the Living

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Cell-free DNA (cfDNA) is evolving into a widely used prognostic and predictive biomarker, particularly in oncology. However, its versatile clinical use precedes a profound understanding of the underlying biology of cfDNA release. There is much evidence to suggest that cfDNA is mainly derived from dying (i.e., apoptotic) cells. However, numerous cancer studies have shown that cfDNA is informative about acquired resistance to given therapies, which is present in living, proliferating tumor subclones. To explain this contradiction, we review current insights regarding cfDNA release, in particular the interplay between apoptosis and proliferation. We describe how improved knowledge about cfDNA biology could be used for novel therapeutic strategies and how this may affect patient management.

Highlights

Cells release DNA into the circulation, which is referred to as 'cell-free DNA' (cfDNA). In healthy individuals, cfDNA is mainly derived from hematopoietic cells.

In patients with cancer, tumor cells release their DNA in addition, which is then present in different proportions as 'circulating tumor DNA' (ctDNA) within cfDNA.

Recently an arsenal of sophisticated methods to extract a multitude of information from cfDNA and/or ctDNA has been developed, which can be used for various clinical purposes, to assess distinct physiologic conditions, or as a tool for basic research.

The release of cfDNA is thought to be tightly linked to programmed cell death or apoptosis; however, at present the underlying biology of cfDNA release remains largely unknown.

Leveraging the full potential of cfDNA as a biomarker will require improved knowledge of all mechanisms and factors involved in cfDNA release.

The Rise of cfDNA in Clinical Medicine

cfDNA is highly degraded DNA fragments, which are detectable in the peripheral blood of every human. In healthy individuals, the vast majority of cfDNA is derived from the hematopoietic system. Under certain physiologic or pathologic states, the composition of cfDNA may change. For example, the blood of pregnant females contains DNA fragments from the fetus and cancer patients have DNA fragments from the tumor in their circulation. Therefore, cfDNA represents an important component of **liquid biopsies** (see Glossary). Liquid biopsies – analyses of non-solid biological sources to obtain information similar to tissue biopsies [1] – are evolving into widely used biomarkers in various medical areas such as noninvasive prenatal testing (NIPT) [2], monitoring of graft rejections after organ transplantations [3], the identification of microbiomes [4,5], and particularly oncology. For the latter, cfDNA includes **ctDNA** shed from tumor cells into the circulation. Furthermore, circulating tumor cells (CTCs), tumor-derived extracellular vesicles, and tumor-educated platelets enable tumor genome characterization by minimally invasive means. Multiple studies have described how molecular information about parent tumors can be extracted from these components and how liquid biopsies can impact precision medicine [6–10].

The potential of liquid biopsies to improve the clinical management of patients with cancer is of utmost importance, as the global number of cancer deaths worldwide has increased from 5.66 million in 1990 to 8.87 million in 2016 (https://ourworldindata.org/how-many-people-in-the-world-die-from-cancer). Furthermore, NIPT is being rapidly and globally adopted in routine clinical practice, with millions of tests conducted per year [11]. In this review, we focus on cfDNA in oncology and circulating placental DNA for prenatal testing. Furthermore, we address analyses of blood samples only, although other body fluids, such as sputum, cerebrospinal fluid, ascites, pleural effusion, and urine, have also been successfully applied for liquid biopsy purposes, particularly in oncology [10].

Recently, the use of liquid biopsies to guide treatment decisions and for the early detection of cancer has come into focus. Methods for early detection of cancer will be essential to reduce cancer morbidity and mortality [12]. This requires ctDNA assays of increased sensitivity. Progress in this field includes targeted deep sequencing [13,14], molecular barcoding approaches [15,16], inclusion of matched cfDNA and white blood cell sequencing to improve somatic variant

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interpretation [17], the combined assessment of circulating proteins and mutations in cfDNA [18], the investigation of plasma DNA fragmentation patterns [19–21], and nucleosome positioning mapping [22]. Furthermore, characterization and monitoring of the evolution of tumor genomes with blood samples could achieve significant improvements in precision medicine and may improve personalized outcome prediction [23]. For this wide spectrum of cancer-related applications, a better understanding of the underlying biology and mechanisms of cfDNA and ctDNA release into the circulation would help in implementing cfDNA diagnostics in the clinic [8,24,25].

The Interplay between cfDNA Release and Apoptosis

Several mechanisms for cfDNA release have been suggested, including **apoptosis**, **necrosis**, and active secretion [26–28]. While necrotic cells release high-molecular-weight DNA [26], which is rarely detected in plasma samples, the relative contribution of necrosis to cfDNA is a matter of debate [27]. Most cfDNA in human plasma is nonrandomly fragmented, which is thought to be due to its origin mainly from apoptotic cells. Notably, the release of apoptotic products into the circulation differs in leukemia or lymphoma compared with solid cancers, as the contact of neoplastic cells with blood is greater and release is more direct.

