Circulating DNA

Its Origin and Fluctuation

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Circulating DNA is present in the blood of all individuals, but it has been found that cancer patients and patients with a variety of other conditions have increased amounts of these circulating DNA fragments in their blood. Even though more than 30 years of research have been done on this subject, the origin of these nucleic acid molecules is still not clear. We present evidence that does not support the general notion that apoptosis or necrosis is the major source of circulating free DNA. Active release of free circulating DNA by living cells may be a plausible mechanism. Disturbance of the equilibrium between the release of DNA by living cells and the mechanisms used for clearing this DNA may play the main role in the appearance of increased amounts of circulating DNA in the blood of individuals with different ailments. Elucidating the origin and the mechanism that cells use to release free circulating DNA into the blood may enhance the diagnostic and prognostic value of these nucleic acid molecules.

Key words: **apoptosis; active release; free circulating DNA; blood plasma**

Introduction

It is widely known that higher concentrations of free circulating DNA can be found, in most cases, in the blood of patients with malignant diseases compared to healthy subjects.[1] Several studies have been performed to establish whether a significant diagnostic and/or prognostic[1] use could be found for circulating free DNA, both in quantity and quality, because of the noninvasive nature in which it can be obtained. Although much work has been done to determine the mechanism whereby these circulating DNA fragments are released into the blood, a definite conclusion could not be reached.

This chapter will address some aspects of the origin of circulating DNA in normal and in pathologic conditions such as cancer, trauma, stroke, pregnancy, autoimmune disorders, and after solid organ transplant in an effort to shed some light on the origin of this DNA. The putative role of apoptosis and necrosis in their origin will be emphasized, and the release of DNA by living cells as well as clearance of these molecules will be highlighted.

Characteristics and Occurrence of Circulating DNA

The double-stranded nature of free circulating DNA was shown as early as 1975 with hydroxyapatite chromatography and with density gradient centrifugation.[2–4] It was also in these early days that the low molecular weight and ladder pattern of circulating DNA was revealed by agarose gel electrophoresis.[4] If this electrophoretic ladder pattern is used as an indication of the size distribution of circulating DNA, it can be accepted that it is in the range of 180 to 10,000 bp.[5] Furthermore, it is possible that the ends of these molecules are capped. Free circulating DNA may also be

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present in the form of nucleosomes or apoptotic bodies.[6,7]

The amount and composition of circulating DNA varies between patients,[5] and many reports agree[8] that cancer patients have much larger amounts of circulating DNA in their blood than do healthy subjects. Early analysis could not detect any DNA in the serum of healthy individuals.[4,9] However, circulating DNA is not confined to serum or plasma of cancer patients, since elevated amounts of circulating free DNA can also be detected in patients with other pathologic conditions such as systemic lupus erythematosus (SLE), rheumatoid arthritis, glomerulonephritis, pancreatitis, cholelithiasis, inflammatory bowel disease, peptic ulcer disease, hepatitis, esophagitis, pulmonary embolism, ulcerative colitis, and miliary tuberculosis (for review see Anker *et al*.[10] and Ziegler *et al*.[6]), which are associated with inflammatory processes. Other cases that involve increased cell death include trauma, stroke, myocardial infarction, angina,[11] sepsis, and septic shock.[12] Furthermore, overtrained athletes[13] also show increased amounts of circulating DNA, whereas fetal DNA can be detected during pregnancy.[14] Can the fact that circulating DNA occurs in increased amounts in so many different conditions point to some correlation between them that may reveal a common mechanism of release or origin?

Many papers report that oncogene mutations and amplifications, microsatellite alterations, and epigenetic changes like DNA methylation (see Refs. 1, 6, 15, and 16 for review) can be found in the circulating DNA similar to that found in tumor tissue of cancer patients. These resemblances suggest that the circulating DNA is most probably derived from the primary tumor or from mature tumor cells.[3,10,17,18] A direct relationship could not be demonstrated between the amount of plasma DNA and the type or clinical status of the cancer.[5]

Two main points of view exist in the literature for explaining the origin of circulating free DNA, that is, these DNA fragments enter the bloodstream following cell death or they are actively released by living cells.[1,4–6,12,19–23]

