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Cell-Free DNA in Serum and Plasma: Comparison of ELISA and Quantitative PCR, *Stefan Holdenrieder,[1*] Petra Stieber,[1] Lisa Y.S. Chan,[2] Sandra Geiger,[1] Andreas Kremer,[1] Dorothea Nagel,[1] and Y.M. Dennis Lo[2]* ([1] Institute of Clinical Chemistry, University of Munich, Munich, Germany; [2] Department of Chemical Pathology, The Chinese University of Hong Kong, Hong Kong SAR, China; * address correspondence to this author at: University Hospital of Munich-Grosshadern, Institute of Clinical Chemistry, Marchioninistrasse 15, D-81377 Munich, Germany; fax 49-89-7095-6298, e-mail Stefan.Holdenrieder@.med.uni-muenchen.de)

Although circulating DNA has generally been referred to as cell-free DNA, it is likely that a significant proportion is bound to protein molecules, possibly as nucleosomes. This conclusion is supported by theory and by observations. Theoretically, circulating DNA is mostly released from degrading cells after cleavage by endonucleases that cut the chromatin into the basic nucleosomal elements *(1, 2)*. Empirically, DNA fragments in circulation are mainly sized in multiples of the nucleosomal DNA *(3, 4)*. Filtration experiments have shown that circulating RNA seems to be associated with particles, whereas DNA is not *(5)*. This might be attributable to the arrangement of DNA in nucleosomes, which conserves them from proteolytic digestion in blood. Nucleosomal complexes consist of duplicate copies of the histones H2A, H2B, H3, and H4 as core proteins, with ~146 bp of DNA on the outside *(6)*.

In various pathologic conditions, qualitative and quantitative changes in circulating DNA have been shown. Only small amounts of serum or plasma DNA have been observed in healthy individuals, whereas high concentrations have been described in patients with various malignancies and in those with several benign diseases, such as infections, sepsis, trauma, stroke, and autoimmune diseases *(3, 7–13)*. Because most of these disorders are associated with increased rates of cell death events, from either apoptosis or necrosis, these mechanisms are considered to be the main sources for circulating DNA. Active release of DNA by lymphocytes is thought to be of minor relevance *(2, 14–16)*. In cancer patients during chemo- and radiotherapy, the kinetics of circulating DNA correlated with tumor response to therapy and also with posttherapeutic tumor recurrence *(13, 17–19)*. Recently, it was shown that initial changes in nucleosomal DNA during chemotherapy are predictive of therapeutic efficacy *(19)*.

Various methods have been established for the measurement of circulating DNA. Quantification of DNA in plasma and serum by real-time PCR is widely accepted as

standard *(20)* and detects all kinds of free and protein-bound circulating DNA. Recently, an alternative method for the direct immunologic detection of nucleosomal DNA in serum and plasma has been described *(21)*. This method quantifies only the histone-bound fraction, which is supposed to be the main part of circulating DNA *(3, 4)*. As the 2 methods potentially detect differing fractions of circulating DNA, we compared their results.

We tested 92 samples, both serum and EDTA-plasma, from 5 patients with pancreatic cancer receiving radiochemotherapy (observation period, 36–48 days) and from 6 patients after cerebral stroke (observation period, 4–6 days). The study was approved by the institutional ethics committee. Informed written consent was obtained from either the patient or a relative in all cases.

For determination of nucleosomes, the serum and plasma samples were centrifuged at 3000*g* for 15 min. Additionally, serum was treated with 10 mmol/L EDTA

