

Nucleosomes in Serum of Patients with Early Cerebral Stroke

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Key Words

Nucleosomes, DNA · Apoptosis · Cerebral ischemia · Stroke

Abstract

Background: Nucleosomes are cell death products that are elevated in serum of patients with diseases that are associated with massive cell destruction. We investigated the kinetics of circulating nucleosomes after cerebral stroke and their correlation with the clinical status. **Methods:** In total, we analyzed nucleosomes by ELISA in sera of 63 patients with early stroke daily during the first week after onset. For correlation with the clinical pathology, patients were grouped into those with medium to slight functional impairment (Barthel Index BI ≥ 50) and those with severe functional impairment (BI < 50). **Results:** Patients with BI ≥ 50 showed a continuous increase in nucleosomes until day 5 (median: 523 arbitrary units, AU) followed by a slow decline. In contrast, patients with BI < 50 showed a steeper initial increase reaching a maximum already on day 3 (869 AU). Both, days after stroke ($p < 0.001$) and BI ($p < 0.001$), had a significant influence on nucleosome concentrations, respectively. Consistently, patients with BI < 50 had a significantly larger area under the curve (AUC/day) of nucleosome values during the first week after stroke (800 AU) than patients with BI

≥ 50 (497 AU; $p = 0.031$). Concerning the infarction volume, nucleosomes showed significant correlations for the concentrations on day 3 ($r = 0.43$; $p = 0.001$) and for the area under the curve ($r = 0.34$; $p = 0.016$). **Conclusion:** Even if nucleosomes are nonspecific cell death markers, their release into serum after cerebral stroke correlates with the gross functional status as well as with the infarction volume and can be considered as biochemical correlative to the severity of stroke.

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Introduction

During cerebral ischemia, hypoperfusion leads to cell death by excitotoxicity, permanent or partially depolarization, Ca^{2+} overload and free radicals, particularly in the ischemic core [1–3]. In contrast, cellular energy mechanisms are partially conserved in the penumbra. In case of continuously insufficient blood supply, delayed apoptotic cell death may result in a considerably larger infarction volume. The extent of cellular demise might be effective for the outcome of patients after long-term recovering processes [1]. As the blood-brain barrier is often disrupted after stroke, the products released by degraded cells gain access to the circulation and can be measured in serum and plasma.

Table 1. Patient characteristics

Median age, years		67.9
Range		32–88
Gender	Male	36
	Female	27
Stroke localization	Middle cerebral artery	50
	Posterior cerebral artery	3
	Thalamus	2
	Posterior cerebellar artery	2
	Brainstem	6
Etiology	Cardioembolic	27
	Arterioembolic	23
	Microangiopathic	6
	Traumatic dissection	4
	Unknown	3
Risk factors	Arterial hypertension	45
	Hypercholesterolemia	22
	Diabetes mellitus	14
	Nicotine abuse	19
	Coronary heart disease	11
BI	75–100	17
	50–<75	14
	40–<50	2
	30–<40	3
	20–<30	7
	10–<20	4
	0–<10	16
Infarction volume	0–<25 cm ³	30
	25–<50 cm ³	8
	50–<100 cm ³	6
	100–<200 cm ³	3
	>200 cm ³	2

Essential products of apoptosis are circulating nucleosomal DNA fragments. They are basic elements of chromatin and consist of a compound of histone core proteins and DNA on the outside. During apoptosis, several endonucleases cleave the chromatin at the easily accessible internucleosomal linking sites specifically into mono- and oligonucleosomal fragments that are released into the circulation after degradation of the cellular membrane [4, 5].

Nowadays it is known that the rate of apoptosis and consequently the release of DNA are increased during various acute and chronic diseases, such as cancer [6, 7], during graft rejection [8], trauma [9], stroke [10] and also during pregnancy [11]. Many of these studies were carried out using real-time PCR for DNA quantification. Using this method, proteins are separated from DNA before measurement of DNA concentration. However, as the as-

sociation of DNA with proteins preserves them from further digestion in serum, most of the extracellular DNA is thought to be present in the form of nucleosomes [4].

In patients with stroke, a recent study has shown that DNA levels in the early ischemic phase possess prognostic value for the 28-day and 6-month mortality [10]. Due to the one-time investigation, it was not clear whether the initial DNA level or later time points would be most effective due to the dynamic development of stroke and its interindividual heterogeneity. Therefore we measured the course of nucleosomal DNA in the blood of patients with cerebral ischemia in a closely monitored manner during the first week after onset of the symptoms. These results were correlated in a first step with the clinical status of the patients objectified by the Barthel Index (BI) [12] and the infarction volume to elucidate whether non-specific biomarkers of cell death are associated with the severity of stroke.