Instrumental to cfDNA fragmentation patterns are caspase-dependent digestion and nucleosomes, which protect DNA from cleavage. Caspases are key mediators of DNA fragmentation. Caspases are the main effectors of the morphological and biochemical hallmarks of apoptosis, such as membrane blebbing, cell shrinkage, chromatin condensation, and, in particular, DNA fragmentation. Caspases can be activated through the extrinsic (death receptor) pathway or the intrinsic (mitochondrial) pathway [29,30] (Figure 1) during apoptosis.

Activated caspase-3 releases caspase-activated DNase (CAD), a DNA-specific, double-strand-specific endonuclease, which can generate double-stranded breaks in internucleosomal chromatin regions [29,31,32] (Figure 1). CAD has been described as a 'professional' apoptotic nuclease and is likely to be the most important player in DNA fragmentation during apoptosis [33]. As CAD lacks exonuclease activity, it is capable only of breaking linker regions between nucleosomes [34]. This nuclear DNA fragmentation, with cleavage of loose parts of internucleosomal DNA, is evidenced by the periodic fragment size of cfDNA matching the apoptotic ladder, with a modal value of DNA fragment length near 167 bp, which corresponds approximately to the DNA wrapped around a nucleosome (~147 bp) plus a linker fragment (~20 bp) [2,35,36]. Evidence that cfDNA reflects nucleosome footprints was recently reported [37,38].

According to this, in cancer patients analysis of ctDNA should inform about dead tumor cell populations. From this, a simple model could be derived in which ctDNA mainly reflects the genomes of therapy-responsive (i.e., dying or apoptotic cells), whereas therapy-resistant (i.e., surviving, proliferating, or living cells) should not be reflected in the circulation (Figure 2A).

In apparent contradiction, a number of publications described that mechanisms of therapy resistance can be identified by employing ctDNA [e.g., in patients with colorectal cancer (CRC) involving *KRAS* mutations under anti-EGFR therapy], in some cases even months before disease progression was clinically noted [39–41]. However, the detection of somatic DNA alterations associated with treatment resistance is hard to reconcile with a scenario as shown in Figure 2A. Hence, it is necessary to reflect on our current knowledge on the links between apoptosis and proliferation.

Apoptosis-Induced Proliferation

Apoptosis-induced proliferation is a well-established phenomenon (for recent reviews, see [29,42]). In the course of normal tissue turnover in humans, approximately 1 million cells die

Glossary

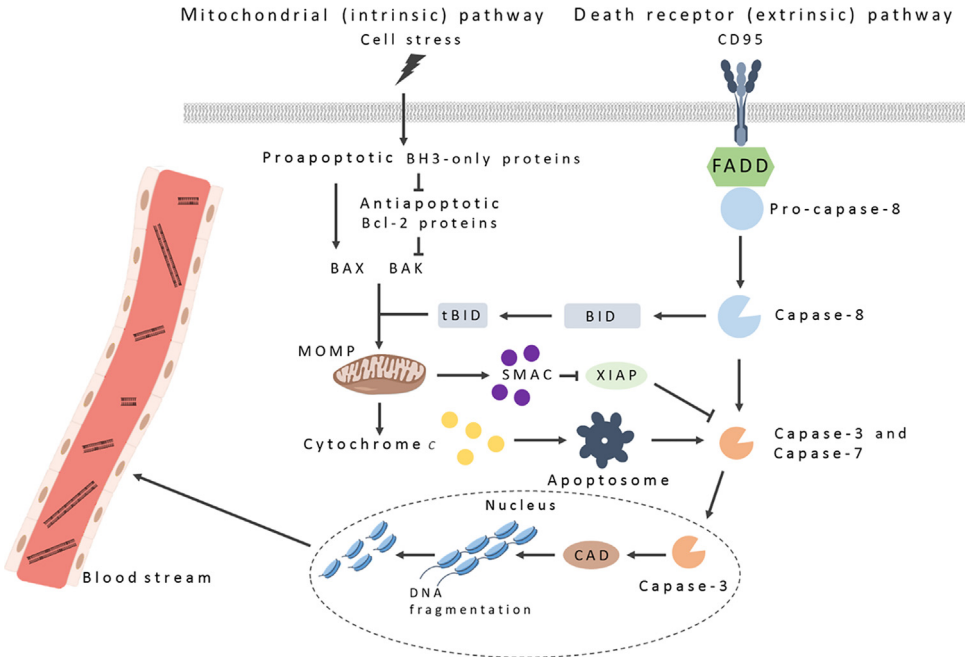
Apoptosis: a highly regulated and controlled process resulting in programmed cell death, which is associated with characteristic cell changes such as blebbing and cell shrinkage and in particular with chromosomal DNA fragmentation. Due to the DNA fragmentation pattern of cfDNA, apoptosis is thought to be the main source of DNA in the circulation. In an adult body, billions of cells die per day due to apoptosis.

