Serum biomarkers of apoptosis

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Biomarkers

Biomarkers, and in particular biomarkers of tumour cell death, the desired endpoint of most therapies, have an important part to play in the clinical management of cancer. The most frequently used definition in the literature of a biomarker is 'a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention' [1]. This chapter will examine the role of novel circulating biomarkers of apoptosis in the care of patients with cancer, and in particular, their use in monitoring response to therapy.

In trials of new anticancer drugs targeting apoptosis, pharmacodynamic effects on gene or protein expression in the tumour, in response to therapy, can be measured. However, blood-based molecular tests that can be performed on circulating cancer cells or serum, rather than tumour tissue-based tests, are less invasive and much more readily applied to most clinical settings. Additionally, serial biopsies of a patient's tumour are rarely feasible; if a biomarker can be detected in the blood and sampled serially it can act as a surrogate to provide a dynamic view of the individual patient's disease course and response to treatment [2].

Following the demonstration of the clinical utility of serum biomarkers there has been a call for the integration of biomarkers as pharmacodynamic (PD) endpoints into the development of new anti-cancer therapies [3–5]. The development of 'targeted' therapies, such as imatinib and trastuzumab, has led to a re-evaluation of the development and licensing of new anti-cancer drugs. The standard first step towards introducing a drug into human use ('Phase I trial') evaluates its toxicity and sets the dose for subsequent studies. Present practice is to determine the maximum dose with an acceptable side-effect profile (the Maximal Tolerated Dose or MTD). Targeted biological therapies often have a larger therapeutic index than cytotoxics; therefore, dosing at the MTD may be exposing the patient to unnecessary toxicity and possibly may reduce the efficacy of the drug [6,7]. This has led to the concept of determining the Effective Biological Dose (EBD), and minimal biological effective dose (MBED) which can be determined by pharmacodynamic endpoints [3,7]. Products derived during apoptosis have the potential to be the basis of a generic assay of response to therapy. Assays to measure circulating products of apoptosis have been developed [8,9] and have shown early promise as prognostic and therapeutic biomarkers [10,11].

Apoptosis markers as a potential generic assay

Apoptosis is an important physiological process of cell death that is meant to occur without the release of intracellular contents and subsequent activation of an inflammatory response. This is important in embryonic development, regulation of the immune system and the response to DNA damage. However, dysregulation of apoptosis is an important process in carcinogenesis, with an imbalance between cell proliferation and cell death being a hallmark of the malignant process [12]. In tumour samples, increased proliferation is associated with an increased number of cells undergoing apoptosis. Certain oncogenic proteins such as c-Myc stimulate both proliferation and apoptosis, although in the tumour, proliferation will predominate [13]. Therefore, in more aggressive tumours of a higher histological grade, more apoptosis is expected and can be observed [14]. This may account for the fact that in certain scenarios, high levels of apoptosis in the tumour independently predict a worse prognosis (e.g. [14,15]).

Cytotoxic chemotherapy can act by a variety of mechanisms affecting DNA and the cell cycle, but may have the final common effect of causing apoptosis [14,