Patients and Methods

Patients

In total, we investigated serum samples of 63 patients with cerebral ischemic stroke. The localization of the occlusion was in 50 patients the middle cerebral artery, in 3 the posterior cerebral artery, in 2 infarctions of the thalamus, in 2 the posterior inferior cerebellar artery and in 6 an artery of the brainstem (table 1). All patients were admitted to the Stroke Unit at the University Hospital Munich-Grosshadern where they received standard stroke care.

In our study, we included all patients with stroke symptoms which occurred within a time frame of maximally 24 h before hospitalization and a BI [11] available at admission. Exclusion criteria were neoplasms, chronic inflammatory diseases and cytostatic therapy at the time of stroke, hemoglobin <9 mg/dl and stroke-specific symptoms that had started earlier than 24 h before admission. The study was approved by the local ethics committee. Informed written consent was obtained either from the patient or a relative in all cases.

The functional impairment at admission was objectified by the BI [11] indicating the everyday ability of self-supply (range: 0–100 points). For our evaluation, patients were grouped according to the median into those with only slight to moderate functional impairment (BI \geq 50; n = 31) and those suffering from severe functional impairment (BI <50; n = 32).

Methods

Nucleosomes were measured in serum at the time of hospitalization and at least once daily during the first week. Because of nucleosome instability, a strict preanalytical protocol was followed. Blood samples were centrifuged within 1–2 h after blood drawing. Subsequently, 10 mM EDTA were added and the samples were stored at -70°C . Quantification of nucleosomes was performed using the Cell Death Detection ELISA^{plus} (Roche Diagnostics, Germany) which is based on a quantitative sandwich enzyme immunoassay principle. Samples were placed into a microtiter plate, and

a buffer solution was added containing two monoclonal antibodies which are directed against DNA and histones (from the mouse clones M-CA-33 and H11-4), respectively, for the specific detection of mono- and oligonucleosomes. The antihistone antibodies were biotinylated and fixed the complexes to the microtiter plate whereas the anti-DNA antibodies were associated with a peroxidase label that reacts with the substrate 2,2'-azino-di-3-ethylbenzthiazoline sulfonate. The resulting color development, which is proportional to the concentration of the nucleosomes captured by the antibody sandwich, was measured photometrically at 405 nm. For measurement of nucleosomes in serum, a standard curve was established using plasma of several healthy donors which was stimulated to induce apoptosis by a mixed lymphocyte reaction as specified in Holdenrieder et al. [7]. Nucleosomes were quantified in relative arbitrary units (AU). Blood samples from each patient were measured within the same run to improve the comparability of the results. Details of the preanalytical sample handling are described in Holdenrieder et al. [7].

The extent of the morphological damage was determined in the first hours after admission to the hospital by computed tomography (CT) and/or during the first days by diffusion-weighted magnetic resonance imaging (MRI), which is highly sensitive to early cerebral stroke [13]. In our evaluation we considered only the first CT or MRI scan as the first days were the most homogenous time points with cerebral images available. The analysis of volume measurements was performed by an experienced radiologist for the MRI imaging on a Linux working station using MedX 3.4.1 software (by Sensor Systems, www.sensor.com); for patients who were imaged by CT, the quantification was performed on the PACS station (Magic View B1, Siemens Medical Systems, Forchheim, Germany). In every instance, the ischemic region was manually outlined on each image slice: the area of the outlined region was multiplied by the thickness for either CT or MR images, plus the interslice gap for MR images (of 0.25), and then summed to give the total lesion volume.

Statistics

For statistical analysis, various variables of nucleosomes were considered, such as the absolute concentrations determined at admission and daily during the first week after stroke, and the area under the curve (AUC/day) that integrated all values of the first week after onset of stroke. For calculation of this variable, at least the nucleosome values of day 1, of days 3 or 4 and of days 5–7 were mandatory. Due to variabilities in the term of hospitalization, the area was normalized by division of the number of days. 55 patients fulfilled the criteria for calculation of the AUC.

Influence of day after stroke and BI (BI \geq 50 vs. BI $<$ 50) on nucleosome concentration was tested simultaneously by two-way Anova using ranks of data. Discriminating power of the AUC/day of nucleosome levels between patients with slight functional impairment (BI \geq 50) and those with heavy functional impairment (BI $<$ 50) was analyzed by the Wilcoxon test. Continuous correlations of nucleosomes and infarction volume as well as of infarction volume and BI were calculated by Spearman's rank correlation together with the 95% confidence interval. A *p* value $<$ 0.05 was considered statistically significant. All *p* values are two-sided. Statistical analyses were performed with software of SAS (version 8.2, SAS Institute Inc., Cary, N.C., USA).

