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REVIEW

Life and death of circulating cell-free DNA

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ABSTRACT

Tumor-specific, circulating cell-free DNA in liquid biopsies is a promising source of biomarkers for minimally invasive serial monitoring of treatment responses in cancer management. We will review the current understanding of the origin of circulating cell-free DNA and different forms of DNA release (including various types of cell death and active secretion processes) and clearance routes. The dynamics of extracellular DNA in blood during therapy and the role of circulating DNA in pathophysiological processes (tumor-associated inflammation, NETosis, and pre-metastatic niche development) provide insights into the mechanisms that contribute to tumor development and metastases formation. Better knowledge of circulating tumor-specific cell-free DNA could facilitate the development of new therapeutic and diagnostic options for cancer management.

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Introduction

Molecular-biological and instrumental diagnostic approaches in cancer management are intended for screening, early detection, risk stratification, effective therapy selection, treatment response monitoring, and disease recurrence prevention. Current decision-making tools in oncology are limited, which leads to overdiagnosis (particularly with mammography),[1,2] false-positives, and low specificity (particularly with serum biomarkers, like CA125).[3,4] As a result, standard imaging modalities typically only provide tumor detection at advanced stages of the disease.[5] Furthermore, the "gold standard" for cancer diagnosis is a tissue biopsy, which is an invasive procedure associated with discomfort and risk of potential complications.[6,7] Moreover, accessibility of tumors for biopsy is limited, and it does not reflect intratumor heterogeneity or the emergence of new subclones during tumor evolution.[8,9] Alternatively, a "liquid biopsy" is a promising approach that could overcome these shortcomings.[10,11]

A liquid biopsy is a minimally invasive approach for detecting prognostically or diagnostically significant tumor-derived markers in body fluids.[7,12] Definition of liquid biopsy applies to:

- circulating tumor cells,[13,14]
- circulating extracellular nucleic acids, including cell-free DNA (cfDNA), mRNA, and microRNA (miRNA),[7,15]
- extracellular vesicles (e.g., exosomes),[16,17]
- nucleosomes,[18,19]
- various glycoproteins and antigens (e.g., PSA, CEA, CA 125, CA19-9, βHCG, αFP, etc.).[3,4]

Although all these components of liquid biopsies have advantages and limitations (for reviews, see refs.[20,21]), the present review will focus on cfDNA. cfDNA carries information about the dynamics of cancer-specific genetic and epigenetic alterations.[22] It was shown that the cfDNA level during treatment was correlated with outcome.[23,24] Some studies suggested that analyses of cfDNA outperformed instrumental methods (such as computed tomography), required a lower tumor burden, and prolonged the time window for adopting clinical decisions.[23,25]

Studies of cfDNA in oncology are predominantly devoted to clinical applications of cfDNA as a tumor biomarker. An association of cfDNA level with treatment outcome, low invasiveness of an assay, implementation of high-throughput techniques make liquid biopsy using extracellular DNA an attractive candidate for a routine test in cancer management. Nevertheless, we have a long way to go in determining reliable markers, estimating prognostic significance, standardizing assays, and validating findings in large-scale prospective clinical trials.[26–28] Moreover, despite the increase in studies that implicate the importance of cfDNA in oncology, a number of unresolved questions remain about the nature of cfDNA, its subtypes, its mechanisms of release, and its clearance in patients with cancer. In addition, it is important to determine the significance of cfDNA in cancer development: i.e., its association with the origin, aggressiveness, and metastatic potential of tumors, in addition to its association with the response to treatment. Current analysis performed by a joint panel of experts of American Society and Clinical Oncology and the College of American Pathologists demonstrated insufficient evidence of clinical validity and utility for the majority of ctDNA assays in advanced and in early-stage cancer, for treatment monitoring, or residual disease detection.[29]

In this review, we consider the dynamics of extracellular DNA, the balance between cfDNA release and clearance, and the roles of various cfDNA subfractions in pathophysiological processes during tumor development. Here, we distinguish three pools of circulating DNA: total circulating cfDNA, circulating tumor-specific cell-free DNA (ctDNA), and circulating mitochondrial DNA (mtDNA).