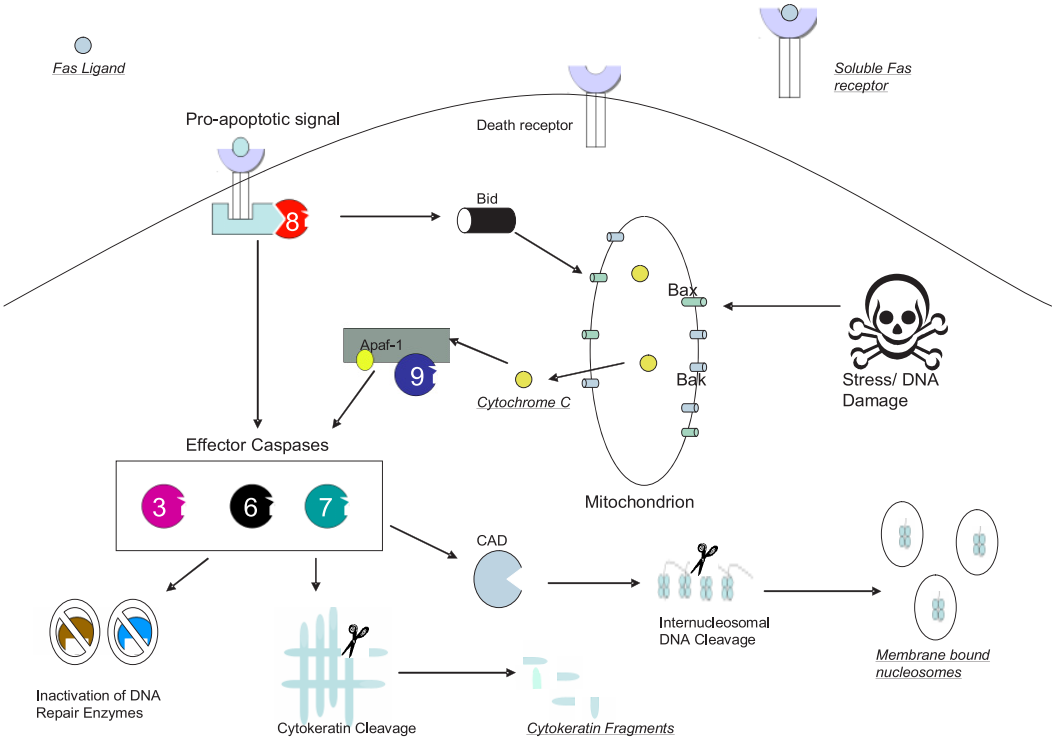

Fig. 1. The machinery of apoptosis. Molecules that have been measured in blood as surrogate markers are italicised and underlined.

16–20], shifting the proliferation:apoptosis balance. Clinical response to chemotherapy is related to the amount of apoptosis induced [21]. One of the mechanisms of chemotherapy resistance is the blockade of apoptotic pathways [22–26], and this may provide cross-resistance to multiple agents. This has led to interest in targeted agents that increase the cancer cell's sensitivity to apoptosis [2]. These drugs have potential both when given as anti-neoplastic agents and administered synergistically with chemotherapy to overcome resistance. Agents targeting the apoptotic machinery by a variety of mechanisms are in clinical development (reviewed in [27]).

The apoptotic machinery

Apoptosis occurs by the activation of a proteolytic cascade of apoptosis-specific enzymes, the caspases [28]; these are cysteine proteases that are constitutively expressed as proenzymes in the cell [29]. They are activated by proteolytic cleavage causing removal of a prodomain and cleavage into large and small subunits to form an enzyme that is specific for aspartate containing sequences.

There are two sub-groups of caspases; the upstream signalling group initiator caspases (caspases 2, 8, 9) which are responsible for the initial recognition and propagation of death signals leading to the proteolytic activation of downstream effector caspases (caspases 3,6 and 7). Apoptosis can be triggered by two separate pathways (see Fig. 1). The intrinsic pathway is initiated at the mitochondria under conditions of cellular stress or in response to DNA damage. This process is under the control of proteins belonging to the Bcl-2 family, which includes both pro-apoptotic and anti-apoptotic members [30]. The balance of these determines the cell response with a pro-apoptotic signal resulting in localisation and integration of Bax and Bak (Bcl-2 family proteins) into the mitochondrial membrane leading to the release of apoptogenic factors [31]. Cytochrome C, one of the mitochondrial proteins involved in the respiratory chain, is released into the cytoplasm and is responsible for the propagation of the apoptotic pathway. It forms a complex with Apaf-1 and procaspase-9 (the apoptosome) leading to homodimerisation and activation of the latter enzyme [32,33].

The extrinsic pathway is activated by pro-apoptotic ligands (such as Tumour Necrosis Factor α, TRAIL, Fas Ligand and apolipoprotein A2) binding to death receptors on the cell surface. Receptor activation recruits adaptor proteins (such as Fadd/Mort-1) which

interact through its C-terminal Death Domain [34]. Pro-caspase-8 then binds to the N-terminal Death Effector Domain of the adaptor protein forming the Death-Inducing Signalling Complex [35].

Homodimerisation of caspase-8 can then occur with resulting autocatalytic activation [36]. Caspase 8 can then activate downstream effector caspases. There is crosstalk between the two pathways; in particular, caspase 8 activates the pro-apoptotic member of the Bcl-2 family Bid [37], leading ultimately to release of mitochondrial apoptogens and subsequent formation of the apoptosome. This is thought to be necessary for amplification of the apoptotic signal in all cells apart from leukocytes [38].