Results

Generally, we observed during the first days after onset of stroke a considerable increase in the concentration of nucleosomes in serum reaching a maximum after 2–5 days. Afterwards the values decreased again slowly. In patients with BI $<$ 50, the increase started already shortly after the ischemic event (medians: day 1, 145 AU; day 2, 524 AU) and reached the maximum after 3 days (869 AU) which was subsequently followed by a decline until day 7 (591 AU). In patients with BI \geq 50, the values increased more slightly (day 1: 125 AU, day 2: 288 AU) reaching the maximum on day 5 (523 AU) followed by a decrease until day 7 (338 AU). When testing simultaneously the influence of days after stroke and BI on nucleosome concentrations by two-way Anova, highly significant results were obtained for both days after stroke ($p <$ 0.001) and BI ($p <$ 0.001). Correspondingly, patients with BI $<$ 50 had a significantly larger AUC/day of nucleosome values during the first week after stroke (800 AU) than patients with BI \geq 50 (497 AU; $p =$ 0.031).

Figure 1 shows typical kinetics of nucleosomes in 2 patients, one with a slight functional impairment (fig. 1a: BI = 100) and one with a severe functional impairment (fig. 1b: BI = 20): the first exhibited only a flat course resulting in a small AUC, whereas the latter had a considerable increase in nucleosome concentrations resulting in a larger AUC (fig. 1). In figure 2, nucleosome concentrations of all patients are expressed as median, quartiles and ranges for every day during the first week after stroke, and for the AUC showing considerable differences in nucleosome levels between patients with slight (BI \geq 50) and those with severe functional impairment (BI $<$ 50; fig. 2).

Concerning the infarction volume, nucleosome concentrations showed significant correlations for day 3 ($r =$ 0.43; $p =$ 0.001) and for the AUC ($r =$ 0.34; $p =$ 0.016) (table 2, fig. 3). In addition, the infarction volume correlated inversely with the BI at admission ($r =$ -0.56; $p <$ 0.0001).

Discussion

Cerebral hypoperfusion leads to various pathological features which are caused by excitotoxicity, peri-infarct depolarization, inflammation and cell death. Concerning the mode of cell death, necrosis might predominantly occur in the ischemic core where cells are damaged most severely and irreversibly. In contrast, apoptosis might be mainly present in the penumbra where cellular mecha-

Fig. 1. Courses of nucleosome concentrations during the first week after cerebral stroke in a patient with infarction of the middle cerebral artery and a BI of 100 points at admission (a) and in a patient with infarction of the middle cerebral artery and a BI of only 20 points (b).