Cell-autonomous nuclease: a nuclease that degrades DNA in a dying cell.

Cell cycle: a rigorously ordered process in which the entire genome is replicated during the S phase and segregated to daughter cells during mitosis (i.e., M phase). Between the S and M phases are the preparatory G1 (between M and S) and G2 (between S and M) phases. Furthermore, a differentiated cell can enter a quiescent state, G0 phase. Both the timing and the order of the cell cycle are monitored by cell cycle checkpoints, mediated by sequential activation and inactivation of Cdks, a family of serine/threonine protein kinases [87]. Intriguingly, both cell cycle progression and apoptosis share several genes, like tumor suppressor genes, such as *TP53* and *RB1*, and the oncogene *MYC*. *TP53* regulates cell cycle arrest and apoptosis while *RB1* is a growth suppressor, and the function of *MYC* is well characterized in both cell proliferation and apoptosis [88]. As these genes are frequently disturbed in cancer, with somatic mutations in *TP53* being one of the most frequent alterations in human cancers [89], tumor cells may be more prone to disturbances of the cell cycle. The co-occurrence of *MYC* amplification and *TP53* mutations indicates distinct cancer subtypes [90].

Circulating nuclease: a nuclease present in the plasma, which may contribute to further cleavage of plasma DNA.

Circulating tumor DNA (ctDNA): DNA fragments released into the circulation from a primary tumor and/or its metastases, which can be found within cfDNA. ctDNA analyses have evolved to become a promising biomarker for a variety of applications, such as detection of minimal residual disease and relapse, identification of resistance markers, or disease monitoring.



Liquid biopsy: analysis of components from biological fluids. In the peripheral blood, such analytes may be CTCs, cfDNA, extracellular vesicles, tumor-educated platelets, proteins, or metabolites. Further biological fluids for analysis include urine, ascites, pleural effusions, and cerebrospinal fluid.

Necrosis: accidental or traumatic cell death due to cell injury, which may result in unregulated digestion of cell components and the release of high-molecular-weight DNA fragments.

Waste-management nuclease: a nuclease that degrades DNA in cells after they have ingested apoptotic corpses or in the extracellular environment.

Figure 1. Apoptosis and DNA Fragmentation. Caspases can be activated through the intrinsic (mitochondrial) pathway (left) or the extrinsic (death receptor) pathway (right). The death receptor pathway is engaged when ligands bind to a subset of death receptors from the tumor necrosis factor (TNF)-related family, such as FAS (also known as CD95) or the TNF-related apoptosis-inducing ligand (TRAIL) receptor (TRAILR). Through adaptor proteins such as FAS-associated death domain protein (FADD), initiator caspases, such as caspase-8, are then activated, which in turn cleave and activate the executioners caspase-3 and caspase-7 [29,30]. Many stimuli, such as DNA damage, proteotoxic stress, and cytokine deprivation, can activate the mitochondrial pathway of apoptosis by engaging proapoptotic BCL-2 homology domain 3 (BH3)-only proteins, which inhibit the antiapoptotic Bcl-2 proteins and activate BAX and BAK to effect mitochondrial outer membrane permeabilization (MOMP). Following MOMP, mitochondrial intermembrane space proteins such as second mitochondria-derived activator of caspases (SMAC) and holocytochrome *c* are released into the cytosol. Cytochrome *c* is involved in the assembly of the apoptosome, which subsequently activates caspase-3 and caspase-7, while SMAC blocks the caspase inhibitor X-linked inhibitor of apoptosis protein (XIAP), which in turn inhibits caspase-3 and caspase-7. Hence, both pathways culminate in activated caspase-3. Furthermore, crosstalk between the two apoptotic pathways exists by caspase-8 cleavage of the BH3-only protein BH3-interacting death domain agonist (BID) [truncated BID (tBID)]. Caspase-3 translocates to the nucleus (broken ellipsoid) where it activates caspase-activated DNase (CAD), which then induces DNA fragmentation by transforming stretches of DNA into mononucleosomal DNA, resulting in the characteristic plasma DNA fragmentation patterns encountered in the circulation.

every second through the highly regulated process of apoptosis [29,43]. To ensure proper cell clearance, apoptotic cells secrete 'find me' signals to attract phagocytes and 'eat me' signals. As a consequence, in healthy individuals, debris from dying cells from normal tissue is efficiently collected by phagocytes and does not reach the bloodstream [31]. Therefore, the vast majority (>90%) of cfDNA in healthy individuals is derived from the hematopoietic lineage [44–46].