The two pathways intersect to form a final common pathway with the activation of the effector caspases, which then have a variety of targets (see Fig. 1 and ref. [29]). They break down cytokeratins and other structural proteins leading to the reorganisation of the cells' cytoskeleton. They also control the inactivation of DNA repair enzymes such as PARP and the activation of apoptotic endonucleases such as CAD leading to condensation and fragmentation of the DNA. There is also a loss of phospholipid asymmetry in the membrane and membrane blebbing. Therefore, caspase activation results in the classical histological hallmarks of apoptosis [39]. Apoptosis leads to the eventual generation of membrane bound DNA fragments. *In vivo* these fragments are then phagocytised by neighbouring cells and macrophages.

Assessment of apoptosis in patients

The gold standard measurement of apoptosis in tumours is histochemical assessment of molecules specific to apoptosis. The TUNEL assay labels the DNA ends generated during apoptosis; labelled bromodeoxyuridine is incorporated by the action of terminal deoxynucleotidyl transferase [40]. IHC can label proteins cleaved by caspases such as Lamin, CK 18 or PARP [41,42]. In addition, antibodies which specifically recognise the activated form of both initiator and effector caspases can be used as tissue biomarkers of apoptosis. IHC allows assessment of early expression of proteins which may not be detectable in biofluids. It also gives information on protein localisation within the cell and allows direct correlation with histological changes [42]. However, serial tumour sampling in the clinic is difficult both practically and ethically.

Annexin V binds to phosphatidylserine, an early biomarker of apoptosis. When recombinant human (rh) Annexin V is combined with a (99 m) Tc-radiolabel, it can be used to image areas of apoptosis within tumours [43]. Belhocine reported that detection of apoptotic signal correlated with response and progression free survival in patients with lung cancer and lymphoma but not in those with breast cancer [44]. However, there are problems differentiating the drug-induced signal from that of physiological apoptosis. In addition, this approach is currently not licensed for clinical use, precluding widespread adoption of this technology.

Measurement of surrogate biomarkers in serum has potential advantages. They allow the development of a cheap, minimally invasive test that can be performed on multiple occasions to give a dynamic picture of response or tumour behaviour. This could guide adjustments of dose and schedule and determine appropriate times for biopsies or imaging. However, it is important that the biomarker is released into the blood, is derived from tumour or reflects tumour behaviour.

All the changes in cellular environment that occur in apoptosis can potentially be measured as surrogates of apoptotic cell death. The proapoptotic FAS ligand (sFasL), which binds onto the death receptors can be measured in blood, as can a soluble form of the receptor (sFas) that prevents ligand receptor binding at the membrane [45–47]. The release of cytochrome C can be detected in extra cellular medium and subsequently in blood [48,49]. Cytoskeletal fragments released by apoptotic cells can be detected both in the cells themselves by IHC and in sera [50–52]. Cleaved DNA fragments are eventually detectable in the blood particularly in pathological processes when the mechanism of plasma clearance may become overloaded [53]. The studies that have been published using these cytokeratin 18 products and nucleosomal DNA to monitor response to chemotherapy are summarised in Tables 1 and 2.

Serum Fas and Fas Ligand

The Ligand for the Fas receptor (sFasL) can be measured in the serum and is pro-apoptotic on binding to the membrane bound receptor. A soluble version of the Fas receptor (sFas) which can compete with the membrane bound version for the ligand and protect the cell from the apoptotic stimulus is also detectable in the serum [63]. These biomarkers (sFas and sFasL) have been reported to be elevated in patients with a variety of cancers compared to healthy controls. These include breast cancer, NHL, colon cancer, gastric cancer and ovarian cancer [64–68,46].

Table 1
Summary of studies reporting the use of cytokeratin 18 (CK 18) products to monitor response to chemotherapy

Paper	Biomarker examined	Disease group	Number of patients	Chemotherapy regimen	Response to therapy	Correlation with response
Pichon 2006 [45]	$CK18^{asp296}$	H&N Breast Others	18 17 7	Various	Increase	Not reported
Kramer 2006 [54]	$CK18^{asp296}$	Prostate cancer	82	Estramustine/Vinorelbine Estramustine/Docetaxel	Increase Increase	Not Reported
Demiray 2006 [55]	$CK18^{asp296}$	Breast cancer	42	Neoadjuvant anthracycline based chemotherapy	Increase	Increase associated with response
Uluyaka 2007 [11]	$CK18^{asp296}$	NSCLC and SCLC	18	Cisplatin + Gemcitabine or Vinorelbine (NSCLC) Cisplatin + Etoposide (SCLC)	Increase	None reported Basal level >43.8 U/L associated with better overall survival
Oloffson 2007 [56]	$CK18^{asp296}$	Breast Cancer	43 18	CEF Docetaxel	Increase Increase	None reported None reported
Oloffson 2007 [56]	CK18	Breast Cancer	43 18	CEF Docetaxel	Increase Increase	Increase>18% associated with overall survival None reported
Kramer 2006 [54]	CK18	Prostate cancer	82	Estramustine/Vinorelbine Estramustine/Docetaxel	Increase Increase	Not Reported