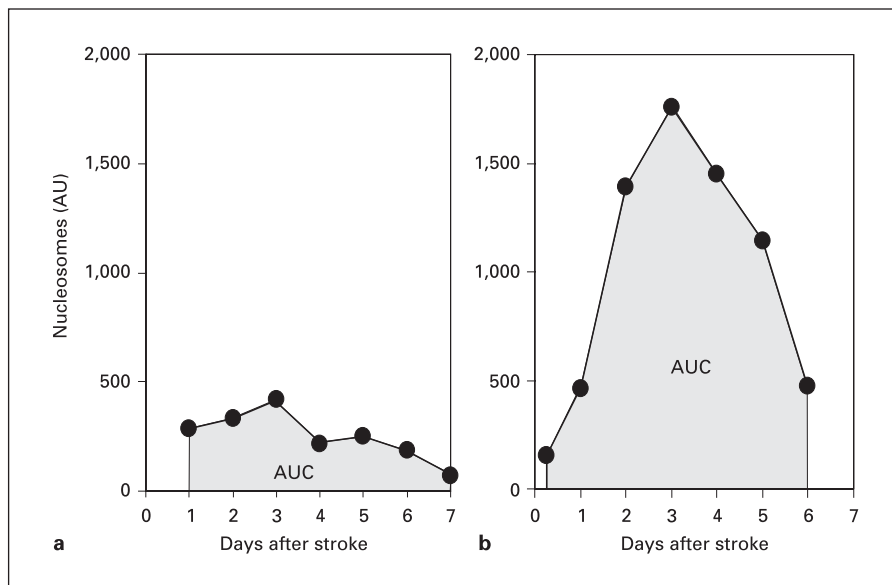
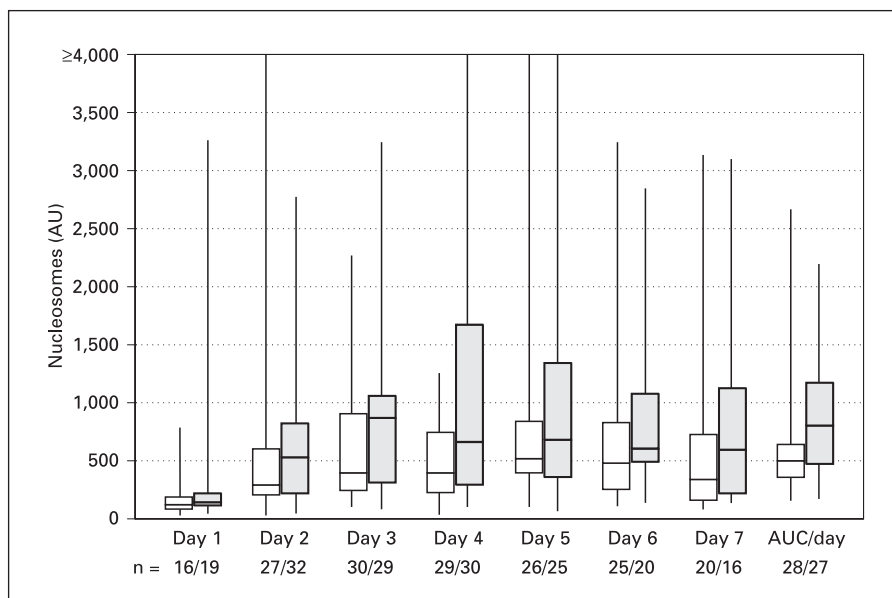


Fig. 2. Nucleosome concentrations (AU; median, quartiles and ranges) during the first week after cerebral infarction in patients with slight (BI ≥ 50) and those with severe functional impairment (BI < 50). Simultaneous two-way Anova testing revealed a significant influence of days after stroke ($p < 0.001$) and BI ($p < 0.001$) on nucleosome concentrations.



nisms are impaired by the restricted blood supply [1]. Presumably, cells are first arrested in their cycles to initiate repair and rescuing systems. Only if they fail, cells will decay after a certain time delay. In addition, some studies report that apoptotic and necrotic features may occur in the same cells at various time levels depending on the properties and demands of the cells, and they suggest the term ‘aponecrosis’ [14].

Biochemical markers reflect the pathophysiological processes and are potentially helpful for the diagnosis, monitoring and prognosis of stroke. Among the serum

parameters that have shown to be released after stroke are more unspecific markers like glucose [15, 16] and C-reactive protein [17, 18], and specific markers for neuronal degradation and glia activation like neuron-specific enolase and S100 protein [19, 20].

Nucleosomal DNA fragments have been described as typical cell death products that are released particularly during apoptosis. However, elevated levels of nucleosomes have also been observed during acute inflammations and during various malignant processes [6]. Therefore it is hardly likely that nucleosomes might be valuable for the

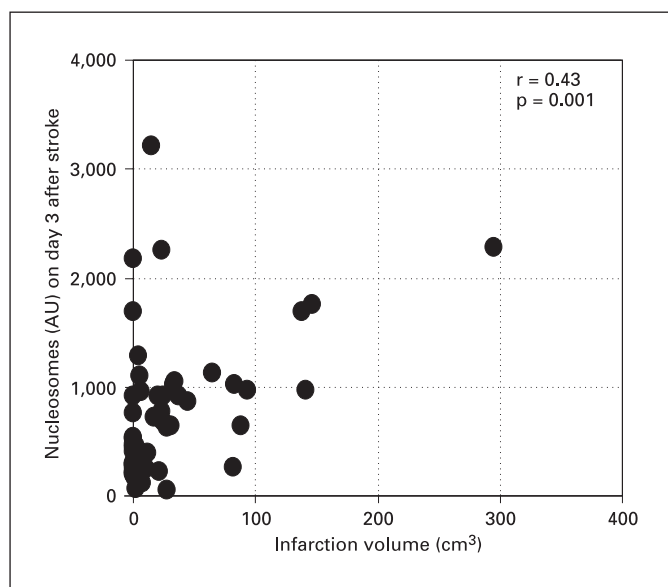


Fig. 3. Correlation of nucleosome concentration on day 3 after stroke with infarction volume ($r = 0.43$; confidence interval 0.21–0.65; $p = 0.001$; Spearman rank correlation).

diagnosis of any specific disease or of stroke. Most impressively, a recent study on circulating DNA determined at early stages of stroke has shown a close association with survival of stroke patients: Rainer et al. [10] reported on 88 stroke patients whose high DNA levels were significantly correlated with an increased mortality rate within 28 days as well as within 6 months after stroke. They found this parameter having a specificity of 74.4% and a sensitivity of 100% for the prediction of hospital mortality [10]. These results are all the more interesting as stroke is known to be a dynamic process with considerable interindividual heterogeneity in its development and manifestation.

