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SHORT COMMUNICATION

Qualification of M30 and M65 ELISAs as surrogate biomarkers of cell death: long term antigen stability in cancer patient plasma

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Abstract
Purpose M30 and M65 ELISAs are proposed as surrogate biomarkers of tumour cell death in patients and are being applied increasingly in the pharmacodynamic (PD) evaluation of anticancer drugs during clinical trials. In the absence of such data, we have studied the long-term stability of the antigens of both assays in plasma of cancer patients stored at −80°C over 2 years.
Results No evidence was detected of degradation in the M65 antigen. However, in a proportion of patients significant increases in levels of M30 antigen were detected
Conclusion Plasma samples for M65 analysis can be stored at −80°C for 2 years; however, caution is recommended when considering long-term storage of samples for the M30 assay.

Keywords M65 ELISA · M30 Apoptosense · Biomarkers · Stability · Plasma · Cancer patients

Introduction

M30 and M65 are relatively new ELISAs that detect different circulating forms of the epithelial cell structural protein cytokeratin 18 (CK18) and have been proposed as surrogate assays of tumour cell death [1]. The M30 antibody recognises a neoepitope (NE) mapped to positions 387–396 of a 21 kD fragment of CK18 (CK18 Asp396 NE) that is only revealed after caspase cleavage of the protein and is postulated as a selective biomarker of apoptotic cell death [2]. M65 ELISA utilises the M5 antibody to detect a common epitope present in the full length protein as well as the 21 kD caspase cleaved fragment [3] and is thus believed to measure, in addition to apoptosis, intact CK18 that is released from cells undergoing necrosis [4]. Both ELISAs are now being increasingly applied in the pharmacodynamic (PD) evaluation of anticancer drugs during clinical trials [3, 5, 6].

We have previously reported that the stability of the M30 and M65 antigens in plasma is limited to 4–6 months after storage at −80°C [7]. However, these studies utilised healthy volunteer plasma spiked with a recombinant human CK18 protein and were conducted over a limited period of time. The present communication extends our previous studies and reports on the long-term stability of the M30 and M65 antigens in plasma collected from cancer patients stored at −80°C.

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Materials and methods

Reagents

The M30 Apoptosense and M65 ELISA kits were both obtained from PEVIVA AB (Bromma, Sweden) and were operated essentially according to the manufacturers instructions (http://www.peviva.com) and as previously described [5, 7].

Collection of plasma samples from cancer patients

Samples were collected at multiple time points spanning the first 3 week treatment cycle from 20 patients who had received AEG35156 (XIAP antisense) at multiple dose levels from 48 to 160 mg/m^2/day during a phase I trial [8]. Within 30 min of collection bloods were centrifuged at 1,000*g* for 10 min to obtain plasma which was transferred to 3 ml polypropylene cyrotubes. Tubes were then frozen at −20°C within 30 min and transferred to −80°C within 3 h.

Design of the stability study

Stability of the M30 and M65 antigens was evaluated by conducting two assays on each cancer patient's plasma sample. The first ELISA was performed on an ongoing basis patient-by-patient over a 26-month period of accrual, always within at least 3 months of sample receipt. After the first analysis, samples were returned to the freezer and stored continuously at −80°C until the second assay. The second assay was carried out on all patient samples as a batch approximately 4 months after the accrual of the last patient. Thus, a variety of samples were effectively incubated at −80°C for between 4 and 26 months. Due to the prolonged period of storage, plasma samples were centrifuged prior to analysis as well as being analysed without any sample preparation, as per the first assay. No single sample was thawed more than three times. The values obtained from the second assay were normalised against the value obtained for the first assay in order to define stability in terms of a percentage.

Results

Results of the long-term stability studies for the cohort of cancer patients investigated are summarised in Fig. 1 for the M65 assay and Fig. 2 for the M30 assay. Here, each bar represents a single patient and the value is the mean percentage [± standard deviation (SD)] obtained after normalising the result of the second assay against the result of the first assay for each of the 3–5 different time points. Also included in Fig. 1 is the 100% line to signify the no change value with time and highlight differences in terms of a percentage increase and decrease above and below the line, respectively. In addition, a typical individual patient PD profile generated from samples analysed during the trial compared to that obtained after prolonged storage at −80°C is shown in Fig. 3 for the M65 assay and Fig. 4 for the M30 assay.

With the M65 ELISA, there was little evidence of a time dependent reduction in antigen levels that would signify instability, where the mean values in 11 of the 12 patients did not significantly deviate from the 100% line (Fig. 1), especially when taking account of the 20% imprecision associated with the technique itself [7]. Centrifugation of samples resulted in a small but significant increase in antigen concentration compared to non-centrifugation (Student's *t* test) in 5 of the 12 patients; however, during the original analyses samples were not subjected to centrifugation. As well as an overall lack of instability, the pattern of individual patient profiles was also preserved after long term storage (Fig. 3).