$CK18^{asp296}$ = CK18 neo-epitope recognised by the M30 assay; H&N = squamous carcinomas of the head and neck, SCLC = Small Cell Lung Cancer, NSCLC = Non Small Cell Lung Cancer, CEF = therapy with cyclophosphamide, epirubicin and 5-FU.

Table 2
Summary of studies reporting the use of nucleosomal DNA to monitor response to chemotherapy

Paper	Disease group	Number of patients	Chemotherapy regimen	Response to therapy	Correlation with response
Holdenreider 2001 [57]	Various	20	Various	Rapid increase followed by slow decrease	Decrease from baseline >50% associated with response
Trejo-Becerril 2003 [58]	Cervical	11	Gemcitabine/Oxaliplatin	Variable	Decrease associated with response.
Holdenreider 2004 [59]	NSCLC	212	Platinum based therapy	Increase then decrease	Lower AUC (1–8) correlated with response
Trejo-Becerril 2005 [60]	Cervical	41	Carboplatin/paclitaxel	Decrease	Decrease from 1st to 3rd cycle correlated with response.
Mueller 2006 [61]	AML	25	Mostly TAD or HAM (two others)	Decrease (following an initial increase in some patients)	AUC (2–4) lower in patients without CR than others
Kremer 2006 [62]	Colorectal	25	Radiochemotherapy with infusional 5-FU	Decrease followed by increase and subsequent decrease	AUC (1–3) higher in patients with PD than others
Holdenreider 2006 [10]	NSCLC	311	Platinum Based therapy	Increase then decrease	Lower AUC (1–8), baseline levels and levels prior to 2nd treatment correlated with response

NSCLC = Non Small Cell Lung Cancer, AML = Acute Myeloid Leukaemia, TAD = therapy with cytarabine, daunorubicin and 6-thioguanine, HAM = therapy with cytarabine and mitoxantrone, AUC = area under the curve.

Serum cytochrome C

As described earlier, cytochrome C is released from mitochondria during apoptosis. Serum levels of cytochrome C have been shown to be elevated in patients with haematological malignancies [48]. Subsequent falls in the serum levels of cytochrome C correlated with response in a study of 21 patients with a variety of malignancies. Liver and epithelial toxicity also produced a detectable rise underlying its potential utility as a marker of apoptosis [49].

Cytokeratins

The breakdown of the cytoskeleton is an important event in apoptosis as this is the point of 'no return'. Cells can retreat from earlier steps in the induction of apoptosis such as release of mitochondrial contents [69,70], whilst dismantling the cytoskeleton occurs after this event [71] but prior to the break-up of the chromatin [41]. Cytokeratins are important members of the cytoskeleton in cell support and in response to stress. Differences in CK expression can be found in chemo-resistant cells [72] and may affect a cell's response to lethal stimuli and the form of cell death that occurs. CK 18 and 19 (members of the intermediate filament family) form hetero-dimers with CK 8 and are expressed extensively in epithelial cells and in solid tumours [73]. CK 18 is a target of the caspases; Caspases 3, 7 and 9 all appear capable of cleaving CK 18 with the final collapse of the cytoskeleton caused by its cleavage by caspase 6 [71]. Cytokeratins are estimated to form 5% of intracellular proteins so apoptosis of small numbers of cells should be detectable [74]. Fragments of CKs are released into the blood where they form relatively stable aggregates that are detectable in patients [75]. Specific antibodies have been developed for use in ELISAs to quantify these fragments. The first assay with clinical potential was TPA (tissue polypeptide antigen), which measured fragments of CK8, 18 and 19. More specific assays have subsequently been developed with TPS (tissue polypeptide specific antigen) measuring solely CK 18 fragments whilst CYFRA21-1 measures CK 19 fragments [76]. CYFRA21-1 is elevated and can predict prognosis in many epithelial cancers (see review [77]), but has been particularly well characterised in NSCLC [78,79]. It has been successfully used to monitor response to therapy in NSCLC, head and neck cancer and cervical cancer [52,80,81]. In 311 patients with NSCLC treated with first-line chemotherapy, responders had lower baseline levels, more rapid falls and lower measurements immediately prior to the second cycle of chemotherapy. In a multivariate analysis, CYFRA21-1 levels predicted response independently from stage, performance status and levels of another apoptotic product nucleosomal DNA (see below) [10].