However, cell death must be swiftly balanced with compensatory proliferation to maintain homeostasis and tissue integrity. Therefore, among the multitude of signals released by apoptotic cells are also mitogens that trigger compensatory proliferation of adjacent cells. Caspases play a role in both the destruction of the cell in which they are activated and the proliferation of neighboring cells, and hence caspases couple intrinsic death and nonautonomous growth [47]. This compensatory process of maintaining tissue homeostasis is referred to as

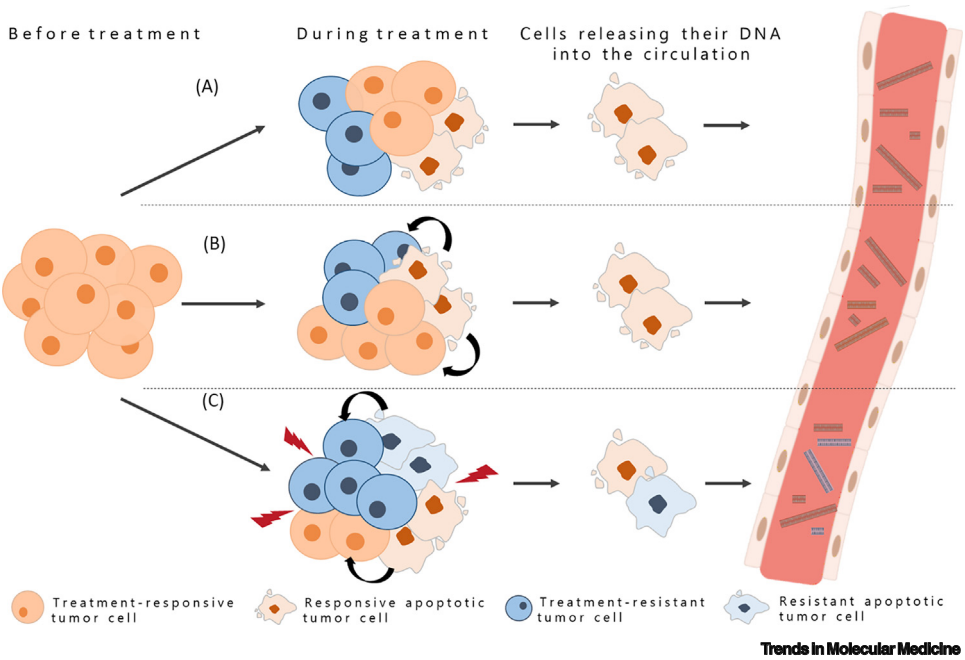


Figure 2. Release of DNA from Apoptotic Scenarios.

For a Figure360 author presentation of Figure 2, see the figure legend at https://doi.org/10.1016/j.molmed.2020.01.012. (A) Treatment-responsive cells (orange) die and undergo apoptosis (pale orange) and release cell-free DNA (cfDNA), while treatment-resistant cells (blue) survive and multiply. According to such a model, mutations representing resistance mechanisms present in the surviving cells should not be detectable by plasma DNA analyses. (B) Apoptosis-induced proliferation is an important mechanism for maintaining tissue homeostasis whereby dying, apoptotic cells send mitogens (indicated as black arrows) to stimulate the growth of adjacent cells. In this apoptosis-induced proliferation scenario, therapy-responsive apoptotic cells (pale orange) may stimulate their adjacent cells to proliferate. This will not result in increased death of therapy-resistant cells (blue), so that the majority of cfDNA released into the circulation is still derived from treatment-responsive apoptotic cells. (C) During proliferation-induced apoptosis, the pool of proliferating, therapy-resistant cells (blue) is exposed to cellular stress such as dwindling nutrients or cell cycle disturbances (indicated by the red jagged arrowheads). In this environment, apoptosis may also be induced in therapy-resistant cells; that is, both therapy-responsive (pale orange) and therapy-resistant (pale blue) cells undergo apoptosis and release their DNA into the circulation, indicated by orange and blue DNA strands in the bloodstream.

apoptosis-induced proliferation [48]. In cancer patients, apoptosis may stimulate tumor growth through this apoptosis-induced proliferation by stimulating adjacent cells to grow (Figure 2B). However, apoptosis-induced proliferation implies that therapy-responsive apoptotic cells stimulate adjacent therapy-resistant cells to proliferate and would therefore also not explain why the latter clones are detectable in plasma DNA (Figure 2B).

