Circulating Nucleosomes in Serum

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ABSTRACT: In the nucleus of eukaryotic cells, DNA is associated with several protein components and forms complexes known as nucleosomes. During cell death, particularly during apoptosis, endonucleases are activated that cleave the chromatin into multiple oligo- and mononucleosomes. Subsequently, these nucleosomes are packed into apoptotic bodies and are engulfed by macrophages or neighboring cells. In cases of high rates of cellular turnover and cell death, they also are released into the circulation and can be detected in serum or plasma. As enhanced cell death occurs under various pathologic conditions, elevated amounts of circulating nucleosomes are not specific for any benign or malignant disorder. However, the course of change in the nucleosomal levels in circulation of patients with malignant tumors during chemotherapy or radiotherapy is associated with the clinical outcome and can be useful for the therapeutic monitoring and the prediction of the therapeutic efficacy.

KEYWORDS: Nucleosomes; Serum; Cancer; Chemotherapy; Dose escalation radiotherapy

STRUCTURE AND FUNCTION OF NUCLEOSOMES

In the nucleus of eukaryotic cells, DNA is associated with several protein components and forms complexes known as nucleosomes. The core particle of those nucleosomes consists of an octamer of the double-represented histone aggregates, H2A-H2B and H3-H4. They are surrounded by 146 base pairs of double-stranded DNA, which are fixed at 14 sites to the surface of this complex. The so-called linker DNA, which varies between 10 and 100 base pairs, connects neighboring nucleosomes to a chainlike pattern. A further histone, H1, is located outside of the nucleosomes and stabilizes the chromatin chain in its tertiary structure.[1–3]

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The arrangement in multinucleosomal order has various advantages. Sequences of DNA that encode important information are located next to the histones and are protected against attacks of proteases and nucleases.[3] In order to enable transcription processes, DNA can be liberated from its close connection to the histones by disrupting the contacts, transferring the histone octamer to another DNA molecule, or sliding the core particle along the DNA.[3] The accessibility of the DNA is regulated by modifications of the histones—for example, by acetyl groups. Histone acetylation and decondensation of the chromatin promote the transcription process, whereas deacetylation and condensation suppress it.[3–5]

NUCLEOSOMES AND CELL DEATH

During cell death, particularly during apoptosis, a set of caspases is activated that leads to the active degradation of cellular constituents.[6,7] Endonucleases [the most popular among them is the caspase-activated deoxyribonuclease (CAD)] bind preferentially to the easily accessible DNA linking sites and cleave the chromatin into multiple oligo- and mononucleosomes.[7] Like other constituents, they are packed into membrane-bound vesicles that are shed from the cellular surface and are engulfed by neighboring cells and macrophages.[8–10] In organisms with enhanced cellular turnover and cell death, such as in cancer or acute infection, as well as after therapeutic induction of extended cell death, such as during radio- or chemotherapy, this mechanism seems to be overloaded. Consequently, nucleosomes are also released into the circulation and can be detected in elevated amounts in serum or plasma.[11–13]

Nucleosome-like DNA fragments can also result from other types of cell death, for example, from oncosis. During this degradation process, the chromatin is mainly cleaved unspecifically by various proteases and leaves the cell in a more disorganized way because of the early rupture of the plasma membrane.[10,14] Nowadays, many types of cell death are known, ranging on a scale from apoptosis on the one end and oncosis on the other end.[15,16] By which way a cell dies depends on many circumstances, like the cell type, the severity of the lesion, the ATP level in the cell, etc.[14,16,17] One can only speculate about the modalities of cell death that result in the release of circulating DNA or nucleosomes, although recently it was reported that they can be discriminated by the determination of DNA size distribution.[18] However, the demonstration of various types of circulating nucleic acids,[19–23] which sometimes exhibit cancerous characteristics[24–31] and correlate with various pathologic conditions,[20–38] therapeutic success[33,34,37] and prognosis,[31,36,38,39] can reveal their enormous diagnostic, monitoring, and prognostic potential.

NUCLEOSOMES IN THE CIRCULATION

Cell-free DNA can circulate in various forms in blood: as naked DNA, associated with histones in nucleosomes, bound to other plasma proteins, or packed in apoptotic bodies. The exact contribution of each form may vary inter- and intraindividually. Experiments in plasma of patients with systemic lupus erythematosus (SLE) revealed that the main part of the circulating DNA is organized in multimeric complexes as mono- and oligonucleosomes.[40,41]

These nucleosomes have an extremely short half-life in the circulation. Hemodialysis experiments have shown that half of the injected mononucleosomes are removed after only 4 minutes.[42] Oligonucleosomes are degraded effectively by endonucleases that are present in the blood[43] and are metabolized in the liver or alternatively eliminated by macrophages or immune cells.[18,32,42,44] Histones are reported to be directly ingested by liver cells, whereas DNA can also be degraded on the surface of Kupffer cells.[42] It remains to be clarified as to whether the nucleosomes are taken up completely by liver cells or are separated before further digestion.