Apoptosis and Necrosis: DNA Release after Cell Death

Apoptosis and necrosis are two distinct mechanisms of cell death and represent two extremes of this phenomenon.[24] During apoptosis, DNA degradation often occurs: chromosomal DNA is first cleaved into large fragments of 50–300 kb and subsequently into multiples of nucleosomal units of 180–200 bp, which is a hallmark of apoptosis.[25–27] Because this ladder pattern is also visible after electrophoresis of circulating DNA, many believe that apoptosis may be the source of the observed DNA fragments in the plasma.[5,22,28] Although the mechanisms are not fully understood, the contents of cells dying by apoptosis are rapidly ingested by professional phagocytes (macrophages and dendritic cells) or neighboring cells,[29] and the DNA is consequently completely digested into nucleotides by DNase II in lysosomes.[25,27] Thus the possibility exists that DNA fragments released by apoptosis are completely removed before they can appear in the circulation.[5,22] If this engulfment of apoptotic bodies is impaired or cell death is amplified, tissue injury or autoimmunity will most probably result.[22,29,30]

Macrophages can be implicated in the generation of circulating DNA as described below. After massive macrophage apoptosis induced by clodronate liposome treatment in mice, a dramatic increase of circulating DNA occurred. The intraperitoneal administration of dead, apoptotic or necrotic, Jurkat cells into mice lacking macrophages caused no further increase in the amount of circulating DNA.[22] However, the administration of the same dead cells into normal mice caused an increase in circulating DNA in the blood of the mice and the characteristic ladder pattern could be observed after electrophoresis for both necrotic and apoptotic cells, respectively. This may indicate that necrotic human cells are engulfed by

mouse macrophages or that DNA from necrotic cells is cleaved by the same enzymes functioning in apoptosis ,thus causing the same ladder pattern as apoptotic cells.[22] PCR analysis of circulating DNA in the blood of these mice showed the presence of both human and murine sequences.[22] It is possible that macrophage apoptosis is caused by engulfment of high numbers of dead cells or by impaired phagocytic function,[22] thus causing release of murine circulating DNA into the blood of these mice. It would have been interesting to know what the levels of circulating murine DNA were before administration of the dead human cells, and if this level stayed the same or increased after macrophage engulfment of the dead cells.

Most proliferating cells lost the ability to become apoptotic,[10] and several ingenious mechanisms have been identified in which cancer cells become resistant to apoptosis[31] in order to escape the immune system.[32] Various targets for therapeutic intervention in cancer have been explored and many of them are thought to proceed via the preferential induction of apoptosis to eliminate cancer cells without affecting normal cells (for review, see Bremer *et al*.[32]).

In contrast to apoptosis, necrosis causes random, nonspecific, and incomplete digestion of DNA and thus a smear is observed in an electrophoresis gel.[21,26,33] By inducing necrosis in cell cultures, Jahr *et al*.[5] demonstrated that necrotic cells produce DNA fragments larger than ~10,000 bp.

If lysis of circulating cells was to be the origin of circulating DNA, many more dying circulating cells should have been present in the blood since the amount of DNA in the plasma undoubtedly exceeds the amount of such circulating cells,[1,10,20] indicating that circulating DNA does not originate from circulating cells dying in the blood. Lysis of T lymphocytes was also examined, but it was shown that T lymphocytes are not the source of circulating DNA. Jahr *et al*. also tested the possibility that normal circulating DNA might originate from endothelial cells by using the methylation status of the endothelium-specific human gene SELE promoter, which is unmethylated in endothelial cells and hypermethylated in other cells, and they found that only a small contribution, if any, is made by endothelial cells of cancer patients.[5]

DNA Release by Living Cells

The possibility that DNA may be released by living cells was suggested by a number of reports,[1,10,12,20,23] but convincing evidence does not exist to prove this hypothesis. It is quite astounding that even though Anker *et al*.[2] realized more than 30 years ago that DNA can be actively released by cells, the mechanism of this active release process is still not elucidated.

