ANTICANCER RESEARCH *19*: 2721-2724 (1999)

# Apoptosis in Serum of Patients with Solid Tumours

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**Abstract.** *Apoptosis, which occurs in highly proliferating tumours spontaneously or during anticancer herapy, may lead to an elevated concentration of circulating nucleosomes in blood. In order to quantify the concentration of nucleosomes, we used the Cell Death Detection$^{plus}$ - ELISA (CDDE) (Boehringer Mannheim, Germany) based on antibodies against histone and DNA, adapted it to the demands of liquid materials and standardized test performance and handling of serum. Furthermore, we investigated serum samples of 185 patients with solid tumours (additionally 24, treated with radio- or chemotherapy), 30 with acute inflammations and 50 healthy persons. Many patients with tumours (78%) and inflammations (77%) showed higher concentrations of serum-nucleosomes (>100 AU), whereas 96% of all healthy persons had low values (<100 AU). Follow up-studies revealed an early peak after initiation of therapy and correlated to the clinical outcome. The concentration of nucleosomes is a sensitive marker of cell death and could be used for monitoring the efficacy of antitumour therapy.*

Cell death as a counterpart to cell proliferation plays an essential role in keeping the balance of a constant cell number in adult organisms, and also in removing cells damaged by irradiation, chemotherapeutic drugs, hyperthermia and other factors. It can occur by two distinct modes: necrosis and apoptosis. The mode of cell death depends less on the type of the stimulus, but more on the dose and the severity of the lesion.

Necrosis is a passive, catabolic process affecting generally groups of cells due to gross injury. It is characterized by cellular and mitochondrial swelling followed by rupture of the plasma membrane and release of the cytoplasmic content into the extracellular fluid. Chromatin is degraded non-specifically by lysosomal enzymes resulting in heterogenous sized fragments visualized as a diffuse smear on electrophoretic gels(1-4). Apoptosis as an active, energy-requiring, physiological mode of cell death is observed in single cells responding to less severe trauma and also in tumours. Characteristics are shrinkage of cell volume, chromatin condensation, nuclear fragmentation (karyorrhexis), membrane budding and appearance of apoptotic bodies. DNA is cleaved by various endonucleases into fragments of 50- 300 kilo base pairs (kbp), later into nucleosome- sized multiples of 180 bp generating the typical "ladder" pattern in agarose gel electrophoresis(1-4). Nucleosomes are formed by an octamere of histones, 146 bp DNA wrapped around it and 15-100 bp linker DNA, the preferential binding site of the endonucleases(5-6).

During apoptosis, mono- and oligonucleosomes can be detected in the cytoplasm indicating the activity of endonucleases. Enclosed by plasma membrane as apoptotic bodies and engulfed by neighbouring cells and macrophages under physiological conditions, this mechanism seems to be insufficient confronted to situations with extended apoptosis, *e.g.* in highly proliferating tumours or during anticancer therapy, resulting in elevated concentrations of nucleosomes in serum.

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*Key Words:* Apoptosis, nucleosomes, serum, tumour, radiotherapy, chemotherapy.

## Methods and Materials

The Cell Death Detection$^{plus}$ - ELISA is based on a quantitative sandwich- enzyme- immunoassay- principle: Monoclonal mouse antibodies directed against DNA and histones detect specifically mono- and oligonucleosomes. The sample is placed into a streptavidin- coated microtiterplate (MTP) and incubated with a mixture of anti- histone-biotin and anti- DNA- POD for 2 hours. The antibodies bind to the histone- and respectively DNA-component of the nucleosomes and fix the immunocomplex to the MTP by streptavidin-biotin-interaction. After the incubation period unbound antibodies are removed by a washing step. Incubating the retained POD-linked complexes with ABTS (2,2'-Azino- di(3-ethylbenzthiazolin-sulfonat)) as substrate permits the photometrical quantification (wavelength 405 nm) of the nucleosomes.