Proliferation-Induced Apoptosis

The release of ctDNA into the blood has been closely related to a combination of tumor volume and proliferative capacity [49] and the context between the rate of cellular proliferation and ctDNA release was meticulously documented in several tumor entities [14,50–53], indicating that proliferation may be associated with apoptosis.

In a proliferating tumor, competition between cells results in the elimination of less fit cells. Due to the heterogeneity of cancers, many different lineages with distinct mutations are present in a tumor [54] and their interaction represents such a competition. Small competitive advantages such as better survival in the presence of a drug can cause one lineage to take over the whole tumor [55].

Hence, proliferating cells constantly compete with each other and certain cells may outcompete other cells. This in turn may result in uncontrolled proliferation of the prevalent clone, which may cause these cells to be stressed. For example, dwindling availability of nutrients and growth factors may affect the ability of a cell to grow [29] and may subsequently result in what we here refer to as 'proliferation-induced apoptosis' (Figure 2C). Further reasons why high proliferation rates may be associated with a high level of apoptosis include disturbance of **cell cycle** and cancer cells' increased sensitivity to apoptosis, as outlined later (Figure 2C).

Evidence for cell cycle disturbances and increased ctDNA release comes from clinical studies. For example, the CDK4/6 inhibitor palbociclib together with endocrine therapy has now evolved to be a standard of care for advanced estrogen receptor-positive breast cancer [56]. Analysis of plasma samples from patients treated with palbociclib and fulvestrant, a selective estrogen receptor degrader used to treat hormone receptor-positive breast cancer, revealed that cancers with incomplete cell cycle arrest on palbociclib continued to proliferate while simultaneously undergoing apoptosis, which enabled them to continuously release tumor DNA into the circulation [57]. By contrast, when cancer cells were capable of completing G0/G1 cell cycle arrest with palbociclib, they no longer underwent cell death and did not release ctDNA. From this, it was concluded that cancer cells under palbociclib selection pressure predominantly undergo apoptosis as a result of a failure to transition from the S and M phases of the cell cycle [57].

Additionally, cancer cells are more sensitive to apoptosis. Cancer cells may express high levels of antiapoptotic BCL-2 proteins in response to the proapoptotic BCL-2 family proteins [i.e., the BH3-only proteins (Figure 1)] [29]. Such a phenomenon is well documented in multiple myeloma and leukemias [58,59] as well as in solid tumors such as ovarian [59] and breast [60] cancer. Cells in such a state are referred to as being 'primed for death'; that is, they are closer to triggering the apoptotic pathway than healthy cells so that, due to the dual upregulation of proapoptotic and antiapoptotic proteins, the cells undergo apoptosis more quickly and easily [61].

In summary, high proliferation rates of aggressive (i.e., therapy-resistant) cell clones may result in stressed systems due to lack of nutrients and growth factors (Figure 2C). As cancer cells are more prone to cell cycle disturbances and are furthermore primed for death, a substantial proportion of proliferating cells may undergo proliferation-induced apoptosis such that resistance mechanisms become detectable in plasma DNA. However, there are many unanswered challenges that need to be addressed.

The Complexity of DNA Degradation during Apoptosis

The complexity of DNA degradation during apoptosis is poorly understood. So far, we have focused our discussion on the role of CAD in DNA fragmentation (Figure 1). CAD belongs to the **cell-autonomous nucleases**, which cleave the DNA in a cell during apoptosis (Figure 3A). While CAD is the main cell-autonomous nuclease in apoptotic cells [33], there are, in addition, other apoptotic nucleases that may participate in the destruction of the DNA of apoptotic cells [33]. One such nuclease is *DNAS1L3* (also known as DNase γ), which has been described to cooperate with CAD to process DNA degradation during apoptosis [62]. Deletion of *Dnase1l3* in mice resulted in aberrant fragmentation patterns of plasma DNA, including an increase of short as well as long, multinucleosomal DNA molecules [63].

If a cell is incapable of degrading its own DNA due to a lack of functioning cell-autonomous caspases, engulfment-mediated DNA degradation will be conducted by phagocytes [33] and their '**waste-management nucleases**' (Figure 3A). Hence, an absence of caspases does not

