Nature and Dynamics of Nucleosome Release from Neoplastic and Non-neoplastic Cells

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Abstract. *Background: Circulating nucleosomes are elevated in the blood of patients with malignant and non-malignant diseases. Here, we investigated the nature and the dynamics of their release in functional cell studies. Materials and Methods: Leukemia blasts were exposed to the intrinsic inducers of apoptotic cell death, cytosine arabinoside (AraC; 10 μg/ml) and etoposide (50 μg/ml), and cell death markers lactate dehydrogenase (LDH) and the nucleosomes were measured in the supernatants at 0, 24, 48, 72, and 96 hours after drug application. In addition, HepG2 cells were exposed to extrinsic apoptosis-inducing tumor necrosis factor-related apoptosis-inducing ligand (TRAIL; 0.5 and 1.0 ng/ml) and the nucleosomes were measured in the supernatants after 0, 24, 48, and 72 hours. Finally, neutrophils preactivated by phorbol myristate acetate (PMA) were co-incubated with platelet-rich plasma (PRP) in the presence of collagen (type I; 8 μg/ml) for 15 or 30 minutes at 37˚C, and the nucleosome release into the supernatant was quantified. Results: During treatment with AraC, cell viability constantly decreased. LDH and nucleosome levels increased at 24 h and peaked at 48 h after exposure to AraC and etoposide. While LDH declined after 96 h, the nucleosomes' levels were still elevated. Similarly, nucleosomes increased dose-dependently 24 h after exposure to TRAIL and reached a peak at 48 h. After 72 h, the nucleosomes' levels decreased again. While there was only a minor release of nucleosomes from PMA-stimulated neutrophils, co-incubation with PRP resulted in a strongly increased nucleosome release after 30 minutes. Conclusion: Nucleosomes are released from cells stimulated intrinsically or extrinsically to undergo apoptotic cell death in a time- and dose-dependent manner. Further mechanisms of release may be their active secretion from stimulated neutrophils when co-incubated with PRP, as may be observed during bacterial inflammation and thrombosis.*

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Circulating nucleosomes are known to be elevated in the sera and the plasma of patients with diverse malignant diseases (1, 2). Furthermore, increased levels of nucleosomes have been detected in the blood of patients with various acute non-malignant diseases, such as acute ischemia, trauma, severe inflammation and sepsis, but also in those with autoimmune diseases (2-5), thus limiting their use for diagnostic purposes. In cancer patients, nucleosomes have shown prognostic potential and the kinetics of circulating nucleosomes have been correlated with the response to cytotoxic chemo- and radiotherapy (6-9). The Nucleosome's levels increased even during the first days of application of antitumor therapy, followed by a rapid decrease (1, 2, 6-12). These changes were relevant for the early estimation of response to chemo- and radiotherapy at the initial phase of the treatment (9-15). In lung cancer cells exposed to irradiation, nucleosome release was dose- and time-dependent and was considerably higher than that of non-malignant cells treated under identical conditions (16).

Nucleosomes are the basic units of eucaryotic chromatin. They are formed by a central protein octamer consisting of two copies of the histones H2A, H2B, H3, and H4 that are surrounded by 147 bp of DNA (17). Nucleosomes are connected with each other by linker-DNA of varying length, forming a chain-like structure (17, 18). Nucleosomes can move along this chain to uncover gene sequences required for the transcription process; this highly dynamic structure is regulated by a set of epigenetic marks, such as specific histone modifications on protruding *N*-terminal histone tails (19, 20).

During apoptotic cell death, nucleosomes are characteristically degraded by endonucleases such as the caspase-dependent DNAse (CAD). After the separation of CAD from its inhibitor ICAD by effector caspases 3 or 7, CAD gains access to the nucleus and cleaves the chromatin, particularly at the easily accessible internucleosomal linking sites, into multiple mono- and oligonucleosomal fragments (21, 22). Under physiological conditions, these final apoptotic products are packed into apoptotic bodies and are engulfed by macrophages and neighbouring cells (23, 24). If the recycling systems are overloaded or defective, the nucleosomes may also reach the blood circulation and can be measured in elevated numbers (2). The nucleosomes are believed to be a major form of circulating DNA in blood (25) and they may also be released during necrotic or other cell death forms. In such cases, degradation is not as orchestrated as during apoptotic cell death (26). As DNA has been reported to be released within neutrophil extracellular traps (NETs) from neutrophils during bacterial invasion (27), it can be assumed that nucleosomes can also be secreted from stimulated cells.