In the case of the M30 ELISA, while there was no time dependent reduction in antigen levels, there was a large increase in levels above the 100% line by 55–114% in 6 of the 11 patients (Fig. 2). Nevertheless, this phenomenon was randomly distributed with time. Centrifugation had less of an impact on M30 compared to the M65 ELISA. Despite the sometimes large increase in values that occurred with storage, individual patient M30 PD profiles were well conserved (Fig. 4).

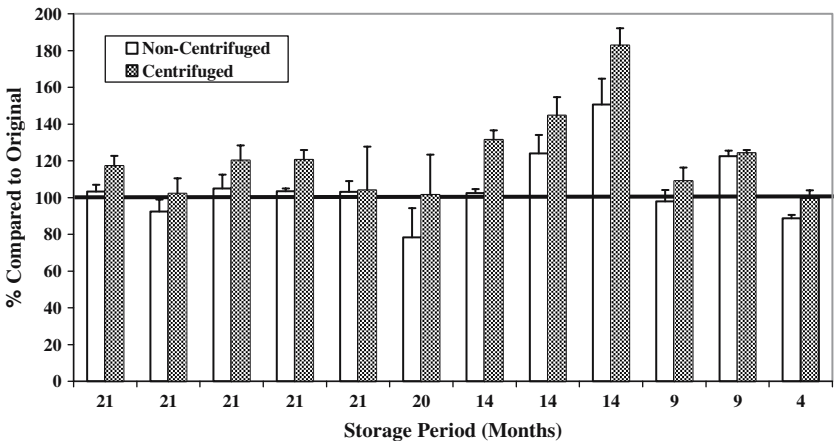

Fig. 1 Long-term stability of the M65 antigen in cancer patient plasma stored at −80°C. Each sample was analysed twice: first, during a phase I trial and second after long term storage. Results from the second assay were expressed as a percentage of the results obtained from the first assay. Each bar represents the mean % ± standard deviation of 3–5 different time point samples taken from a separate patient

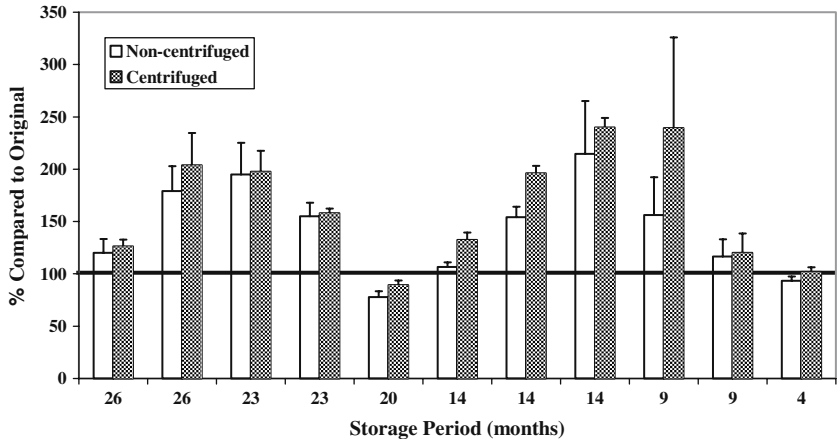

Fig. 2 Long-term stability of the M30 antigen in cancer patient plasma stored at −80°C. Each sample was analysed twice: first, during a phase I trial and second after long term storage. Results from the second assay are expressed as a percentage of the results obtained from the first assay. Each bar represents the mean % ± standard deviation of 3–5 different time point samples taken from a separate patient

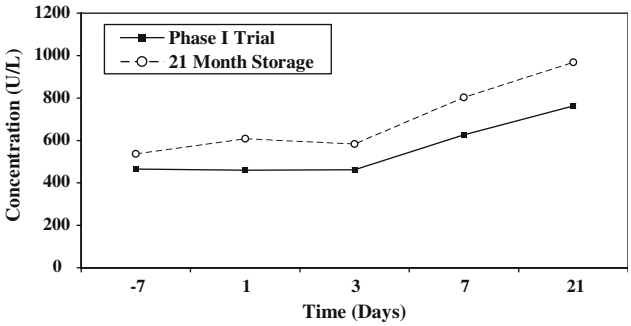

Fig. 3 Pharmacodynamic profiles of the M65 antigen in cancer patient (07), plasma analysed during a phase I trial and after a 21-month storage period at −80°C