Four lines of evidence to support the hypothesis that living cells release DNA were highlighted by Chen *et al*.[23]: (*a*) Instead of increased circulating DNA, which is expected if apoptosis is the mechanism of release, Leon *et al*.[9] found circulating DNA to be significantly decreased in response to radiotherapy. This may be because of the inhibitory effect of radiation on the proliferation of the cancer cells and thus less DNA is released. (*b*) Even with no cells dying in culture, DNA is still observed in the supernatant, and the concentration increases proportional to the proliferation of cancer cells; this unpublished observation of Chen *et al*.[23] agrees with Anker *et al*.,[2] who observed in 1975 that human blood lymphocytes actively release double-stranded DNA into their culture medium until a certain concentration is reached, no matter how long the incubation lasts, and that newly synthesized DNA is released preferentially. Work by Anker *et al*.[34] on frog auricles corroborated this observation. Anker also stated that the quantities of DNA that were released were similar, irrespective of whether a quarter of the cells or none at all die, except for cancer cells, which can release more DNA than normal cells.[10] This shows that cell death is not responsible for the free DNA observed in the plasma. Furthermore, Stroun *et al*.[20] showed that the characteristic ladder pattern on an electrophoresis

gel can also be observed for actively released DNA. (*c*) In the early stages of cancer, when seemingly little cell death is occurring, circulating DNA may already be present in higher than normal levels. As the cancer burden increases, so does cell death; however, the amount of proliferating cancer cells and thus the DNA levels increased significantly because of the increased amount of proliferating cells and not because of the amount of cells that die. (*d*) Lymphocytes are not the only cells that spontaneously release DNA into culture media when stimulated; release may also occur during division of other cell types, which include normal and malignant cells in the body. Additional observations from work on frog auricles are that the transfer of auricles to a new medium caused renewed release to the same concentration as in the previous medium, purified frog DNA did not inhibit release, and damaged auricles did not yield more free DNA.[34] Further evidence for preferential release of DNA by viable cells is given by Stroun *et al*.,[35] when they compared the proportion of *Alu* repeat sequences to the *ß-globin* gene in serum and lymphocytes.

Additional Sources of Circulating DNA

On the basis of the observations made by Raptis *et al*.[36] in the early 1980s, an exogenous source of free circulating DNA was excluded. However, these observations were shown to be wrong by the presence of viral DNA circulating in the plasma of some patients with cancer associated with viral infection, such as nasopharyngeal carcinoma, where Epstein–Barr virus DNA can be detected in 96% of cases, cervical cancer, where human papilloma virus DNA can be detected in 50% of cases, and hepatocellular carcinoma, where hepatitis B virus DNA can be detected.[37,38]

Cells that have lost their nuclei but remain functional undergo a process termed denucleation or terminal differentiation. According to Bischoff *et al*.,[7] this may be another source of circulating DNA, but many tumors do not express enough of the molecular or morphologic markers of the terminally differentiated state[39] to prove the existence of this phenomenon in cancer. Thus terminal differentiation is unlikely to provide a significant contribution to the origin of circulating DNA.

Clearance of Circulating DNA

More than one mechanism may be responsible for the clearance of free DNA from plasma since Lo *et al*.[40] observed that the clearance of fetal DNA after delivery occurs in different phases. First an initial rapid phase is observed, which is followed by a slower second phase. In most of the women in their study, all DNA was cleared 2 hours after delivery.

Regardless of the rapid clearance rate, with a mean half-life estimated to be at only 16.3 minutes,[41] it is known that fetal DNA is present in large amounts in the maternal circulation during pregnancy. This means that fetal DNA must be released in large quantities to maintain the high concentration which is continuously detectable in the maternal circulation during pregnancy. Thus, the rate at which fetal DNA is released exceeds its clearance rate. The concentration of fetal DNA consequently provides an almost real-time picture of the interaction between DNA release and DNA clearance.[40] Free circulating DNAs found in healthy people, cancer patients, and organ transplant recipients most likely have the same clearance mechanism(s) as in maternal plasma. Therefore DNA will also be rapidly removed and display a real-time picture of release, which may be useful in monitoring disease and transplant efficiency.[40]

Although the mechanism of clearance has not been elucidated, a few possibilities can be proposed. Many reports state that free DNA can be detected in urine,[18,41] and thus it can be expected that the kidneys may play a role in clearance. Animal studies also suggested that the liver, spleen, and kidneys may be responsible for removal of circulating DNA.[40]

Lo *et al.*[40] explored the possibility that plasma nucleases may have a function, but showed that these nucleases only have a partial role in the removal of circulating fetal DNA. Tamkovich *et al.*[42] showed that neutral deoxyribonuclease I activity is inhibited in the blood of cancer patients, thus causing a higher concentration of circulating DNA, while DNase I activity could be detected in healthy patients. DNase I activity, then, may play a significant role in the clearance of circulating DNA.