The assays described above are not specific to apoptosis. Equilibrium exists between insoluble CKs in the skeleton and a soluble intracellular pool. Necrosis with its subsequent membrane disruption will cause release of this soluble pool [82], which will be detected by these assays. However, in 1999, Leers and colleagues described a monoclonal antibody (M30), which is specific to a neo-epitope only expressed during apoptosis. Caspases 3, 7 and 9 cause cleavage of CK 18 at aspartate 238 and 396 [83]. The M30 antibody recognises a ten amino acid neo-epitope ($CK18^{asp396}$) that is exposed following cleavage at the sequence 393 DALD-S [41]. M 30 was initially used attached to a peroxidase as an IHC stain for apoptotic cells. It was further developed into an ELISA that can detect the cleaved CK 18 product in biological fluids [51].

$CK18^{asp396}$ is detectable in low levels in normal subjects [8,11,45,84], but higher levels are detectable in patients with a range of solid malignancies [45,51, 74]. *In vitro* the half life of these CK 18 fragments appears to be approximately 2.5 days [85]. *In vivo* the $CK18^{asp396}$ fragments are found in a complex with other CKs (i.e. CK 7, 8 and 19) protecting the small fragments from rapid clearance by the kidneys [56]. The actual half life *in vivo* has yet to be determined but removal from the blood appears to be performed by the spleen as it resumes its embryonic role (when large numbers of cells are undergoing physiological apoptosis) and accumulates apoptotic fragments [86].

The exact mechanism by which $CK18^{asp396}$ reaches the circulation remains unclear. It is not known whether the fragments are derived directly from the tumour itself, circulating tumour cells or 'mosaic' cells (tumour cell inserted into the endothelial lining of the tumour vasculature). CK 18 fragments are released, but with a delay after generation, *in vitro*, probably due to membrane disruption of apoptotic fragments [71].

A similar ELISA using a monoclonal antibody (M65) that detects all forms of CK 18 has also been developed (see Fig. 2). This will detect CK 18 released not only during apoptosis but also in other forms of cell death such as necrosis. The use of the two assays in conjunction offers the potential to assess the balance between apoptosis and necrosis [82]. These assays could potentially be a generic biomarker of apoptosis and necrosis and, in particular, of the response to therapy.

The M30 Apoptosense® and M65® assays have now been thoroughly validated [85] and initial stud-

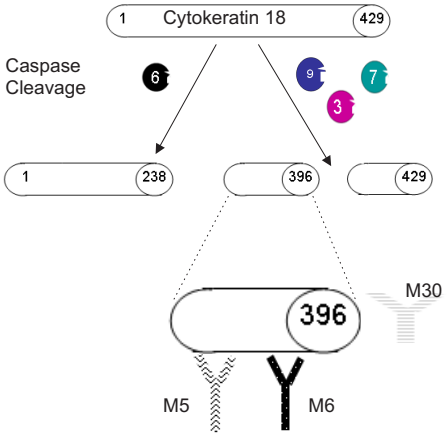

Fig. 2. Cleavage of CK18 by caspases demonstrating the epitopes on CK18 of M6 (capture antibody for M30 and M65 assays), M30 (detector antibody for the M30 assay) and M5 (detector antibody for the M65 assay). Adapted from Cummings and colleagues [87].