Based on these results we conducted a study with serial determinations of nucleosomal DNA fragments in blood to see (a) whether there exist typical kinetic patterns of DNA release after stroke, (b) whether they are related to the clinical status of stroke patients and (c) whether they are related to the infarction volume.

For the measurement of nucleosomes we used an ELISA that has been developed recently and has shown a good methodological performance [5]. It detects specifically the nucleosomal complexes of histones and DNA in the blood. Even if Rainer et al. [10] quantified DNA by a PCR technique which includes the separation of DNA from histone proteins by proteinase K digestion, the results should be comparable as it is known that most of the

Table 2. Correlation of nucleosome concentrations during the first week after stroke and infarction volume (Spearman rank correlation)

	Correlation coefficient	p value
Day 1	0.14	0.433
Day 2	0.02	0.875
Day 3	0.43	0.001
Day 4	0.25	0.068
Day 5	0.26	0.081
Day 6	0.21	0.178
Day 7	0.19	0.288
AUC/day	0.34	0.016

circulating DNA in blood occurs in the form of nucleosomes and only a small amount as free DNA [21, 22].

Our results showed heterogeneous kinetic patterns of DNA release into serum after stroke. However, we observed in most patients an increase during the first days after onset of the symptoms reaching the maximum on days 3–5. These findings are in accordance with the kinetics of apoptosis induction that is known to be strongest after 48–72 h after severe cell damage [23]. However, inflammatory processes might contribute to the elevation of nucleosome values at that time, too. When dividing the patients according to the severity of the functional impairment into those with $BI < 50$ and $BI \geq 50$, the initial nucleosome concentrations did not show significant differences between both groups. In the further follow-up of patients with severe functional impairments, the nucleosome levels increased earlier and more strongly than in those having only slight to moderate functional lesions. Consistently, the area under the curve of nucleosome values during the first week after stroke differed significantly between both groups. For the concentrations on day 3 and the AUC, we also found a significant correlation with the infarction volume.

Our results indicate that nucleosomal DNA as typical cell death marker is released in a time- and severity-dependent manner after stroke which can also be followed in serum. Whether the increase in nucleosomal amount in blood after several days is only the result of the initial cell damage or whether it mainly reflects further pathophysiological processes that are still taking place days after the onset of stroke remains to be clarified. The individual dynamics of stroke development was not mapped by imaging techniques either as they were only performed at the initial

phase of stroke. This might have led to an underestimation of the stroke volume particularly in those cases where the dimension of the infarction increased later on.

Concerning the source of circulating nucleosomal DNA, several hypotheses might be taken into account: First, they could derive from cell death within the injured brain. As nucleosomes correlate with infarction volume and clinical status of the patients, this explanation sounds reasonable; however, it is not necessarily deductable. Alternatively they might be released by apoptotic inflammatory cells like polymorphonuclear leukocytes or lymphocytes which are known to migrate into the cerebral circulation and infiltrate the injured area [1]. As the severity of the ischemic event may be related to the quantity of infiltrating leukocytes, circulating nucleosomal DNA could also simply reflect the extent of leukocyte apoptosis. Finally, as leukocyte activation is also seen in the periphery after stroke, they could at least contribute to the release of circulating DNA. Performing correlations of nucleosomal DNA with leukocytes and C-reactive protein as inflammatory parameters as well as with neuron-specific enolase and S100 protein as markers of

neuronal degradation and glia activation might be appropriate to address these hypotheses.

Nevertheless, the correlation of the kinetics of circulating nucleosomal DNA in serum with the extent of functional lesions and one-time determination of the infarction volume might be valuable for upcoming investigations on the heterogeneous development and manifestation of stroke as well as potentially for the individual monitoring of stroke in future. Besides the comparison with more specific markers like neuron-specific enolase and S100 protein, the next essential steps in the evaluation of the power of nucleosomes in stroke patients will include the prognostic analysis to distinguish different stroke populations, etiologies and treatment effects.

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