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Levels of cell-free DNA

cfDNA was first described in immune complexes derived from patients with systemic lupus erythematosus in 1948,[30] but serum cfDNA levels from patients with cancer were characterized for the first time 30 years later.[31] It was shown that the total amount of circulating DNA was higher in patients with cancer than in healthy subjects.[31,32] However, an increased cfDNA content was not specific to malignancies; elevated levels were also detected in the plasma of pregnant women and in patients that received transplantations.[33,34] Elevated cfDNA might also reflect physiological (e.g., exercise)[35,36] and non-malignant pathological processes,[7] such as inflammation, diabetes, tissue trauma, sepsis, and myocardial infarction.[37–39]

The concentration of cfDNA in blood varies significantly; it ranges between 0–5 and >1000 ng/ml in patients with cancer and between 0 and 100 ng/ml in healthy subjects.[7,24] There is also a marked variation in blood ctDNA levels among patients with different tumor types. For example, the ctDNA detection frequency in patients with advanced pancreatic, ovarian, colorectal, gastroesophageal, breast, melanoma, and some other malignancies was higher than in patients with primary brain, renal, and thyroid cancers.[40,41] Khier and Lohan[42] hypothesized that this variability could be explained by tumor localization; for example, the blood-brain barrier and the capsules surrounding some organs could limit the release of ctDNA into the body fluids.

It was observed that patients with benign lesions or with early-stage cancer have lower amounts of cfDNA compared to patients with advanced or metastatic tumors of comparable size.[24,43] That finding suggested that the level of ctDNA shed by tumors differed at various stages of cancer; thus, the level could reflect tumor interactions with the microenvironment or the various metabolic properties of progressing cancer.[44,45] Therefore, although the variability in ctDNA is typically attributed to the tumor burden,[46] it actually might reflect tumor metabolism.[47,48] For example, in patients with melanoma, ctDNA levels were correlated with metabolic disease volume, estimated with ^{18}F-labelled fluorodeoxyglucose positron emission tomography.[49,50] Therefore, the ctDNA level was a complex reflection of tumor biology, rather than simply associated with tumor burden or the number of dying cells. This finding suggested that ctDNA measurements might be more relevant to advanced stages of the disease and less relevant to precancerous lesions. However, the use of multianalyte tools (e.g., CancerSEEK) and combinations of several marker types (i.e., ctDNA and tumor-related glycoproteins) represent promising approaches for early tumor detection.[51]

Tissue origin of cell-free DNA

The source of cfDNA is an intriguing question in cancer and in other pathological conditions. The identification of the cfDNA origin could contribute to revealing the affected tissues or organs and provide information about the mechanisms of cfDNA shedding. Several approaches have been used to investigate this question, including *i*) identification of tissue-specific patterns of promoter methylation;[52–55] *ii*) analysis of tissue-specific modifications in circulating nucleosomes, *per se*;[18,19] and *iii*) identification of tissue-specific DNA fragmentation patterns or nucleosome occupancy.[53,56,57] In cancer, all these approaches could aid in defining the cfDNA tissue of origin without requiring a preliminary search for genetic differences.[27]

It can be postulated that circulating tumor cells are not the main source of cfDNA. Indeed, the amount of cfDNA in blood corresponds to several thousand genomic equivalents, and much fewer circulating tumor cells are present in blood.[41,58,59] Next, a significant part of total cfDNA consists of non-mutated DNA. According to various studies, the fraction of ctDNA constitutes ~0.1–89% of cfDNA,[41,49,60] but it may increase with disease progression.[59] Consequently, it can be speculated that the bulk of cfDNA originates from cells in the tumor microenvironment, destroyed in hypoxic conditions, or from cells involved in the antitumor response.[61] Indeed, analyses of DNA fragmentation patterns and nucleosome occupancy showed that the nucleosome footprint in healthy subjects corresponded to hematopoietic lineages, but in patients with cancer it also aligned with the cancer type.[62] Interestingly, whole-genome array cfDNA analyses of tissue-specific methylation patterns in healthy individuals demonstrated that most cfDNAs were of hematopoietic origin (55% white blood cells and 30% erythrocyte progenitors).[54,55,63] In addition, a fraction of cfDNAs from solid tissues were derived from vascular endothelial cells (~10%), neurons (~2%), and hepatocytes (~1%).[54,55] Methylation profiles of patients with cancer (n = 4 with metastatic colon cancer, n = 4 with lung cancer, and n = 3 with breast cancer) also showed that cfDNA levels were elevated compared to levels observed in healthy individuals (>20-fold increase). The largest fraction of cfDNA in patients with cancer was derived from the tumor tissue of origin.[29,54]