ies in patients receiving cytotoxics and apoptotic targeting therapies have been performed. Biven and colleagues reported that a treatment index (calculated by the maximum increase from baseline /pre treatment $CK18^{asp396}$ level) correlated with response in 32 patients receiving chemotherapy for recurrent breast cancer [74]. Interestingly, no correlation with response was seen in patients with a high pre-treatment $CK18^{asp396}$ result. This may be an artefact of their method of calculating the treatment index or the test may be unable to detect a treatment-induced effect if masked by high background rates of apoptosis. However, they reported that *in vitro* paclitaxel induced death in a breast cancer cell line which led to the release of approximately 10^{-5} U of $CK18^{asp396}$ [74]. This led to their estimation that death of only 10^8 cells should cause an increase in M30 antigen levels of 330 U/l given a circulating volume of 3 L. This should have been easily detectable. Recently, Pichon and colleagues reported their experience with 42 patients receiving differing regimens of chemotherapy in different tumour types (18 patients with head and neck cancer and 17 with breast cancer) [45]. They found no relationship between baseline $CK18^{asp396}$ levels and tumour site, tumour histology or presence of metastases. They observed an increase in patients receiving chemotherapy and this was most pronounced in patients with adenocarcinomas. Their data would imply the $CK18^{asp396}$ release is tumour related rather than a function of chemotherapy-induced toxicity.

In patients with breast cancer it has been demonstrated that $CK18^{asp396}$ level correlated with the number of organs involved with metastatic disease and with performance status [51]. It has now been demonstrated that in patients receiving neoadjuvant chemotherapy for breast cancer that a statistically significant rise in $CK18^{asp396}$ levels in responding patients was seen. The $CK18^{asp396}$ level measured 24 h after chemotherapy in this small study was able to predict future response with a sensitivity of 70% and specificity of 67% [55]. Unusually, baseline $CK18^{asp396}$ levels in this study were low and sometimes even undetectable prior to therapy so that analysis of the percentage increase (as reported) may not be the most appropriate statistical method.

Kramer and colleagues were the first to report their clinical experience utilising both CK 18 assays in 82 patients with hormone refractory prostate cancer with palliative chemotherapy (estramustine in combination with either docetaxel or vinorelbine) [54]. They found that docetaxel in particular caused an increase in the $CK18^{asp396}$ when measured 2 days after administration, although with a considerable spread between patients. This increase was maintained throughout eight cycles of docetaxel therapy. Similar increases were seen following the administration of vinorelbine chemotherapy although they were not as large or as well maintained as the docetaxel-induced changes. They hypothesised that this was a reflection of docetaxel's higher activity in this tumour type. Rises in $CK18^{asp396}$ were correlated with the baseline PSA and the baseline $CK18^{asp396}$ levels. Given that PSA reflects tumour volume, Kramer suggested that the $CK18^{asp396}$ is being derived from tumour cells rather than normal epithelial cells suffering toxicity. The administration of estramustine did not result in an increase in $CK18^{asp396}$; a rise in total CK 18 was seen but did not correlate with PSA suggesting that this may be a toxicity related signal. There was no attempt reported to correlate changes in CK spectrum with clinical responses or toxicity.

In summary, the studies published so far have demonstrated, using small numbers of patients, that it is feasible to detect changes in the M30Apoptosense® and M65® assays in patients receiving chemotherapy. These changes may reflect outcome, however, given the different treatments, patient populations and sampling times, firm conclusions are difficult to draw.

Nucleosomal DNA

Chromatin is found in the nucleus as the DNA strand wrapped around histone protein complexes. A nucleosome consists of one octomer of histone proteins, with a single 150 base-pair loop of DNA around [88]. During apoptosis the chromatin is cleaved

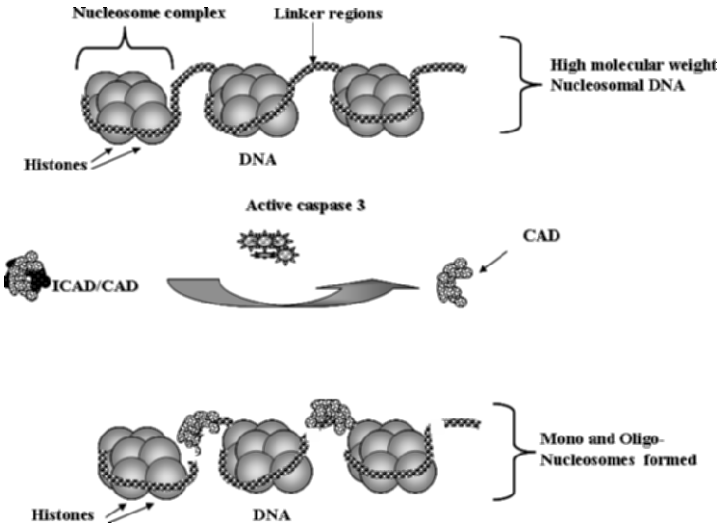

Fig. 3. Cleavage of nucleosomal DNA by caspase activated DNase (CAD). Intact nucleosomes are cleaved at the inter-nucleosomal linker region by CAD releasing mono and oligo nucleosomes.