As all of these elimination mechanisms have saturable capacity, the half-life of nucleosomes in serum or plasma increases in a concentration-dependent manner and thus is prolonged under pathologic conditions.[42,45,46] Whereas the activity of endonucleases in the circulation is often reduced in malignant diseases,[11] the association of the C-reactive protein, which is elevated, for example, during infectious diseases, to the histone component can delay the elimination of the nucleosomes.[45,46]

IMPORTANCE OF CIRCULATING NUCLEOSOMES

The role played by nucleosomes in the pathogenesis of diseases is only partially known. In autoimmune diseases like SLE, circulating nucleosomes might be one of the elements that initiate and maintain the upregulation of the immune system. This observation is supported by the fact that antinucleosome antibodies were reported to precede the appearance of anti-DNA antibodies in the circulation.[47] In malignant diseases, the presence of large amounts of circulating nucleosomes might be a part of the tumor counterattack that overwhelms the immune system.[48] On the other hand, others speculate on the function of cancerous nucleosomes in the circulation for the development of metastases.[49]

DETECTION OF NUCLEOSOMES

At present, various assays that use different antibodies against DNA-histone complexes[50,51] are available for the quantification of nucleosomes. The Cell Death Detection ELISA^{Plus} from Roche Diagnostics is a sandwich immunoassay that is based on two monoclonal mouse antibodies against DNA and histones. This test was used by several groups for the quantification of nucleosomes in tissue of mouse models,[52,53] as well as in plasma from patients with breast cancer.[38]

Because of some shortcomings of the original version of the assay, it was modified and standardized by our group so that the assay is more applicable to liquid materials, with reproducible results.[54] As a crucial step, we identified the preanalytic handling of the blood samples, which could influence the test results considerably. Due to the presence of endonucleases in serum and plasma, the preanalytic procedure was standardized and the nucleosomes were stabilized against further *in vitro* degradation. Facing the probable automation of the assay in the future and a sufficient stability of nucleosomes in serum for at least 6 months, serum was the chosen matrix for the determination of circulating nucleosomes.[54]

Elevated levels of DNA in the circulation have been reported to occur under various conditions: in patients with various tumors, autoimmune diseases, and other

benign disorders.[34–37] However, because of its lack of specificity, the determination of DNA was abandoned until the discovery of cancer-specific characteristics.[24–26] Since then, the spotlight has been particularly focused on the qualitative aspects of circulating DNA, such as microsatellite instability, loss of heterozygosity, promoter hypermethylation, and mutations of the ras gene.[28–31]

However, despite its obvious nonspecific nature, the presence of nucleosomes in serum and plasma might yield important information for the diagnosis and differential diagnosis, the therapeutic monitoring, and the prediction of the therapeutic efficacy and prognosis in patients with various pathologic conditions.

SPONTANEOUS OCCURRENCE OF NUCLEOSOMES IN SERUM

High levels of spontaneous release of circulating nucleosomes are expected in situations of enhanced cellular turnover and cell death. In order to estimate the value of the amount of nucleosomes for diagnosis of various diseases, we investigated sera of 590 persons (63 healthy persons, 109 patients with acute benign diseases, and 418 patients with various malignant tumors) before the start of therapy.[55]

The group of healthy persons had homogeneously low values that were significantly different from those of the group of patients with benign diseases as well as those with malignant tumors, both of whom exhibited a broad range of values. However, the difference between the concentration of nucleosomes in patients with malignant tumors and patients with benign diseases as the relevant control group was not significant.[55]

One reason for the high levels of circulating nucleosomes in patients with benign diseases was acute inflammation, which corresponded with stimulation of the immune system, high rates of cell death, and reduced removal of the nucleosomes from the circulation.

Within the group of patients with malignant tumors, the pattern of the values was very similar for most of the tumor types investigated. Only two entities were different. First, patients with cancer of the prostate showed only low values in a similar range to those of healthy persons and, second, patients with lung cancer had markedly higher values.[55]

In terms of the tumor stage, in many tumors we noted a tendency for the association of higher concentrations of nucleosomes with advanced tumor stages. In patients with gastrointestinal tumors, the difference between the later stages and early ones was highly significant (FIG. 1). However, in other tumor entities, (e.g., breast cancer), the levels of nucleosomes did not bear any relationship to the tumor stage.