In order to elucidate the modes and the dynamics of nucleosome secretion, we investigated the time-dependent release of nucleosomes from leukemia blasts treated by cytotoxic drugs that are known to intrinsically induce apoptotic cell death after DNA damage (28). We also investigated their dose-dependent release from cancer cells upon extrinsic stimulation with the apoptosis-inducing agent tumor necrosis factor-related apoptosis-inducing ligand [TRAIL; (29)] and, finally, their release from neutrophils after their co-incubation with platelet-rich plasma, as is physiologically observed in inflammatory and in thrombophilic processes (30).

Materials and Methods

Exposure of leukemia blasts to cytosine arabinoside (AraC) and etoposide. Leukemia blasts that were previously used in cell experiments (28) were diluted to a density of 1×10^6 cells/ml in Iscove's modified Dulbeccos's medium (IMDM), supplemented with 20 mmol/l HEPES, 100 μg/ml streptomycin, 10 mmol/l L-glutamine and 10% fetal calf serum (FCS) (all from Gibco Life Technologies, Eggenstein, Germany), as described earlier (28). Cells were exposed to 10 μg/ml AraC (Pharmacia, Erlangen, Germany) or to 50 μg/ml etoposide (Sigma, Deisenhofen, Germany) and were incubated at 37˚C, in an atmosphere with 5% CO_2 and 95% humidity for up to 96 hours. Cell viability was tested with the WST cytotoxicity assay (Roche, Manheim, Germany), as described earlier (28). In the cellular supernatant, that was harvested every 24 hours, the concentration of lactate dehydrogenase (LDH) and the nucleosomes were measured before, as well as at 24, 48, 72, and 96 hours after, drug exposure.

LDH was quantified enzymatically by an Olympus AU 2700 analyzer (Hamburg, Germany). The nucleosomes' concentrations were measured by the Cell Death Detection ELISA plus from Roche Diagnostics (Mannheim, Germany), as described earlier (31). All samples were measured within one run in order to avoid interassay variations.

Exposure of HepG2 to TRAIL. HepG2 cells were maintained in culture medium as described elsewhere (29). They were diluted to 1×10^7 cells/ml and were exposed to 0.5 and 1.0 ng/ml TRAIL (Sigma). The supernatants were exchanged every 24 hours and the nucleosomes were quantified at 0, 24, 48 and 72 hours after drug exposure.

Nucleosome release from activated neutrophils. Freshly isolated human neutrophils (Ficoll isolation) preactivated with phorbol 12-myristate 13-acetate (PMA; 100 nM) plus aprotinin (20 μg/ml) were co-incubated with platelet-rich plasma (PRP) in the presence of collagen (type I; 8 μg/ml) for 15 or 30 minutes at 37˚C. Nucleosomes were quantified in the supernatants recovered from the mixture, as well as from neutrophils and PRP alone.

Results

Exposure of leukemia blasts to AraC and etoposide. The cell viability of leukemia blasts increased from 1.0 to 1.9 after 24 h and to 2.2 after 96 h in untreated cultures, as measured by the WST cytotoxicity assay, while it decreased from 1.0 to 0.9 after 24 h and to 0 after 96 h in the AraC-treated cultures. Correlating inversely, the general cell death marker LDH increased 4.2- and 2.7-fold after 48 h in cultures treated by AraC and etoposide, respectively, and returned to pre-therapeutic concentrations after 96 h (Figure 1A). The nucleosomes started to increase as early as 24 h 2.6-fold (AraC) and 2.3-fold (etoposide) to reach a maximum release after 48 h (AraC: 5.6-fold; etoposide 5.3-fold increase, respectively) and remained elevated after 96 h (AraC: 3.7-fold; etoposide 2.4-fold increase, respectively) (Figure 1B).

Exposure of HepG2 to TRAIL. Similarly, nucleosome concentrations in the supernantants started to increase after 24 h in HepG2 cells exposed to TRAIL. The relative increase was stronger after exposure to 1.0 ng/ml TRAIL (3.8-fold) as compared with 0.5 ng/ml TRAIL (2.5-fold). Again, the maximum release was observed after 48 h (4.3- and 4.2-fold increase for 0.5 ng/ml and 1.0 ng/ml TRAIL, respectively). After 72 h, the nucleosomes' concentrations were almost at pre-treatment levels (Figure 1C).

Nucleosome release from neutrophils. The nucleosomes' levels in neutrophils that were activated with PMA only were quite low. The same applies to PRP, which is not supposed to contain nucleosomes. When activated neutrophils were co-incubated with PRP, in the presence of type I collagen, for 15 minutes, nucleosome levels were increased in the supernatant. A more pronounced increase in nuceosome release was observed with 30 minutes incubation time, at the level of 11.1-fold (Figure 1D).