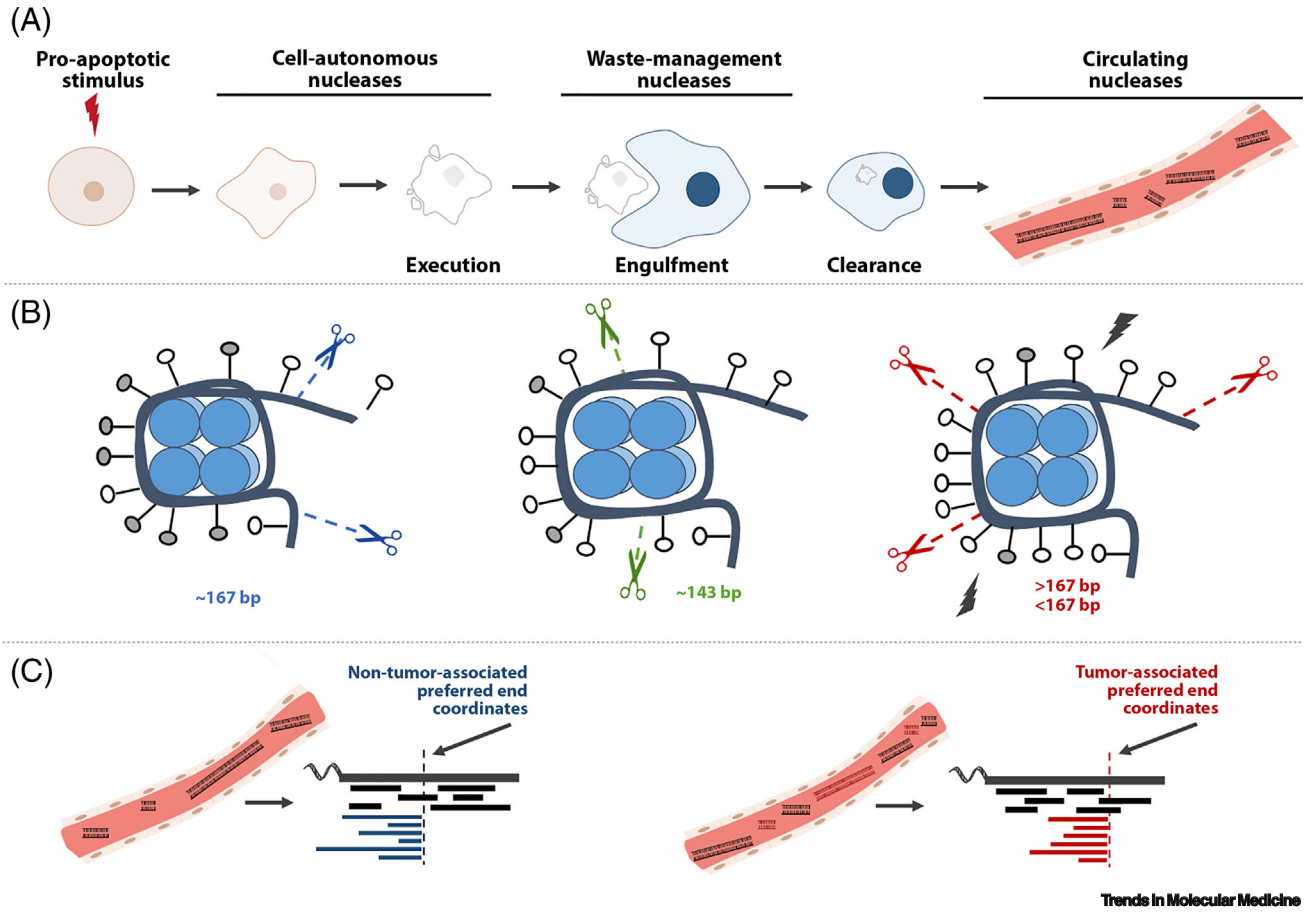


Figure 3. The Origin of Fragmentation Patterns. (A) DNA is cleaved consecutively through different nucleases. After proapoptotic stimuli, DNA is cleaved by cell-autonomous nucleases in the apoptotic cells. Subsequently, the apoptotic cells are engulfed by phagocytic cells and the DNA is further digested by proteases and waste-management nucleases in lysosomes. The fragmented DNA is released into the circulation and further exposed to **circulating nucleases**. (B) DNA wrapped around a nucleosome in a noncancerous somatic cell (left), a placental cell (center), and a tumor cell (right). In noncancerous somatic cells, nucleosome-bound DNA is more methylated than flanking DNA (gray circles, methylated DNA; white circles, unmethylated DNA), which induces tighter wrapping of DNA around nucleosomes so that the DNA is frequently cleaved by caspases in the linker regions between nucleosomes (blue scissors), resulting in frequent fragments of ~167 bp. Placental tissue is genome-wide hypomethylated enabling looser DNA wrapping and hence alternative cleavage sites within nucleosomes (green scissors), resulting in an increased number of DNA fragments of shorter length, ~143 bp. Cancer cells may also exhibit hypomethylation but there may be additional factors, such as cell cycle disturbance and altered proliferation (black jagged arrowheads), so that multiple cleavage sites (red scissors) may be employed, resulting in DNA fragments of various lengths (i.e., fragments smaller or greater than 167 bp). (C) Different DNA cutting positions result in an increase of distinct plasma DNA fragmentation ends. The left panel shows non-tumor-associated preferred end coordinates (blue broken line), which differ from the tumor-associated preferred end coordinates (red broken line) in the right panel. Adapted in part from [76].

prevent DNA fragmentation, so that apoptotic DNA fragmentation may occur both caspase dependently and independently [34].