into strands of one or more nucleosomes. This is initiated by caspase-activated deoxyribonuclease (CAD) an endonuclease normally found complexed to its inhibitor protein ICAD. The cleavage of the inhibitor of CAD (ICAD) by caspase 3 releases and subsequently activates CAD. This endonuclease preferentially cleaves DNA at the linker sites between the nucleosomes generating fragments of DNA 180 base pairs in length and multiples thereof (see Fig. 3). These mono- and oligo-nucleosomes in membrane bound apoptotic fragments are phagocytised by macrophages. This process is vital for the subsequent release of the nucleosomal DNA into the blood, probably as a result of secondary apoptosis of the macrophages [89]. The histone proteins partially protect the DNA from further degradation by nucleases in the serum.

Although theoretically specific to apoptosis, necrotic processes will also lead to the presence of nucleosomal DNA in the blood due to apoptosis of overwhelmed macrophages [89]. Circulating nucleosomal DNA levels are lower in cancer patients following tumour resection than in pre-operative patients suggesting that circulating nucleosomal DNA is at least partly tumour derived [62].

A sandwich Elisa has been developed that measures circulating nucleosomal DNA (Cell Death Detection ELISA^{plus}, Roche) [57] and has been validated in our laboratory (Nigel Smith, Paterson Institute). *In vivo* this nucleosomal DNA appears to have an extremely short half-life, of the order of minutes [90,91]. However, circulating levels are elevated in cancer patients and in patients with autoimmune conditions, suggesting either very high levels of production, altered metabolism or both [57,91].

Nucleosomal DNA appears to have similar potential to the cytokeratin assays to monitor therapy [57]. In an early study on 18 breast cancer patients receiving treatment with docetaxel, a peak in nucleosomal DNA was observed 24 h post chemotherapy. The size of this peak correlated with the extent of chemotherapy-induced myelosuppression whilst the subsequent fall in levels correlated with response to therapy [92].

Several studies looking at serum levels in patients receiving chemotherapy and radiotherapy have been published [59,61,62,93,94]. They initially reported that falls in nucleosomal DNA correlated with response to chemotherapy and radiotherapy. The rate of decline in the nucleosomal DNA appeared particularly important [57]. In further studies on 212 patients receiving chemotherapy treatment for Non Small Cell Lung Cancer, they described a number of parameters that differed in responders compared to non-responders [59]. Data was plotted using the results pre-treatment and on days 3, 5 and 8. A calculation of the area under this graph (AUC), which reflects both the baseline pre-chemotherapy level and changes in nucleosomal DNA after chemotherapy, was significantly correlated with response. The nucleosomal DNA levels immediately prior to treatment in the second and third cycles were also significantly different between responders and non-responders. In multivariate analysis, the level of nucleosomal DNA was an independent factor predicting response. More recently, a further study measuring nucleosomal DNA in 311 patients with stage III and IV NSCLC

reported by this group confirmed these findings [10]. Responding patients could be distinguished from those with progressive disease on treatment by the pre-therapeutic level, the value at Day 8 of the first cycle of treatment, the AUC over the first 8 days as described above and the value immediately prior to the second treatment. Nucleosomal DNA levels independently predicted response from stage, performance status and CYFRA21-1 level. Using nucleosomal DNA and CYFRA21-1 in combination it was possible to achieve 100% specificity for response with a sensitivity of 29% suggesting that these tests could safely be used to aid clinical judgements in patients [10].

A study in patients with acute myeloid leukaemia showed that nucleosomal DNA could be used to predict response in this setting; however, patients who responded had a higher (rather than lower) AUC over days 2–4 than patients who progressed [65]. Nucleosomal DNA levels also correlated with leukocyte count; it is unclear whether this is because they are both surrogate markers of efficacy or if the leukocytes affect the generation and/or breakdown of nucleosomal DNA. In summary, the early studies suggest that the assay holds potential but the kinetics of the release of nucleosomal DNA require further examination in larger patient series.