Mechanisms of cfDNA release into the circulation and subfractions of cfDNA

Mechanisms of cfDNA release can be deducted by analyzing cfDNA subfractions. cfDNA comprises mainly double-stranded (ds) nuclear DNA and mtDNA. Some studies have also described small extrachromosomal circular DNA (eccDNA), microDNA, and single-stranded (ss), viral, bacterial, or food-derived (plant and meat) DNAs.[64–67]

Most cfDNA that originates from the nucleus is packaged in the form of mono- or oligonucleosomes.[68] cfDNA is present both on the surface and in the lumen of vesicles.[69] However, some studies have suggested that over 90% of the cfDNA was associated with exosomes.[70] Vagner et al. (2018) demonstrated that most ctDNA is packaged in large (1–10 μm diameter) extracellular vesicles. Those DNA particles are is chromatinized, and can comprise up to 2 million base pairs.[71] The association of ctDNA with extracellular vesicles was confirmed by the observation that patients with cancer had elevated levels of exosomes and nucleosomes in peripheral blood.[12,67,68] *In vitro* analyses also showed higher amounts of exosomes secreted by tumor cells compared to the amounts secreted by cultured fibroblasts.[72] Wang et al[73] demonstrated that ctDNA released from cultured cancer cells did not correlate with the level of cellular apoptosis or necrosis; instead, it was correlated with the percent of cells in G1 phase. They

suggested that increased release of cfDNA from differentiated cells might be due to the active release of cfDNA packaged inside exosomes or in other forms that are protected from degradation in the blood. It is worth noting that the profile of free nucleic acids in plasma (DNA and miRNA) differs from that found in exosomes,[15] and profiles vary among various subtypes of vesicles.[69] Thus, exosome isolation can be used to enrich ctDNA.

The estimated size of cfDNA varies from ~40–200 base pairs (bp), with a peak at about 166 bp.[56,74,75] However, individual cfDNAs might carry thousands of base pairs (>20–30 kb).[24,64] Separation of extracted cfDNA with gel electrophoresis has displayed fragment ladders that ranged from ~160 or 180 bp to 1000 bp. The size of these fragments is due to multiple DNA lengths in nucleosomes and predominantly corresponds to mono- and oligonucleosomes. This feature is characteristic of caspase-dependent cleavage; consequently, it is assumed that the bulk of cfDNAs found in healthy and ill individuals is released during apoptosis.[16,56,65,76]

Longer DNA fragments (i.e., >10 kb) are considered to result from necrotic cell death; for example, from cells in necrotized parts of tumors.[25] Experiments *in vitro* have demonstrated that the amount of extracted DNA fragments depended on the type of necrosis-inducing agent applied.[77,78] Moreover, blood sampling can affect the type of cell death; indeed, the collection of serum stimulates a release of necrotic DNA from blood cells; this mechanism could account for the higher levels of total cfDNA found in serum compared to levels found in plasma samples.[23] Unexpectedly, some studies have observed that radiation therapy, which potentially induces necrosis, could result in a reduction of cfDNA levels (up to 90%) in the plasma of patients with cancer.[31,79] This finding has raised questions about cell death mechanisms and DNA release during various therapeutic interventions.