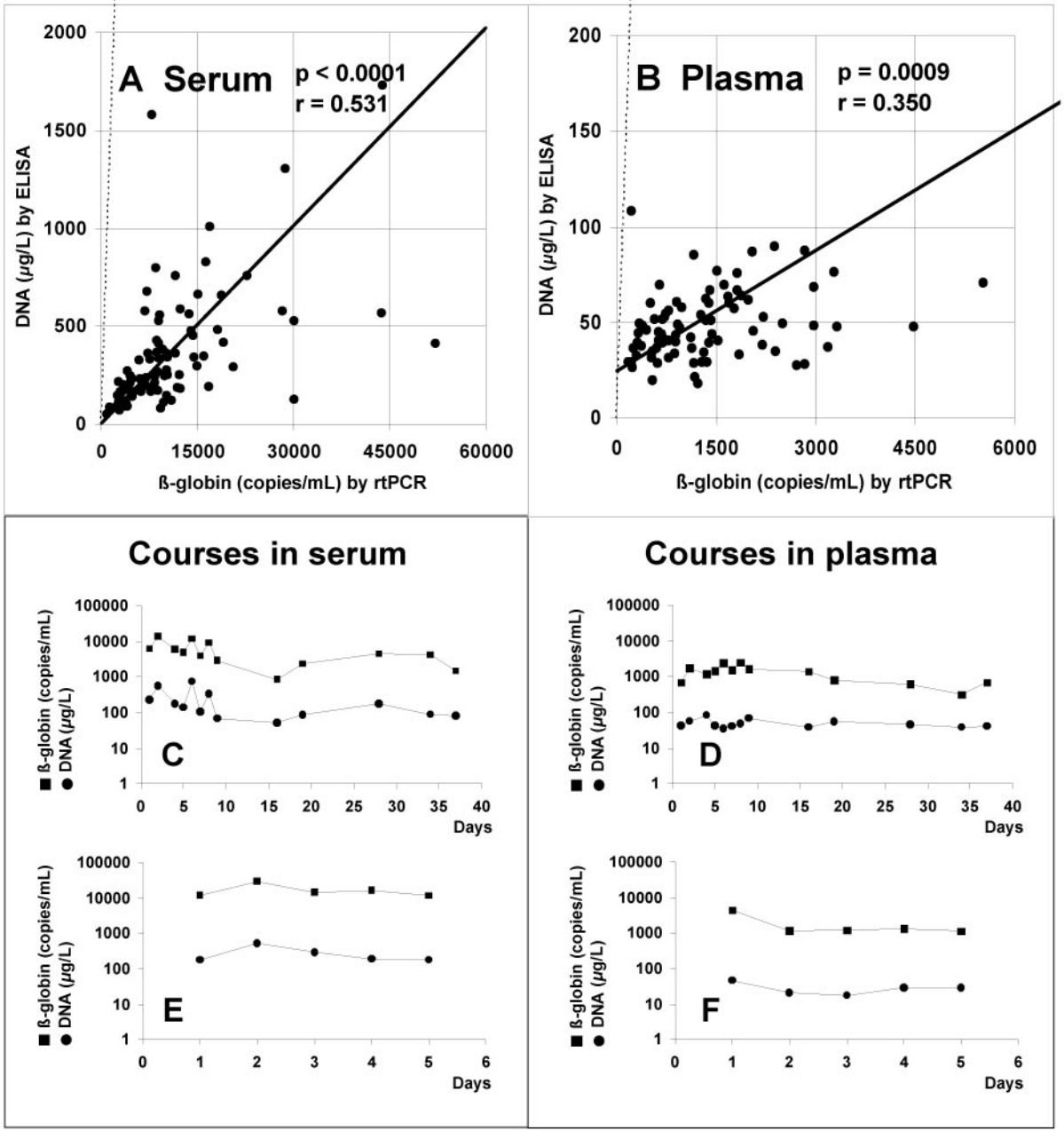

Fig. 1. DNA quantification by real-time PCR and ELISA.

(*A* and *B*), correlation of DNA quantification by real-time PCR (β-globin; copies/mL) and ELISA (DNA; µg/L) in serum (*A*; $r = 0.531$; $P < 0.0001$) and plasma (*B*; $r = 0.350$; $P = 0.0009$). (*C–E*), courses of DNA determined by real-time PCR (β-globin; copies/mL; ■) and ELISA (DNA; µg/L; ●) in sera (*C*) and plasma (*D*) of a patient with pancreatic cancer receiving radiochemotherapy and in sera (*E*) and plasma (*F*) of a patient after cerebral stroke.

(pH 8) immediately after centrifugation. Subsequently, both samples were divided into aliquots, stored at −70 °C, and analyzed in batches containing all samples from a single patient. The nucleosome ELISA (Cell Death Detection ELISA^{plus}; Roche Diagnostics) is based on a quantitative sandwich enzyme immunoassay principle. Monoclonal mouse antibodies directed against DNA (single-stranded and double-stranded) and histones (H1, H2A, H2B, H3, and H4) detect specifically mono- and oligonucleosomes. The anti-histone antibody additionally binds to the microtiter plate, whereas the anti-DNA antibody labeled with peroxidase reacts with 2,2′-azinobis(3-ethylbenzothiazoline sulfonate) *(21)*. The amount of captured nucleosomes is proportional to the resulting color development and enables quantification in nanograms per milliliter DNA by use of a calibration curve constructed with known amounts of nucleosomal DNA.

For measurement of DNA by PCR, serum and plasma samples were stored at −70 °C immediately after centrifugation. Subsequently, they were transferred to the Chinese University of Hong Kong for analysis by real-time PCR quantifying β-globin gene sequences *(20)*.

Correlations between the methods were tested by the Spearman rank test. A P value <0.05 was considered statistically significant. Concordance rates of parallel increases and decreases in results obtained by ELISA and real-time PCR were calculated for both serum and plasma formats, respectively.

Results of the nucleosome ELISA and real-time PCR assay were highly correlated in serum ($r = 0.531$; $P < 0.0001$). In plasma, DNA concentrations were considerably lower, but a significant correlation between both methods was found ($r = 0.350$; $P = 0.0009$).

In sera of patients during radiotherapy, both methods showed high peaks in the first week of therapy and mainly decreasing values during the following weeks. In patients after stroke, increases in the values in the first days after infarction were detected by both ELISA and real-time PCR. When the occurrences of parallel increases and decreases in serum were tested, ELISA and real-time PCR showed a concordance rate of 77.3%. In plasma, the concordance rate of both methods was 66.7% (Fig. 1; also see the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol51/issue8/).

Our results suggest that the nucleosome ELISA and real-time PCR methods detect similar DNA fractions in serum. The correlation of single values as well as the concordance rate in kinetic investigations is considerable taking into account that the 2 methods use completely different quantification systems. Although DNA concentrations in serum were notably higher than in plasma, possibly because of additional release of DNA during the clotting process, we minimized the error caused by interfering factors before and after centrifugation by following a strict preanalytical protocol for the handling of serum samples *(21)*. The 2 methods also showed a considerable correlation for plasma samples, although the imprecision of both assays is higher in the low concentration ranges *(21)*.

In conclusion, the high concordance in the kinetic observations indicates that both nucleosomal ELISA and real-time PCR methods have applications in monitoring cell death events inside the body and have the potential to monitor patients during therapy. Because the ELISA technique has advantages in cost, time, and labor, it may be an alternative to quantitative PCR, particularly for serial measurements.

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