The use of cell death assays to assess toxicity

A potential problem with all serum apoptotic markers is that changes seen could reflect normal tissue toxicity rather than tumour response. CK 18 is not expressed in haematological cells and therefore changes in $CK18^{asp396}$ and total CK 18 release should only reflect response to therapy in epithelial or endothelial cells or non- haematological side effects. Changes in biomarkers caused by normal tissue toxicity could still correlate with outcome, given that patients experiencing more toxicity are known to sometimes have improved outcomes from cytotoxic therapy [95]. All cells release nucleosomal DNA and therefore an increase in circulating nucleosomal DNA could be detected in response to both haematological and non-haematological toxicity. Moreover, in situations where malignancies of non epithelial origin are studied, combining the Nucleosomal assay with the M65 assay for CK18, could potentially be very informative. The Nucleosomal assay will give total cell death in both tumour and normal tissue whilst a M65 signal can only be generated by epithelial tissue and therefore will inform on toxicity. Interestingly, Trejo-Becerril found no nucleosomal DNA toxicity related signal in non-tumour bearing mice given cisplatin [58] and no signal was seen in adjuvant patients (where the tumour has been surgically removed) receiving chemoradiotherapy [62]. This suggests that changes in nucleosomal DNA in response to chemotherapy reflect tumour death rather than reflecting toxicity.

Problems with the use of apoptosis assays

A major problem with all biomarkers is determining what magnitude of change should be regarded as significant. Knowledge of the variability inter- and intra- subjects is vital for the interpretation of this data. Cummings and colleagues measured the $CK18^{asp396}$ and total CK 18 levels in 23 phase I cancer patients at two time-points, pre-treatment, with a 5–7 day gap between collections. They found a mean between day variation of 14.1% for the M30 apoptosense® assay and 12.9% for the M65® assay. They suggested a change from baseline of twice the noise (the between sample variation) could be considered as a significant change in these ELISAs [87]. Holdenreider used serial measurements to generate an AUC, which may reflect both changes in apoptotic rate and in tumour burden over time [59,61,62,93,94].

A problem that has emerged is that the conventional view of cell death as being either apoptotic or necrotic is too simplistic and that there are more than just two mechanisms of cell death [96]. A spectrum exists between apoptosis and necrosis, with organelles such as the lysosymes, mitochondria and endoplasmic reticulum being important in the process of cell death. Pathways of programmed cell death that are caspase-independent have been described [97]. Lysosomal enzymes such as the cathepsins can cooperate with the caspases in apoptosis (either as a downstream effector or an upstream activator) but can also mediate cell death in a caspase independent manner. Chemotherapy may cause death by these pathways without activation of the caspases [98], and cell death by these mechanisms may not be detected by the assays described above.

New apoptotic biomarkers

Given the number of therapies targeting apoptosis presently under investigation in the clinic, fully validated serum biomarkers of apoptosis are urgently needed. Demonstrating that a biomarker adds clinical worth is difficult to demonstrate in early trials. Often, patients enrolled in these trials have already progressed

on several lines of previous chemotherapy and therefore have treatment resistant disease and response rates are low. Also, new non-cytotoxic agents may have unexpected mechanisms of action and toxicities as well as having a biological effective dose well below their maximum tolerated dose. Chemotherapy agents used in standard practice at major cancer centres have been previously assessed in large phase III trials. Their mechanisms of action, response rates and expected toxicities are well known. Validation and interpretation of novel biomarker data in patients receiving these drugs will add to the results coming from the use of these assays in phase 1 trials of novel agents. Armed with knowledge of the kinetics of the biomarker in the conventional chemotherapy setting, assessment of the clinical utility of novel targeted agents should become both feasible and informative. Analysis of the proteome (the whole spectrum of proteins expressed in a tissue) offers an extremely powerful potential tool in cancer biomarker research. The changes in cellular composition and intracellular signalling associated with the response to novel therapies can be detected in the serum. However, there are large technical problems to be overcome with clinical proteomics, not least the reliability and reproducibility of the data generated. Also, the proteome is difficult to analyse due to the large dynamic range in the protein signal. Signals of interest are likely to be of low abundance and may be in a concentration of 10^{-10} compared to albumin [99]. However, proteomics' offers the potential of discovering many more novel markers of tumour response to both apoptosis and cell signalling targeted agents.

Although clinical studies with these novel biomarkers appear promising, it is unlikely that a single assay will be specific enough to guide clinical management or influence the regulatory approval of novel agents. The use of multiple apoptotic assays in conjunction with imaging biomarkers is likely to prove more informative and offers the potential to improve patient care and speed the licensing of anti-apoptotic therapies.

Conflict of interest statement

None declared.

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