The anti- histone- antibodies show specific affinity to the histones H1, H2A, H2B, H3 and H4, the anti- DNA-antibodies bind ss- and ds- DNA. The assay is able to detect the DNA-content of $10^3$ cells. The interassay-comparability could be enhanced by standardizing the incubation time of

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Table I. *Median, mean CNS- values and concentration of nucleosomes in serum above 100 and 500Au (absolute numbers and percentages) for healthy persons (HP), patients with solid tumours - patients with lung cancer (LC), breast cancer (BC), ovarian cancer (OC), colorectal cancer (CC) and other gastrointestinal carcinomas (OGIC) - and patients with acute inflammations (AI).*

| | N | Median | Mean | >100 AU | % | > 500 AU | % |
|---|---|---|---|---|---|---|---|
| **HP** | **50** | **23** | **30** | **2** | **4** | **0** | **0** |
| **Tumours** | **185** | **284** | **424** | **145** | **78** | **62** | **34** |
| LC | 31 | 410 | 582 | 30 | 97 | 14 | 45 |
| BC | 43 | 197 | 376 | 36 | 84 | 12 | 28 |
| OC | 35 | 307 | 453 | 28 | 80 | 14 | 42 |
| CC | 42 | 229 | 390 | 28 | 67 | 13 | 31 |
| OGIC | 34 | 223 | 351 | 23 | 68 | 9 | 26 |
| **AI** | **30** | **221** | **323** | **23** | **77** | **7** | **33** |

ABTS to 30 min, by establishing a standard curve with highly apoptotic material and by introducing arbitrary units (AU) - 1000 AU indicating the highest standard value (about 2500 mE).

As matrix we used serum. After centrifugation of the blood sample we added 10 mM EDTA (pH8) to the serum and stored it at -20°C.

*Patients.* We investigated sera of 50 healthy persons, 30 patients with acute inflammations and 185 patients with solid tumours: 31 with lung cancer (LC), 43 with breast cancer (BC), 35 with ovarian cancer (OC), 42 with colorectal cancer (CC) and 34 with other gastrointestinal carcinomas (OGIC). Additionally, we observed the follow up of 12 radiotherapy patients (4 with lung cancer, 4 with head and neck cancer and 4 with lymphomas) as well as of 12 chemotherapy patients (4 with colorectal cancer, 2 with pancreatic cancer, 2 with sarcomas and 4 with lymphomas).

## Results

*Methodological results.* To get reliable results, we had to identify factors which might interefere causing falsly high or low values. Whereas haemolysis - induced by shaking the blood sample and prolongation of the period before centrifugation > 2 hours - resulted in elevated values, delayed addition of 10 mM EDTA led to falsly low values. These effects could be avoided by careful handling of the blood sample, centrifugation within 2 hours and addition of EDTA directly after centrifugation.

Serum- samples stored at -20°C showed a good long term stability at low and high extinction- levels, even after 6 months, with a CV between 4,2 and 9.2%.

The imprecision, as a criterion for the reliability of the ELISA, ranged in the intraassay comparison (n=10) between 3,0 and 4,1 %, in the interassay comparison (n=14) between 8.6 and 13.5%.

*Clinical results.* 48 of 50 healthy persons (= 96%) had very low values below 100 AU. The median ranged at 23 AU and the mean at 30 AU. Patients with solid tumours showed levels ranking from low to very high. 145 out of the 185 tumour patients (= 78%) had concentrations of nucleosomes in serum (CNS) above 100 AU, 62 (= 34%) even above 500 AU. The median of all tumour patients was 284 AU and the mean 424 AU. A similar pattern of values we observed in patients with acute inflammations: 23 of 30 (= 77%) had values above 100 AU, 7 (= 33%) even above 500 AU. The median reached for these patients 221 AU, the mean 323 AU (Figure 1, Table I).

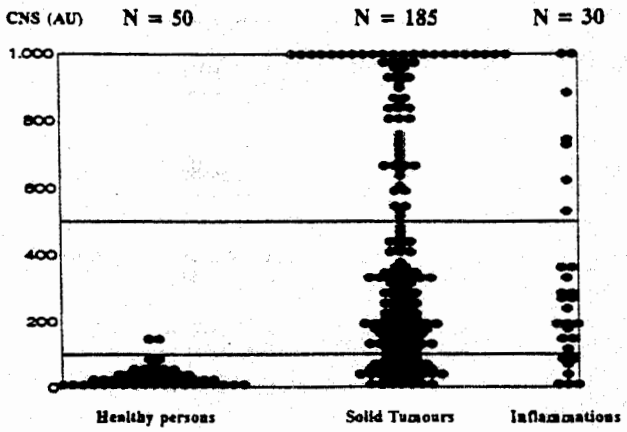


Figure 1. *Distribution of CNS- values for healthy persons, patients with solid tumours and patients with acute inflammations.*