In contrast to cell-autonomous nucleases, waste-management nucleases digest DNA that does not originate from the nucleus of the cell in which the nucleases were made [33]. Instead, waste-management nucleases destroy DNA in cells that ingest apoptotic corpses (Figure 3A) or act as a secreted enzyme in the extracellular compartment to degrade DNA released as a consequence of cell death. An important waste-management nuclease is *DNase I*, a secreted protein, which mainly functions in the blood and alimentary tract to degrade extracellular DNA. Hence, this nuclease has no access to nuclei during the initial stages of apoptosis [33] and consequently, deletion of *Dnase1* in mice did not result in observable changes in the size profile of circulating DNA [64].

Furthermore, nucleases exist in the plasma of humans [65] (Figure 3A). In pregnant women, the clearance of circulating placental DNA was used to establish that circulating placental DNA has a short half-life of less than 30 min [66]. However, when blood samples had been collected before Cesarean section and the plasma incubated at 37°C for 2 h, the plasma placental DNA was only incompletely eliminated, suggesting that plasma nucleases play only a partial role in the degradation of and removal of cfDNA [66]. This suggests that, beyond DNase degradation, other DNA removal systems, such as kidney and liver function or binding to other blood components [67], play a pivotal role in the complex cfDNA biology.

To improve our knowledge about these processes, appropriate model systems will be needed. For example, caspase-8 plays a central role in initiating death receptor-mediated apoptosis (Figure 1) and a xenograft mouse model of caspase-8-deficient CRC was developed to obtain apoptosis-resistant CRCs [68]. Another model could be to mimic primed-to-die cancer cells by coexpression of proapoptotic BH3-only proteins and antiapoptotic BCL-2 proteins [69], an approach that is also applicable *in vivo* by injecting such primed-to-die generated cells subcutaneously into mice as syngeneic hosts [70]. If such, or other, models were extended to include detailed cfDNA analyses, they could provide novel insights on how certain apoptosis-related features may impact plasma DNA release and their associated fragmentation patterns.

However, besides nucleases and their cofactors, there may be additional features impacting DNA fragmentation patterns. For example, nucleosome-bound DNA is more methylated than flanking DNA [71]. However, placental DNA is globally hypomethylated, so that nucleosomal DNA in placental tissue has a more open chromatin structures than the methylated maternal somatic tissue [72]. Therefore, nucleosome-bound placental DNA has increased accessibility to endonucleases during apoptosis and hence alternative cleavage sites compared with maternal DNA, which may explain why placentally derived DNA is shorter than maternally derived DNA in the plasma of pregnant females [73] (Figure 3B). Similarly, the size distribution of DNA from cancer cells has been reported to be shorter than DNA fragments from nonmalignant cells [20,21,74] and as tumor genomes may also exhibit genome-wide hypomethylation [75], similar/analogous mechanisms may be involved in generating shorter ctDNA fragments (Figure 3B). As another possible explanation, the shortness of ctDNA could stem from cfDNA released during cell proliferation rather than apoptosis as discussed earlier [50]. Fragmentation patterns have recently raised so much interest that the term 'fragmentomics' was coined to describe their study [76].

Another interesting concept is 'cfDNA preferred ends', describing the nonrandom fragmentation of cfDNA indicated by enrichment of genomic regions at the ends of cfDNA [77] (Figure 3C). The genomic localization of the preferred ends reflects the nucleosome pattern from which a particular cfDNA fragment originated. Accordingly, the fraction of cfDNA fragments possessing placental DNA preferred ends correlates positively with the fetal DNA fraction in the plasma DNA of pregnant women. Similarly, ctDNA fragments may have distinct preferred end sites, as recently shown in hepatocellular cancers [78].

Given the increasing interest in plasma DNA as a liquid biopsy and the recent observations about different plasma DNA fragmentation patterns [19–21,79–81], it will be mandatory to intensify apoptosis research and related areas to understand the underlying biology and to leverage this for diagnostic purposes and precision medicine.

Clinician's Corner

Particularly in oncology, analysis of cfDNA will be an integral part of precision medicine, as both cfDNA and ctDNA have great potential as predictive biomarkers and will be increasingly used for clinical decision making.