Chelobanov *et al.*[43] summarized the available data on various DNA binding proteins which were detected on the cell surface of many different cells and cell lines. It was suggested that these DNA binding proteins recognize and transport DNA across the plasma membrane into the cell for possible degradation to mononucleotides or transportation into the nucleus. Binding of DNA to the cell-surface receptors is pH- and temperature-dependent and can be inhibited by a number of substances. It was found that serum of SLE patients competitively inhibits binding of DNA, which may be the result of an increased amount of circulating DNA in SLE serum. One study observed between 810 and 2600 molecules of bound DNA per cell and another study observed expression of DNA receptors by 67% of lymphocytes and 98% of monocytes.[43] Thus, depending on the rate of uptake by these cells and the amount of DNA bound to receptors, cells with surface receptors for DNA may contribute and possibly play a major role in the clearance of free circulating DNA.

The mechanism(s) whereby clearance of circulating DNA is achieved is currently still poorly understood,[44] but binding and uptake by different cells as well as DNase activity in the blood may be the main mechanisms of clearance.

Other Conditions That May Give Rise to Circulating DNA

The concentration of circulating DNA in the plasma of patients after undergoing hemodialysis was shown to be significantly higher than before undergoing hemodialysis or compared to controls, and the typical DNA ladder pattern associated with apoptosis was observed in agarose electrophoresis for the circulating DNA isolated from patients after undergoing hemodialysis.[28]

Patients with untreated active systemic lupus erythematosus have much higher concentrations of circulating DNA in their plasma than do healthy individuals, but this decreases to normal levels after treatment.[36] Organ rejection also caused an increase in the amount of circulating DNA, in the urine in this case, and after treatment the amount of free DNA rapidly decreased.[41] When comparing plasma DNA concentrations in healthy individuals to those in patients having received bone marrow transplants, no significant difference could be found, but when the total circulating DNA was split into two factions—originating either from the bone marrow or from the rest of the body—it was found that a significantly higher concentration originated from the bone marrow.[45]

Chang *et al.*[46] observed a 10-fold increase relative to controls in the amount of circulating DNA in patients suffering from myocardial infarction. They attribute this to widespread apoptosis followed by necrosis in the infarct. Comparison of the electrophoretic pattern of circulating DNA from healthy individuals, cancer patients, and patients who suffered a myocardial infarction showed a more diffused ladder pattern for the latter.[46] However, it is possible that this may be an artifact of the study, as all the samples were not analyzed on the same gel.

The presence of fetal DNA in the maternal circulation was demonstrated in 1997 by Lo *et al.*[40] Furthermore, it was found that fetal DNA increases with gestational age, and a sharp increase can be observed during the last 8 weeks of pregnancy.[14,47] In case of complications during pregnancy, such as pre-eclampsia, even more fetal DNA is present, which may be because of impaired clearance or some form of cell injury or placental breakdown.[7] An

interesting observation is that circulating DNA in pregnant women has a much wider size distribution than circulating DNA in nonpregnant women.[48] According to Bianchi *et al*.,[47] the majority of fetal DNA during pregnancy originates from the placenta. Free DNA in the fetus may also originate in the same manner as in normal individuals, and this fetal free DNA will cross the placenta to be released in the maternal blood, causing the maternal blood to contain both maternal free DNA as well as fetal free DNA.

It has been shown that exercise overtraining can cause increased amounts of plasma DNA, which can be related to the training load; it can increase 9- to 17.5-fold after long distance running and remain increased even after 96 hours.[13] An increase in oxidative stress was also observed after exercise overtraining.[13] Can the exercise-induced increase in DNA-damaging reactive oxygen species (ROS) be implicated in the production of circulating DNA?