Among the various tumour types patients with lung cancer showed the highest values with a median of 582 AU and a mean of 410 AU, patients with breast cancer the lowest ones

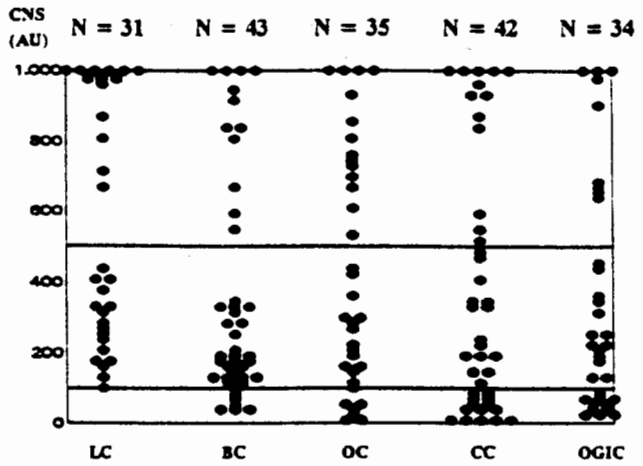


Figure 2. *Distribution of CNS- values for patients with lung cancer (LC), breast cancer (BC), ovarian cancer (OC), colorectal cancer (CC) and other gastrointestinal carcinomas (OGIC).*

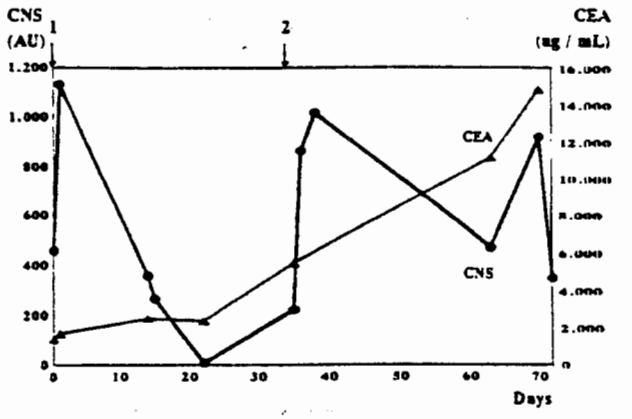


Figure 4. *Follow up of a patient during chemotherapy: Patient H.K. with pancreatic carcinoma (T3 N1 M1) was treated with gemcitabine 1000 mg/m² = 1700 mg total dose (day 1, 8, 15) and cisplatin 50 mg/m² = 85 mg total dose (day 1 and 15) (= therapy 1). Because of progression of disease therapy 2 with 5-fluoruracil 500 mg/m² and folinic acid 300 mg bolus (both day 1 to 5) was initiated. Progression of disease was indicated by increase of CEA.*

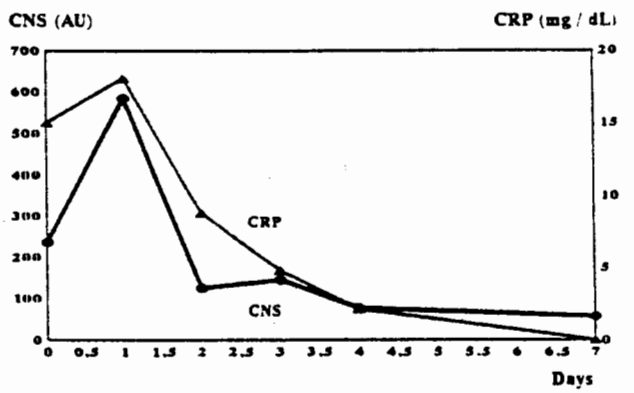


Figure 3. *Follow up of a patient with acute inflammation: Patient S.F. with cholangitis and cholestasis showed good correlation between CNS, CRP and clinical outcome.*