Novel multiparameter strategies together with sophisticated machine learning tools to detect disease-associated cfDNA signatures in liquid biopsies will transform molecular diagnostics.

Besides oncology, other clinical applications include noninvasive prenatal testing (NIPT) for fetal aneuploidies from maternal blood and improved diagnosis of acute diseases such as myocardial infarction or monitoring of chronic diseases such as diabetes.

The utility of cfDNA/ctDNA in clinical practice is often hampered by the low abundance of the target signals (i.e., in oncology, the frequently volatile presence of DNA fragments from tumor cells, which may be present at very low allele frequencies, even in advanced tumor stages [24]). Correct interpretation of these signals will critically depend on elucidation of the biology of ctDNA release.

Our incomplete knowledge about the biology of cfDNA release is one factor contributing to why at present unequivocal demonstration of the clinical utility of cfDNA analysis is outstanding in oncology [86].

Concluding Remarks

We have described how cfDNA is evolving into a powerful biomarker; however, we still lack a solid understanding of the fundamental mechanisms of its origin. Although it is already frequently used as biomarker in oncology as well as in other medical areas, an improved understanding of the biology of cfDNA release is mandatory for a wide range of applications.

Future research will reveal more on fragmentation patterns, their association with nucleosomes, and the involved enzymes, such as nucleases. This not only will be of relevance to biologists but will represent truly translational research, as improved insights into the respective apoptotic mechanisms could also contribute to novel treatment options. In vertebrates, cell death is mostly mediated by the mitochondrial (intrinsic) pathway [82] and most conventional cytotoxic agents act by activating the intrinsic pathway. For example, DNA-damaging agents (i.e., etoposide or alkylating agents) as well as γ-irradiation induce apoptosis by TP53-mediated activation of PUMA [82]. Caspase-8-deficient tumor cells responded in the aforementioned mouse model to a mitochondria-derived activator of caspase mimetic treatment by inducing massive cell death and regression of tumors [68]. In the primed-to-die cancer cell mouse model, mice were treated with the BH3 mimetic ABT-263, which led to rapid cell death [70]. However, beside cancer cells, bystander stromal or immune cells affect treatment response and resistance development, so that the tumor microenvironment should be included in appropriate models.

Furthermore, instrumental to these efforts will be plasma DNA tissue deconvolution [45] (i.e., determining the cell or tissue of origin of cfDNA), which has been done by employing methylation marks [45,46,83,84] or cfDNA fragmentation patterns [19,37,85]. It is likely that combinations of these various parameters with machine learning and artificial intelligence will provide information on cfDNA composition with unprecedented resolution [8].

Importantly, improved knowledge about DNA release could pave the way for further liquid biopsy applications in biology or medicine, such as investigating the plasma DNA composition in certain physiologic conditions like aging or the identification of increased release of DNA from a certain organ as an early disease indicator. In addition, elucidating cfDNA biology should facilitate the interpretation of liquid biopsy results, particularly cases in which plasma DNA alterations are observed at very low allele frequency. Given that the underlying biology of cfDNA release has yet to be elucidated, addressing the outstanding questions will be mandatory for liquid biopsies to fulfill all of their promise for many clinical applications (see Outstanding Questions).

Outstanding Questions

Do forms of cell death other than apoptosis, such as parthanatos, pyroptosis, ferroptosis, NETosis, mitochondrial permeability transition-regulated necrosis, necroptosis, or immunogenic cell death, contribute to the cfDNA pool and if so, to what extent?

With what resolution will detailed tissue deconvolution become possible when factors such as methylation, cfDNA fragmentation patterns, preferred ends, and nucleosome positioning are combined and to what extent will improved knowledge about the tissue of origin yield novel insights into cfDNA biology?

What are the exact impacts of cell-autonomous nucleases, waste-management nucleases, and circulating nucleases on cfDNA fragmentation?

What is the underlying biology of cfDNA release and what parameters of the primary tumor, metastases, or microenvironment determine the amount of tumor DNA released into the circulation?

What is the interplay between apoptosis and proliferation? Are the mechanisms of and interplay between proliferation and apoptosis different in early and late-stage cancers?

In patients with cancer, to what extent is ctDNA release influenced by given therapies? Are there differences between responders and nonresponders that can be used to predict who will benefit from a therapy being administered?

The dose- and time-dependent dynamics of apoptosis and other cell death types after drug administration may be pivotal to identify correct time points for monitoring response in clinical trials. How long can the direct therapy effect be measured (e.g., the cfDNA peak and subsequent decrease after application) and when is the tumor biology and progression itself more relevant?

What is the explanation for the significant variability of ctDNA that is observed in cancer patients?

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