Discussion

The release of cell death products such as intracellular enzymes and structural proteins, or degraded cytosolic or nuclear constituents such as nucleosomes, is a sign of, or a

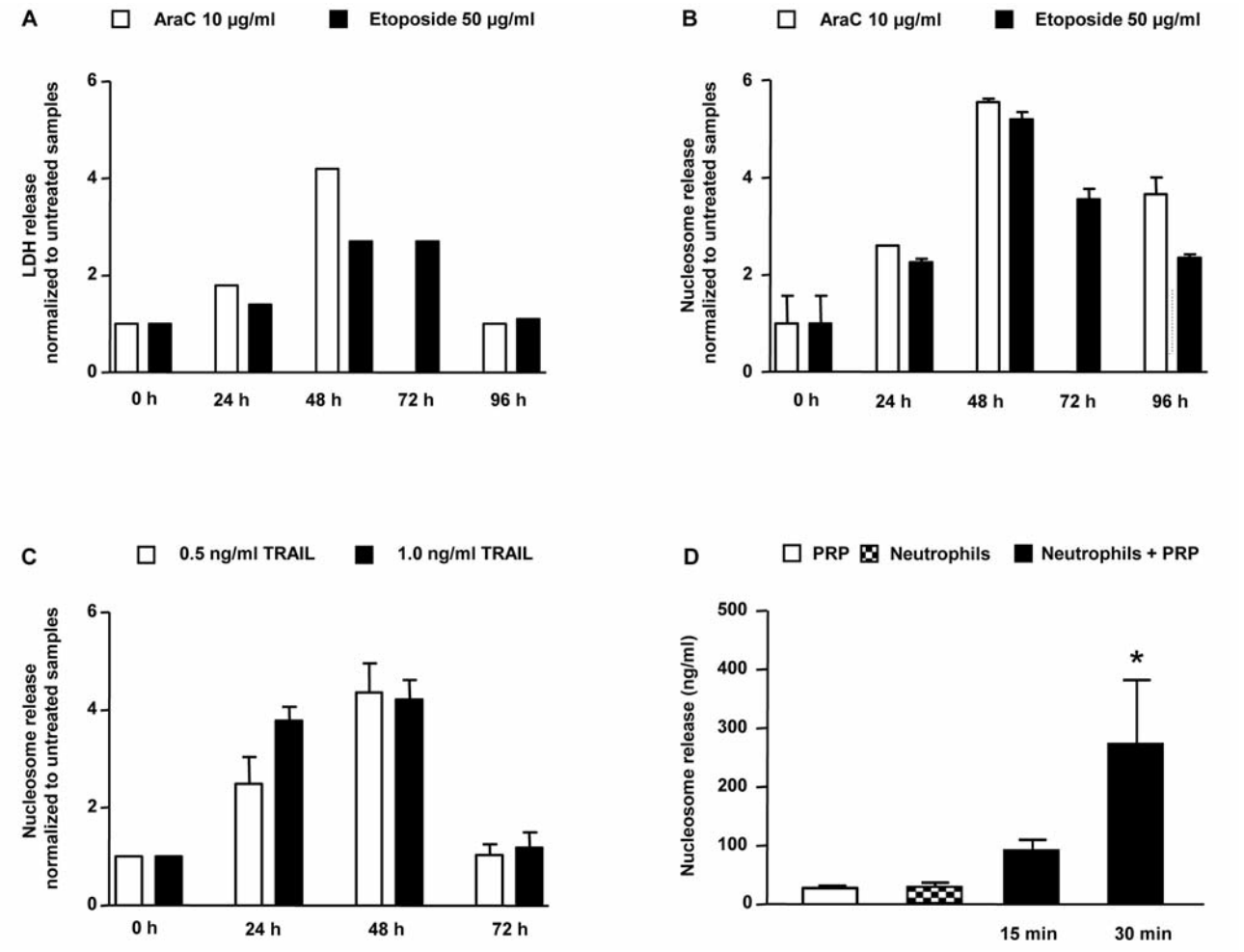

Figure 1. *Time-dependent release of lactate dehydrogenase (LDH) and of nucleosomes from leukemia blasts into the supernatant after exposure to the intrinsic apoptosis-inducing agents cytosine arabinoside (AraC) and etoposide (VP16) (A, B). Time- and dose-dependent release of nucleosomes from HepG2 cells into the supernatant after exposure to the extrinsic apoptosis-inducing agent tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (C). Release of nucleosomes from phorbol myristate acetate (PMA)-stimulated neutrophils co-incubated with platelet-rich plasma for 15 and 30 minutes (D).*