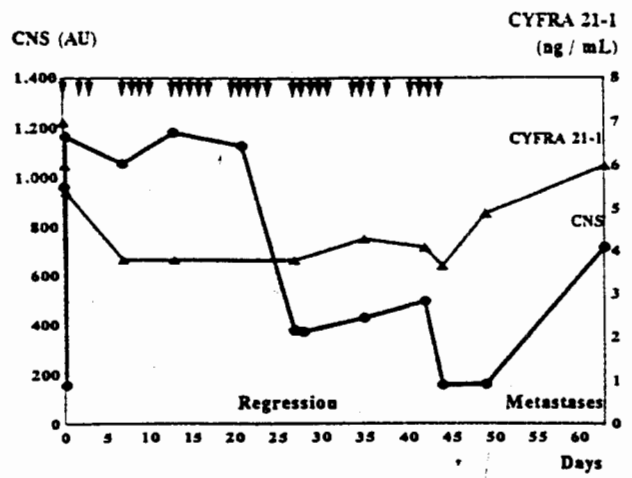


Figure 5. *Follow up of a patient during radiotherapy: Patient E.K. with metastatic carcinoma of the lung was irradiated with a total dose of 60 Gy (30 fractions each 2,0 Gy, volume: 9,91). About 6 hours after the first fraction, CNS reached high levels (about 1000 AU) and decreased only after 3 weeks going along with regression of the tumour (x-ray proven). With appearance of new metastases (x-ray proven) and malignant pleura exsudate (cytological proven) CNS increased again.*

with a median of 197 AU and a mean of 376 AU. Patients with ovarian cancer (median 307 AU, mean 453 AU), colorectal cancer (median 229 AU, mean 390) and other gastrointestinal tumours (median 223 AU, mean 351 AU) were intermediate (Figure 2, Table I).

The follow up of patients with acute inflammations revealed a correlation between the concentration of nucleosomes in serum (CNS), the C reactive proteine (CRP) and the clinical outcome (Figure 3).

Most of the patients undergoing chemotherapy showed a rapid increase of CNS 1 to 3 days after initiation of therapy followed by a decrease of the values. Sometimes infectious complications led to temporary elevations of CNS and overlayed the therapeutic effect (Figure 4).

High amounts of nucleosomes were detected also in serum of most of the irradiated patients with a period of delay less than one day after start of therapy. Decreasing values often were correlated to x-ray proven regression of the tumour (Figure 5).

## Discussion

The intra- and interassay- imprecision of the CDDE range within the limits demanded for a hand- performed assay (CV

< 10% respectively CV < 15%) indicating a good quality of the test material. Standardized handling of the blood samples and longterm stability of serum under storage conditions are prerequisites to determine reliably the concentration of nucleosomes in serum and enhance the comparability of the results.

As acute inflammations often induce massive necrosis and most of spontaneous cell death in proliferating tumours occurs *via* apoptosis, we investigated these entities of diseases to determine the specificity of the CDDE for apoptosis. As expected, and as seen by the similar patterns of values, the CDDE could not distinguish between apoptosis and necrosis. After desintegration of the plasma membrane at a late stage of cell death oligo- and mononucleosomes appear in the circulating blood regardless of whether being cleaved by endonucleases during apoptosis or accidentally degraded by lysosomal enzymes during necrosis. Thus, nucleosomes in serum can be derived from various origins.

However, the differentiation between apoptosis and necrosis loses significance when evaluating clinically the activity or mass of tumours. After exclusion of an acute inflammation the concentration of nucleosomes in serum can indicate the proliferation, mass or invasiveness of a tumour - taking into account the interindividual immunological variability of cells to undergo apoptosis. Other studies concerning the concentration of DNA in plasma and serum, although measured by different techniques, show comparable results for various tumours(7-10).

When monitoring patients during chemo- or radiotherapy, the increase of concentration of nucleosomes in serum at initiation of therapy can correlate with the sensitivity of the patient's cells to cytotoxic drugs or irradiation. However, the follow up of the basic values of CNS determined before each cycle of therapy seems even to be more important. After exclusion of infectious complications, a complete decrease of the basic values often correlates with regression of the tumour and good prognosis whereas constantly high values or only partial decrease of basic CNS often indicates tumour residues or bad prognosis.

Although further studies investigating the therapeutic effect on the CNS in relation to the patients' sensitivity to treatment and prognosis of various tumours are necessary, these results show that the concentration of nucleosomes in serum as a sensitive marker of cell death might be used for monitoring the efficacy of antitumour therapy, possibly in combination with well- established tumour markers.

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*Received January 22, 1999*
*Accepted April 20, 1999*