Shorter fragments of DNA (<100 bp) are enriched with ctDNA, mtDNA, and bacterial DNA.[65,74,80,81] This observation can be useful in developing methods for enhancing ctDNA detection, and it has led to questions about the nature of these short fragments and the mechanisms of their release. Short fragments comprise DNA that is protected from nuclease degradation by nucleosomes in blood. They may form by transcription factor positioning (35–85 bp) or they may represent harmful DNA excreted by exosomes.[16,26,56,82]

The distribution of DNA fragments of different lengths has clinical significance because it reflects cfDNA integrity. cfDNA integrity is determined by the ratio of long to short PCR product amplified from the same locus (for example, the ALU1 locus). Patients with cancer have a significantly elevated level of cfDNA integrity compared to healthy individuals and patients with benign diseases.[83,84] High integrity is explained by augmented levels of necrotic death in large tumors at advanced stages, and it is also associated with tumor aggressiveness.[76,83]

Apart from apoptotic and necrotic cell death, other DNA release mechanisms have been described, including oncosis, pyroptosis, phagocytosis, active secretion, neutrophil extracellular trap release (NETosis), and excision repair.[24,85–89] In contrast to various forms of cell death, active secretion occurs in live, functional cells. Examples of active secretion include the expulsion of nuclei by maturing erythroblasts,[90] vital NETosis,[91] and egestion of mtDNA.[92,93] These activities give rise to several questions: why do live cells get rid of DNA, and how do they live without it? Are there some pathways that compensate for secreted DNA, and what is the biological significance of active secretion?

Activated neutrophils release nuclear DNA in response to various stimuli. Such DNA forms neutrophil extracellular traps (NETs). Although NETosis was first described in neutrophils, it was also shown to occur in mast cells, basophils, and macrophages.[94] The fibers of NETs comprise nuclear DNA strands decorated with proteins that possess anti-bacterial characteristics (myeloperoxidase, pentraxin 3, neutrophil elastase, MMP9, and others).[91,95] The release of NETs usually takes hours, and it eventually leads to neutrophil lysis (suicidal NETosis).[96] On the other hand, another fraction of polymorphonuclear leukocytes can rapidly excrete vesicular NETs to provide an effective, rapid response to bacteria. The remaining anuclear neutrophils are not lysed but retain the ability to crawl and engulf bacteria (vital NETosis).[91,95] Platelets can trigger NETosis. The importance of platelet/neutrophil interplay was underscored by a study that showed that inhibiting platelet activation with aspirin significantly reduced NET formation.[97] Platelets induce NETosis by releasing thromboxane A2; then, in turn, NETosis leads to thrombin generation.[37,98]

Lymphocytes, monocytes, and neutrophils can also release mtDNA strands.[93] In the blood, mtDNA can be present in both particle-associated and non-particle associated forms.[99] mtDNA webs are different from NETs; they are not decorated with pathogen-slaying proteins.[93] However, these two types of DNA webs can be connected: mtDNAs can reside in NETs[100] and mtDNA can potently induce the formation of NETs.[101]

Clearance of cfDNA

The level of extracellular DNA in the circulation is determined by a balance between DNA release and DNA clearance processes. cfDNA clearance can occur in the "home" tissue, in blood or other body fluids, and in organs, such as the liver, spleen, kidney, or lymph nodes.[102] Healthy individuals have low levels of circulatory cfDNA because apoptotic cells and cfDNA are rapidly cleared. In malignancies, chronic inflammation, or excessive cell death, clearance is insufficient, and cfDNA accumulates. Insufficient clearance might explain the correlation between high cfDNA levels and pathological conditions. The exact mechanisms of cfDNA accumulation remain obscure, but it can be speculated that an excess of dying cells could overload the clearance system and surplus cell content is released into the medium.

The estimated half-life of cfDNA in circulating blood varies from several minutes (e.g., 4 min after hemodialysis cessation) to 1–2 h.[103–106] Interestingly, clearance of fetal DNA from maternal blood occurs in a bi-phasic manner: first, a rapid phase occurs with a mean half-life of ~10 min to 1 h; then, a second slow phase occurs with a mean half-life of ~13 h.[107] The half-life of the ctDNA level after surgical tumor resection in a pre-clinical rabbit model of head and neck cancer was 23–52 min.[25] A serial analysis of ctDNA in patients with colorectal cancer showed a half-life of 114 min.[108] The short half-life of cfDNA is convenient for

"real-time" analyses of cfDNA; it facilitates treatment response evaluations and dynamic tissue status assessments in various pathophysiological conditions; for example, tissue damage or regeneration.