reaction to cellular damage, that occurs spontaneously during acute pathological conditions including trauma, ischemia and sepsis, during malignant diseases with high cellular turnover, and, to a lesser extent, during degenerative and chronic autoimmune diseases (2, 32-34). Cell death products can also be detected at elevated levels after therapeutic interventions including cytotoxic treatments for cancer (2, 6-15, 32). Beyond the increased release of cell death products from damaged cells, the reduced capacity of local recycling systems and the restricted elimination from blood circulation, contribute to the resulting blood levels of these parameters (6, 32, 35). Recent data have shown the externalization of DNA from neutrophils upon stimulation with bacterial lipopolysaccharides in the form of NETs, as being important physiological mechanisms for the compartmentalization of the infectious process, in order to better cope with it (27, 36). Thereby, NETs may be formed by neutrophils that are purely stressed (27), by those commiting beneficial suicide (nettosis) distinct from apoptotic and necrotic cell death (36, 37), while NETs have been also observed to promote some autoimmune diseases (38).

Concerning cytotoxic drugs, AraC and etoposide are known to intrinsically induce apoptosis by efficiently damaging DNA, mostly in terms of single-strand breaks. AraC inhibits polymerase α and is incorporated as a false metabolite into the DNA, thereby provoking DNA chain termination (28). Etoposide stabilizes the DNA strand complex with topoisomerase II, resulting in a DNA strand break. Both types of DNA damage finally act as pro-

apoptotic signals and lead to loss of cell viability, to activation of caspase-3 and to morphologically apoptotic features (28). AraC is used as a potent drug in acute myeloid leukemia (AML) (39), while etoposide is part of a diverse chemotherapeutic regimen for hematopoetic and solid tumor diseases. In line with an earlier functional analysis of these drugs in leukemia blasts (28), we found a partial reduction of cell viability after 24 h and a total loss after 96 h of exposure to AraC. Furthermore, a considerable release of LDH and nucleosomes was observed as early as 24 h after drug exposure, which peaked at 48 h to 5.6-fold (AraC) and 5.3-fold for the levels (etoposide) of nucleosomes released into the supernatant. Taking into consideration the time delay until cell disintegration, these results correspond well with the finding that caspase-3 activity was elevated 6 h after drug exposure in the cellular lysates (28). However, we could not confirm stronger effects of induction of apoptosis and of nucleosome release by etoposide, as compared to AraC (28). Interestingly, in an earlier clinical study on patients with AML, undergoing primary chemotherapy, serum concentrations of nucleosomes increased during the first days of therapy and these changes were correlated with the clinical outcome after induction chemotherapy (8).

Beyond the well-investigated apoptosis-induction by FAS/CD95, the interaction of TRAIL and its receptors has recently gained more interest as an efficient extrinsic apoptosis-inducing system that has been used for therapeutic approaches and plays a role in immunosurveillance and, if deficient, in resistance to cytotoxic drugs (40, 41). As osteoprotegerin, a transcriptional target of the Wnt/β-catenin signal transduction pathway, acts as a decoy receptor for TRAIL, this survival factor is relevant for carcinogenesis through the development of resistance to TRAIL-mediated apoptosis (29). While significant apoptosis induction, 24 h after TRAIL exposure, was observed only after doses of more than 5 ng/ml (29), we measured considerable increases of the released nucleosomes with 0.5 and 1 ng/ml TRAIL after 24 h and 48 h, assuming a high sensitivity of the HepG2 cell line to TRAIL, and that nucleosomal release is a sensitive measure for cell death in cell cultures.

Earlier studies on exposing normal and malignant lung cells to diverse doses of irradiation have also shown dose- and time-dependent increases of nucleosome release. The nucleosomes' concentrations in the supernatants peaked after 24 h and 48 h and decreased to almost normal values at 120 h. Thereby, the release in cancer cells was significantly more pronounced when compared with normal lung cells, exposed to similar irradiation doses (16).

The release of nucleosomes from stimulated neutrophils co-incubated with activated platelets has already been described, thus indicating a role of circulating nucleosomes in collaboration with neutrophil serine proteases in thrombosis formation, by degradation of tissue factor pathway inhibitor (30). Here we show that nucleosomes are strongly released from preactivated neutrophils as early as 30 minutes after co-incubation with PRP. As neutrophils are still alive at the time of nucleosome release and as this effect is considerably faster than the release observed from cancer cells treated by cytotoxic agents, an active secretion of nucleosomes upon PRP stimulation is assumed.

Conclusion

Nucleosomes are released from cells which are stimulated intrinsically or extrinsically to undergo apoptotic cell death in a time- and dose-dependent manner and may be used as a correlating factor of cell death, induced by cytotoxic drugs. Further mechanisms of release include active secretion from stimulated neutrophils when these are co-incubated with PRP that may play essential roles in generation of thrombosis and in inflammation.

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