These observations led to the conclusion that the concentration of circulating nucleosomes in serum was not useful for the primary diagnosis and differential diagnosis of patients with malignant tumors.[55]

THERAPY-INDUCED OCCURRENCE OF NUCLEOSOMES IN SERUM

Many antitumor therapeutic strategies have been developed with the aim to effectively destroy and eliminate the rapidly proliferating tumor cells.[56–59] However, the extensive cell damage and death caused by, for instance, chemo- and radiotherapy is

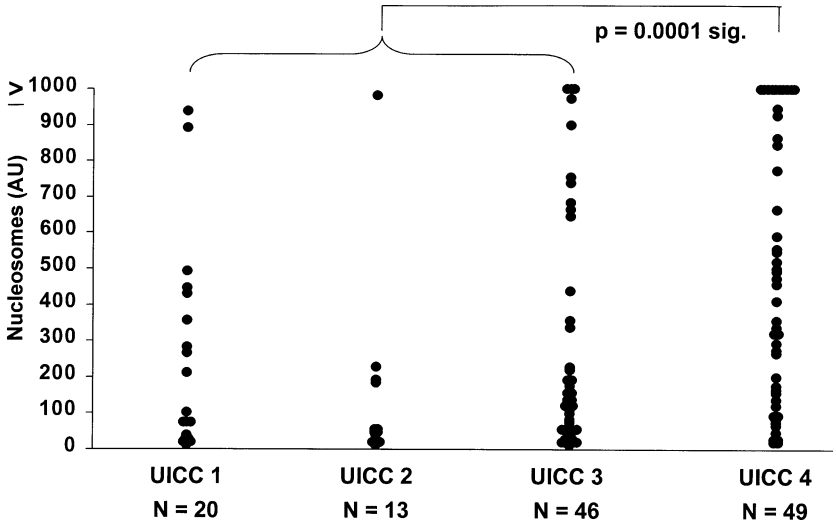

FIGURE 1. Spontaneous concentrations of circulating nucleosomes in arbitrary units (AU) according to the tumor stage in patients with gastrointestinal tumors (n = 128).

not limited only to tumorous tissue, but also affects normal tissues with highly regenerative properties, such as the gastrointestinal mucosa or lymphocytes,[60] and may lead to elevated amounts of nucleosomes in circulation. Despite the nonspecific origin of the circulating nucleosomes, we investigated the course of change during chemo- and radiotherapy, with the aim of determining whether the concentration of nucleosomes could be useful for therapeutic monitoring and whether it correlates with the clinical outcome.[55]

Chemotherapy

During chemotherapy, we followed the courses of 42 patients (with colorectal cancer, lung cancer, and lymphoma), who received primary, adjuvant, or second-line therapy, for a period of 3 to 12 months. As chemotherapy was applied in cycles, we expected the release of a considerable amount of nucleosomes during therapy, resulting in elevated levels in serum, with subsequent decrease in the treatment-free interval. In most patients, this hypothesis was confirmed. The concentration of nucleosomes increased rapidly 1 to 3 days after the start of the therapy and declined in the treatment-free period. The subsequent cycles of chemotherapy produced similar peaks that started from different levels. As these baseline values (which were determined before each new cycle) reflected the spontaneous occurrence of nucleosomes and possibly corresponded to tumor mass or activity, we took these levels into consideration for the estimation of the clinical outcome. In 23 of 25 patients with remission of disease according to the UICC criteria, we observed a decrease for more than 50% of the baseline values. A typical course of a patient with complete remission after chemotherapy for Hodgkin's disease is illustrated in FIGURE 2. In

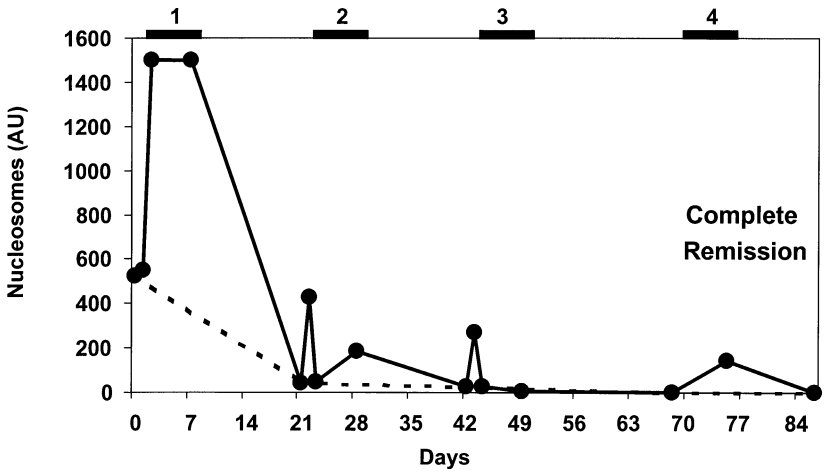

FIGURE 2. Course of circulating nucleosomes in a patient with Hodgkin's disease IIa who received 4 cycles of chemotherapy according to the BEACOPP regimen. The values increased during the cycles and decreased in the treatment-free interval. The baseline values decreased constantly and completely, corresponding with the high efficacy of the therapy.

contrast, 8 of 14 patients with progression of disease showed an increase by more than 50%; 4 patients with progressive disease had strongly alternating values. TABLE 1 demonstrates the changes of the baseline levels of circulating nucleosomes in relation to the clinical efficacy of chemotherapy.