The half-life of cfDNA depends on various factors, including its association with molecular complexes that prevent rapid cfDNA degradation, the type and stage of the tumor, the treatment modality, etc.[24,102] In blood, cfDNA degradation is carried out essentially by circulating enzymes, such as DNase I, plasma factor VII–activating protease (FSAP), and factor H.[109,110] For example, the level of DNase I was inversely correlated with the concentration of cfDNA in patients with cancer.[82,111]

Elimination of cfDNA occurs in the liver, spleen, and kidney.[107,112] The liver is the main organ for nucleosome clearance: 71.0% to 84.7% of the nucleosomes are removed from the circulation within 10 min.[106] Kupffer cells in the liver and macrophages in the spleen were shown to be responsible for trapping and clearing DNA and nucleosomes.[113] Studies on extracellular ssDNA removal by the kidneys showed that the clearance rate of naked DNA through glomeruli depended on DNA size. Short fragments (160–200 bp) were present in the kidney even after 24 h, but longer fragments (2–6 kb) were not detected.[103] Those data suggested that the kidney might be selective in DNA clearance, but that study did not completely reflect the situation *in vivo*, because cfDNA is mainly double-stranded, coiled around histones, or bound with other multimolecular complexes. Moreover, experiments in animals have shown that chronic renal failure was associated with low cfDNA uptake and low plasma cfDNA levels, which suggested that the kidney was only partially involved in cfDNA clearance.[103] In addition, the kidney has moderate-to-high deoxyribonuclease activity, and urine has the highest enzyme activity. These properties might explain why urine samples have low DNA concentrations and high DNA fragmentation.[103,114]

Potential biological significance of cfDNA

Extracellular DNA can be considered a "passive", transient passenger or even a waste molecule in body fluids collected in pathological or physiological conditions. However, a plethora of research has supported the notion that cfDNA plays active roles. Indeed, cfDNA is a heterogeneous, complex entity, which includes different types of DNA that can appear in various forms and can be included in multimolecular complexes. The particular subtype and distribution of cfDNA in blood might determine its activity. Here, we will describe the key functions of different cfDNA subtypes as distinct entities, but they are definitely interconnected.

Immunomodulation and tumor-associated inflammation

Molecules released upon cell death or cell damage act as damage-associated molecular patterns (DAMPs), which mediate immunomodulatory effects. Proinflammatory effects can be mediated by the active secretion of DNA (for example, in vital NETosis). This secreted cfDNA is carried in extracellular vesicles in the blood, and leukocytes can take it up into the cytoplasm through endocytosis. Proinflammatory effects can also be induced by intrinsic DNA, which was leaked from the nucleus or mitochondria into the cytoplasm after DNA damage or during alterations in genes that control DNA-damage repair. Strictly speaking, in these cases, the cell's own DNA is not properly considered cfDNA, but its "precursor".

Extracellular histones, nucleosomes, and naked cfDNA differ in terms of cytotoxicity and proinflammatory action.[94] Histones elicit proinflammatory signaling via toll-like receptors (TLR2/4), which results in the production of TNF-α, IL-6, IL-10, and MPO. Histones also exhibit TLR-independent cytotoxicity.[115,116] They can also induce NET formation, which in turn releases more histones.[115] Histones are cytotoxic to the endothelium and can induce macro- and microvascular thrombosis and renal dysfunction.[116] Antibodies to histones mitigated mortality in various mouse models of sepsis.[116] In contrast, nucleosomes stimulate different inflammatory pathways, and they do not have the cytotoxic effects displayed by histones.[94] The rapid elimination of nucleosomes through hepatocytes decreases the likelihood that more harmful nucleosome components will be present in blood.[94] mtDNA, which is similar to bacterial DNA, is recognized by immune cells as a DAMP.[93] However, in contrast to bacterial DNA, mtDNA does not induce IL-6 production.[117–119] Nevertheless, cfDNAs of mitochondrial, nuclear, and bacterial origins have similar procoagulant and platelet-stimulating potentials.[119] These examples highlight observations that the origin and type of cfDNA determine the various types of cellular reactions.