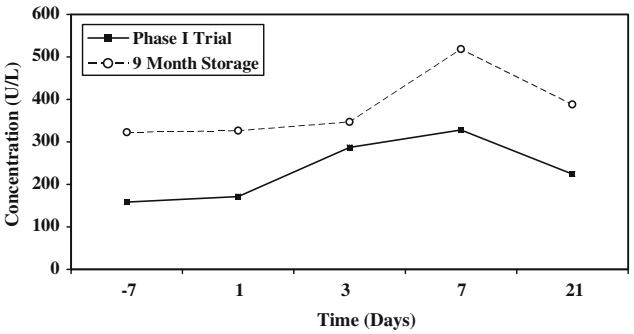

Fig. 4 Pharmacodynamic profiles of the M30 antigen in cancer patient (18), plasma analysed during a phase I trial and after a 9-month storage period at −80°C

Discussion

In the present study, we have demonstrated an apparent lack of downward instability in the M30 and M65 antigens in cancer patient plasma when stored at −80°C in contradiction to our recently published data in healthy human volunteer plasma [7]. However, in that previous study more rigid criteria were applied to define instability, where significance was defined by a statistical reduction in concentration between two consecutive time points, and these studies were not extended over the long term. Moreover, analogous stability studies with the long established biomarker of apoptosis, circulating DNA nucleosomes, in many cases utilising a sandwich ELISA assay, have also demonstrated increased stability in cancer patient serum compared to healthy subject serum, due to reduced circulating endonuclease activity [9].

Unlike the recombinant CK18 protein used in our previous stability studies [7], both the endogenous M30 and M65 antigens are believed to consist of polymeric complexes which result in enhanced stability over their monomeric counterparts [4, 10]. Despite that, these complexes clearly remain soluble in plasma, since centrifugation of plasma samples often produced an increase in concentration of antigen available for detection by the M30 and M65 assays, presumably due to removal of particulate matter that accumulated on storage and partially interfered with the sandwich ELISAs.

Keratins in general and CK18 specifically are known to exhibit good chemical stability, especially in the case of the caspase cleaved fragments of CK18 [11, 12]. We have also confirmed in our previous stability studies that caspase cleaved CK18 (CK18 Asp396 NE) appears to be more stable than the intact protein [7]. Therefore, it was surprising that, in the present study, more variability in measurements were observed with the M30 assay, in the form of significant rises in antigen concentration. Since the effect was randomly distributed throughout the 26-month storage period, it would appear that it was not time dependent, but that other factors must have been responsible. By incorporation of in-house quality controls (QCs) in all our M30 and M65 analytical runs of patient samples, we were able to

conduct independent quality control assessment on individual kit performance [5, 7]. During the time course of the patient accrual and sample analysis as many as five different production batches of kits were utilised, giving rise to the possibility that the variability we observed in the M30 assay results may have been due to variable kit to kit QC. However, the data obtained with the independent QCs revealed that kit to kit and batch to batch variation never exceeded more than 10%, and therefore could not account for increases in excess of 50% observed in the cancer patient samples after storage.

Investigations on CK18 breakdown and reorganisation during apoptosis have shown that the cleaved product is retained in impermeable apoptotic bodies, until it is eventually released by an efflux mechanism that remains poorly characterised [4]. Apoptotic bodies can be detected in human serum/plasma and care has to be taken when preparing serum for the nucleosomal assay to prevent artefactual disruption of cellular components including apoptotic bodies [13, 14]. Thus, it is possible that varying levels of apoptotic bodies were present in patient plasma samples and that upon prolonged storage and two rounds of freeze thawing disruption occurred resulting in a release of soluble CK18 fragments.

An alternative explanation is that in some patient's plasma, but not others, variable and trace caspase activity may have been present promoting in situ conversion, either during thawing or less likely on prolonged exposure at −80°C, of intact CK18 into CK18 Asp396 NE, the M30 epitope. Elevated circulating caspase activity has been reported in cancer patients compared to normal healthy subjects, although marked inter-patient variations were noted [15, 16]. In the cohort of patients studied the M65 assay always detected values two to fivefold higher than those determined by the M30 assay, although both are calibrated with the same recombinant CK18 peptide fragment and are directly comparable. Thus in relation to the caspase cleaved fragment the intact protein was present in excess. However, caspase cleavage of CK18 would not necessarily result in a reduction in the signal detected by the M65 ELISA, since the cleavage product CK18 Asp396 NE would also register in this assay.

In conclusion, we demonstrate that M65 and M30 antigens do not degrade with time in cancer patient plasma stored at −80°C for over 2 years. Centrifugation of plasma samples stored long term is recommended for purely practical reasons. However, caution is recommended when considering long-term storage of samples for the M30 assay. Although overall PD profiles are preserved in a significant proportion of patients after long-term storage artefactual elevations in levels of M30 antigen occurred.

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