Early Estimation of the Therapeutic Efficacy

Due to the rapid increase in the concentration of nucleosomes during therapy and their short half-life in the circulation, we proposed that it could be a promising marker for the early prediction of the therapeutic efficacy. Such information would be valuable for the physician to adapt the regimen, early in the course of treatment, on an individual basis or to employ other therapeutic options. We further investigated this by following the course of change in concentration of nucleosomes in sera of patients with advanced lung cancer, during the first cycle of chemotherapy. These patients exhibited the highest initial values in our previous studies and would particularly benefit from the early adjustment of therapy because of the poor clinical prognosis and the limited efficacy of the therapeutic possibilities.

Blood was collected several times a week in order to monitor the immediate biochemical response to therapy. Staging investigations including CT were performed after every 2 cycles. Preliminary results indicate that patients with partial remission after the first staging examination showed lower values during the first week than patients with static or progressive disease. This means that higher peaks and insufficient decrease in the nucleosome levels would correspond to a poor response to therapy, possibly due to highly aggressive tumors and less effective elimination of nucleosomes from the circulation. On the other hand, lower levels of nucleosomes in serum before and during therapy were associated with better response of possibly

TABLE 1. Correlation of the baseline values of circulating nucleosomes and the clinical outcome of patients during chemotherapy

Baseline levels of circulating nucleosomes	*n*	Remission (*n* = 25)	Progression (*n* = 14)	No change (*n* = 3)
Decrease > 50%	26	23	1	2
Increase > 50%	8	0	8	0
No change	4	2	1	1
Alternating	4	0	4	0

less aggressive tumors and with a well-functioning system for the removal of nucleosomes.

Radiotherapy

During radiotherapy, we followed the courses of treatment of 16 patients (with lung cancer, head and neck cancer, lymphoma, and colorectal cancer). As radiotherapy was applied daily, 5 times a week over 4 to 6 weeks, we expected a different pattern of nucleosomal release with an initial increase that would be followed shortly by a decrease in the values, if tumor and other radiosensitive cells were eliminated effectively by therapy. However, if high numbers of those cells remained or nucleosomes were removed ineffectively from the circulation, we would expect a constant high level during therapy.

We observed that, in most patients, there was a very fast increase within 6 to 24 hours after the first fraction of radiotherapy. In 9 of 10 patients with remission of disease, the values declined early within 1 day after the peak and reached minimum values in the range of healthy persons during or after the therapy. In contrast, 4 of 5 patients with progression showed a delayed start of decrease and higher minimum values.[55]

For the interpretation of the values, severe inflammatory complications had to be excluded as they can produce considerable temporary elevations of the nucleosomal level in serum.

Patients receiving radiotherapy could benefit particularly from the early estimation of the therapeutic efficacy. Very often, the radiation dose applied is an average of the dosage that is effective for most patients. Consequently, some patients with radiosensitive tumor may unnecessarily receive a dangerously high dosage that can lead to severe side effects, whereas more radioresistant patients are treated suboptimally.[61] For the estimation of the individual response to irradiation, physicians have only a few options, like imaging techniques, which only show late and macroscopic changes. In contrast, the change in nucleosome concentration in serum could indicate the biochemical response early in the course of therapy and could contribute to the individual optimization of the chosen therapeutic regimen. As a method for the early appraisal of the risks and effectiveness of radiotherapy, it would be especially useful for studies dealing with radiation dose escalation over 100 Gy using IMRT techniques.

PERSPECTIVES

If the results regarding the influence of chemotherapy and radiation treatment on circulating nucleosomes in serum are confirmed in larger numbers of patients, they might inspire further prospective and detailed investigations in sera of patients with various tumors, including those with better prognosis, such as patients with colorectal cancer, breast cancer, or lymphoma. Further promising approaches certainly include the comparison with other markers of cell death and established tumor-associated antigens. Finally, the prognostic relevance of the nucleosomes remains to be elucidated.

As circulating nucleosomes are shown to participate in the pathogenesis of autoimmune diseases and might reflect the extent of cell death under various traumatic conditions, such as stroke, multiple trauma, and sepsis, their determination could be useful for many other purposes. In particular, the course of change in the nucleosomal levels in circulation deserves further evaluation as a possible marker for the therapeutic monitoring and prediction of treatment efficacy for a vast variety of diseases. The handicap of its nonspecific nature could, in fact, be its most valuable property.

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