Extracellular mtDNA activates white blood cells (e.g., neutrophils, dendritic cells) via TLR9.[117–120] TLR9 activation and the activation of AIM2 and NLRP3 inflammasomes induce the secretion of proinflammatory cytokines and stimulate an immune interferon response.[93,96] Cytokines secreted as a result of cfDNA-TLR9 signaling are implicated in tumor-associated inflammation. These signals recruit monocytes and induce their transformation into pro-tumorigenic M2 macrophages.[121,122] Thus, cfDNAs can contribute to the rewiring of tumor microenvironments.

When oxidized mtDNAs or damaged nuclear DNAs (i.e., damaged by chemotherapeutics or created in FA/BRCA $^{-}$ or mismatch-repair pathway-deficient cells) leak into the cytosol, they are sensed by the cGAS-STING (stimulator of interferon genes)-IRF3 or the STING–NF-kB pathway.[120,123] In turn, STING induces the production of proinflammatory cytokines and chemokines, which participate in tumor development. DNA located either on the surface or inside vesicles can also initiate activation of the interferon type I (INFI) response through the cGAS-STING pathway. For example, DNA can be transferred from T-lymphocytes to dendritic cells through an immunological synapse.[69] Activation of the INFI pathway through the transfer of mtDNA is part of a mechanism that increases the resistance of dendritic cells to viral infection.[69]

Another effect of STING activation is the upregulation of the immune checkpoint protein, PD-L1, found in tumor cells, in microenvironment cells, and on exosomes.[124–126] DNA repair defects or DNA-damaging therapy can upregulate PD-L1, including the PD-L1 molecules located on exosomes, to induce immunosuppression.[124,127–129] Moreover, changes in extracellular cfDNA levels correlate with the response to anti-PD-1 immunotherapy drugs (Nivolumab or Pembrolizumab), as shown in patients with non-small lung cancer, uveal melanoma, or microsatellite-unstable colorectal cancer.[130–132]

Maintenance of cell homeostasis

One of the functions of active DNA release is the removal of damaged DNA, such as oxidized mtDNA found in vesicles.[69] Damaged DNA is harmful to cells; it is expelled via exosomes to maintain cellular homeostasis and prevent aberrant immune responses.[133] The secretion of damaged mtDNA through exosome biogenesis is necessary to maintain mitochondrial homeostasis and mtDNA metabolism.[69] Cellular DNA is partly degraded in the cytosol by TREX1 (DNase III).[88] However, this DNA might also be excreted through exosomes, although the exact pathway remains unknown. Moreover, the mechanism for secreting normal, undamaged DNA from living cells remains obscure.

Observations in cell lines and affected patients have shown that nucleosome leakage into the cytoplasm can be induced by DNA-damaging agents, such as chemotherapeutics,[125,126] or it can result from inherited DNA-damage repair defects (e.g., BRCA1/2 or ATM deficiency). Studies of ATM-deficient cells showed that predominantly ssDNA was released into the cytoplasm, although dsDNA also contributed to cytosolic DNA.[126] The fate of DNA that is leaked into the cytoplasm remains unclear, but we assume that part of this DNA can be secreted in extracellular vesicles.

Transforming ability and functional modulation of other cells

The ability of cfDNA to transform cells was demonstrated when NIH3T3 murine cells were treated with serum from patients with colon cancer or with the supernatant of SW480 human cancer cells.[134,135] The observation that cfDNA could integrate into the genomes of healthy cells led to the development of the genometastasis hypothesis.[134–138] However, further studies showed that the uptake of vesicles that contain DNA depends on the condition of the cell recipient. For example, transformed cell line variants are more sensitive to exosome-mediated uptake than other cell lines. In addition, serial passages might result in the disappearance of genomic DNA present in vesicles. Thus, this type of "external" DNA might not integrate into the host genome, and its effects on cell behavior might be transient.[139]

In addition to the integration of cfDNA into the genomes of cells, the horizontal transfer of genomic DNA might mediate intercellular communication and influence the functions of affected cells.[140] Moreover, tunneling nanotubes and extracellular vesicles, which are used to transfer mitochondria to neighboring cells, might also be used to transfer mtDNA to other cells.[69,141–143] Intact exogenous mtDNA transferred in this manner was shown to act as an oncogenic signal that induced endocrine therapy resistance in OXPHOS-dependent breast cancers.[92]