A highly significant difference between the concentration of plasma circulating DNA was found between healthy individuals and those with minor or moderate trauma and major trauma early after injury, and it was found that patients with adverse outcomes, including death, had much higher plasma DNA concentrations than did those who did not develop complications, showing that DNA may be a valuable prognostic marker in trauma patients.[44]

Circulating plasma DNA concentrations were shown to be increased in the first 24 hours after acute stroke, and the amount measured in patients within the first 3 hours after the event was 5-fold higher in those who died than in those who survived. In general it appears that higher circulating DNA concentrations are present in patients with more dramatic clinical presentations, suggesting that it may be a useful indicator for predicting disability and mortality in stroke patients.[49] Although the origin of the circulating DNA in stroke is unknown, ROS are also produced[49] and may be involved in the generation of free circulating DNA, as alluded to above.

Patients with severe sepsis or septic shock had significantly higher circulating plasma DNA concentrations than in normal controls, and even higher concentrations were found in those who did not survive intensive care and those who needed renal or inotropic support within the first 24 hours. When the concentration of circulating DNA was used as a predictor of intensive care survival, a sensitivity of 92% and specificity of 80% was observed, again suggesting that it can be used as a prognostic marker of mortality and sepsis in intensive care patients.[12]

Conclusions

Almost every paper on circulating DNA states that apoptosis and/or necrosis is the source of free circulating DNA in serum and plasma. Many investigators use the fact that a ladder pattern is evident after electrophoresis of free circulating DNA as proof that apoptosis is the source of these fragments. However, this ladder pattern can also be found for the DNA in the culture medium in which lymphocytes grow. Furthermore apoptotic cells are ingested by macrophages and their DNA is digested into nucleotides. If macrophage ingestion fails on a scale large enough to produce the amount of circulating DNA in the blood, inflammation would definitely be a problem, and autoimmunity would occur frequently in cancer and the other conditions mentioned. The fact that many cancer cells are resistant to apoptosis argues against the notion of it as a mechanism for generating circulating DNA. Radiotherapy or irradiation, chemotherapy, and other cancer treatments cause cell death by apoptosis,[50] and the amount of circulating DNA is less in cancer patients under treatment than it is in those patients before treatment, also disproving apoptosis as a source of circulating DNA. Necrosis, on the other hand, produces large DNA fragments, and the ensuing inflammation would

also be a problem if this were to be a source of large amounts of circulating DNA. Thus, we conclude that apoptosis and necrosis are not the main source of circulating DNA in the blood, although it may play a contributing role.

The possibility that circulating DNA may be liberated by living cells was already observed in the late 1970s,[9] and evidence that DNA is released *in vitro* by human blood lymphocytes was presented in the mid 1970s.[2] To our knowledge it has not been proven that DNA can be released into the circulatory system *in vivo* by living cells, but we do not anticipate a reason why it is not a possibility. Additionally, even though the mechanism by which clearance of DNA from plasma is achieved is poorly understood, and only a few papers address this issue, the appearance of increasing amounts of circulating DNA in the blood may be because the equilibrium between the release of DNA by living cells and the clearance of DNA is disturbed by an adverse condition. The low concentration of circulating DNA in the blood of normal individuals may thus be due to a lower rate of DNA release by cells, or a rapid removal of DNA by the optimal functioning of clearance mechanisms, and as soon as this equilibrium is disturbed, an increased amount of circulating DNA can be observed in the blood of an individual.

Circulating DNA can be found in a variety of conditions and, even though these conditions are unrelated, the presence of circulating nucleic acids is a common feature and thus some kind of correlation ought to be found that may point to a similar mechanism of origin. Although researchers have been looking for the origin of circulating DNA for more than 30 years, and quite a few possibilities have been explored, the mechanism of release still has to be elucidated. The possibility that more than one mechanism may be involved is feasible, but the factors influencing their relative contribution and the interaction between the mechanisms need to be understood for optimal utilization of this very valuable, noninvasive prediction and prognostic marker.

More work needs to be done to determine the mechanism of clearance and the mechanism cells use to release DNA, as well as to understand the significance of and the effect that these circulating DNA fragments have in the body. In-depth comparative characterization of circulating DNA obtained from patients with various pathologic conditions or compromised cells may also be useful in elucidating the origin of these molecules.

Conflicts of Interest

The authors declare no conflicts of interest.

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