Tumor growth and metastatic niche establishment

Apart from their role in antibacterial defense, NETs are found in tumors at sites of neutrophil accumulation. It is thought that these NETs might influence the cancer microenvironment, promote tumor growth, and contribute to the establishment of a pre-metastatic niche.[144] When a contact pathway activates DNA release by NETosis, it induces a strong procoagulant response. This response was observed in patients with breast cancer after chemotherapy.[145] The deposition of NETs in the microvasculature was shown to aid in the trapping and immobilization of tumor cells, protects them physically or by the generation of tumor-promoting thrombi. The combination of vascular dysfunction and NETosis creates convenient conditions for tumor cells to enter into surrounding tissues.[95,146]

NETosis may contribute to metastatic niche development and facilitate metastases, particularly on a background of post-surgical infection in patients with cancer. Among patients undergoing liver resections for metastatic colorectal cancer, surgical stress, accompanied by increased NET formation, was associated with a >4-fold reduction in disease-free survival.[147] In addition, metastatic breast cancer cells can induce neutrophils to form metastasis-supporting NETs, even in the absence of infection.[148] Particular components of NETs, such as MMP9, might also contribute to metastasis formation by supporting extracellular matrix remodeling and an angiogenic switch.[149] Recently, it was shown that NETs, released in response to inflammation, through proteases, MMP9, and neutrophil elastase, might stimulate proliferation of dormant cancer cells.[150]

NETosis can be stimulated by a hypoxic microenvironment and tumor-derived exosomes. Increased plasma levels of G-CSF, IL-8, and TGFβ predispose neutrophils to NET formation in patients with cancer.[146,149,151]

Understanding the role of NETs in tumor progression could suggest potential therapeutic directions. Inhibiting G-CSF and IL-8 production diminished NET-induced vascular dysfunction.[95,97,146] Degradation of NETs with DNase restored vascular function, suppressed inflammation, and reduced tumor cell invasion and metastasis.[148,152] NETosis could be reduced with an antibody block of P-selectin or P-selectin glycoprotein ligand 1 (PSGL-1), which is involved in neutrophil–platelet interactions; with the inhibition of PAD4, which is implicated in histone citrullination (marker of NETosis); or with DNase treatment. These approaches can be considered attractive interventional options for NET-associated pathologies.[97,146,152–154]

It should be noted that, in addition to promoting tumor growth, NETosis also aids neutrophils in killing tumor cells. The exact role of NETs can be defined by the cytokine landscape.[155] Moreover, various therapeutics may stimulate NET production differently: an analysis of estrogen receptor modulators showed that Tamoxifen induced[156] and Raloxifene inhibited NET formation.[157]

Discussion

cfDNA has attracted increasing attention as a promising component of the liquid biopsy. cfDNA carries information about genetic and epigenetic tumor-specific alterations. The presence of cfDNA, its amount, integrity, and the fraction of ctDNA, appear to comprise a marker of tumor sensitivity to treatment and correlate with cancer aggressiveness (Figure 1).

An analysis of data in the literature has revealed many unclear aspects connected to cfDNA biology. The origin of increases in cfDNA observed in patients with cancer has not been sufficiently investigated. A variety of DNA release mechanisms have been described; some are consequences of various cell death pathways; others are variants of active

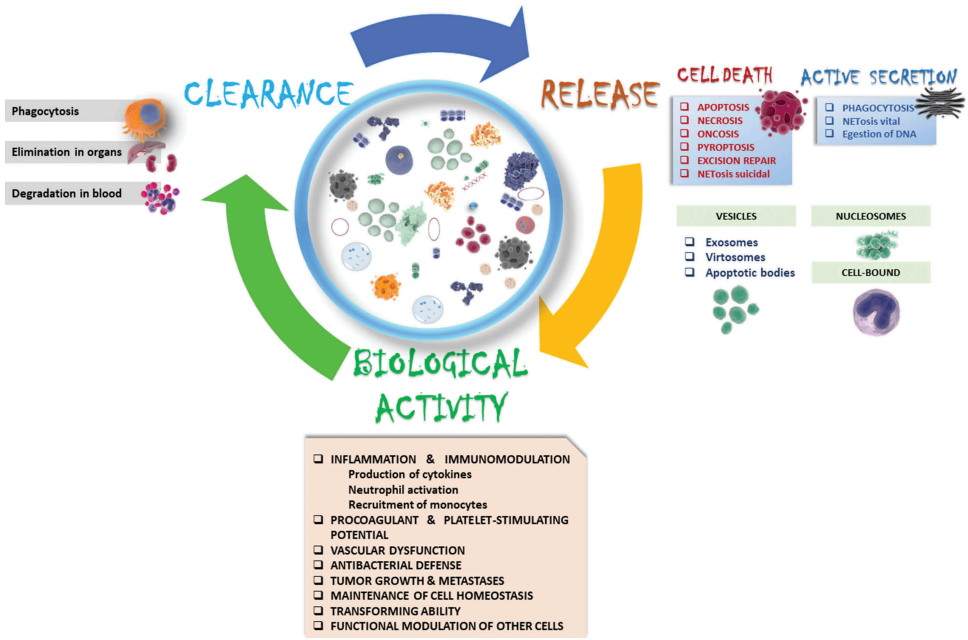

Figure 1. Cell-free circulating DNA life-cycle.

The three main components of cfDNA life are presented: release, biological activity, and clearance. The figure summarizes the well-described mechanisms of each component. Although in most clinical settings naked nuclear cfDNA is analyzed, it travels in various forms in body fluids: free, inside exosomes, bound to histones (nucleosomes), protected by transcriptional factors or are as part of immune-related components (such as NETs).cfDNA is not a passive biomarker of pathophysiological conditions, but plays an active role in multiple processes such as inflammation, immunomodulation, tumor growth promotion, etc..

secretion. Most cfDNA is associated with macromolecules (proteins, lipids, other nucleic acids) or packaged in vesicles. cfDNA size might be indicative of the mechanisms and inducers related to DNA release.

The ctDNA fraction is typically extracted from blood and treated as a single entity. One must remember that cfDNA is a heterogeneous mix, comprised mainly of nuclear and mitochondrial DNA. The content and profile of nucleic acids differ in various compartments; cfDNA might be free, oxidized, damaged or undamaged, present in exosomes, or present in other extracellular vesicles. The patterns of DNA integrity reflect various mechanisms of cell death and tumor interactions with the microenvironment. Therefore, addressing each cfDNA subfraction may potentially be indicative of therapeutic effects, after taking into consideration the contributions from DNA release mechanisms.

Increasing evidence has suggested that cfDNA plays active roles. Nuclear and mitochondrial DNA are actively secreted in response to various external stimuli. In physiological conditions, DNA released into the blood by NETosis participates in antimicrobial immune responses. In pathological conditions, DNA release can lead to sterile inflammation or even promote metastases and the establishment of a metastatic niche. DNA released by NETosis facilitates vascular dysfunction and induces thrombotic complications in patients with cancer.

The secretion or leakage of damaged DNA is a homeostatic mechanism that responds to DNA-damaging agents, mutations in DNA-damage repair pathways, or mitochondrial oxidation. This DNA is sensed as a DAMP, and it plays a role in antibacterial or antiviral innate immune reactions. In the context of tumor cells with DNA-damage repair mutations, these DAMPs sustain tumor-associated inflammation and support the reprogramming of tumor infiltrating cells.

Some fascinating studies showed that cfDNA played a role in the functional rewiring of cells, and they demonstrated that cfDNA had the ability to transform tumor DNA.

In conclusion, despite the increasing number of studies on the clinical utility of cfDNA, little is known about the biology of cfDNA. Future studies on the mechanisms of cfDNA release and clearance might shed light on tumor biology and aid the development of more sophisticated assays for cfDNA in the field of clinical oncology. Studies that investigate cfDNAs will provide important insights into intracellular communication, cell turnover, body defense mechanisms, tumor growth promotion, and metastatic niche formation.

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