Review

Nucleic acids in circulation: Are they harmful to the host?

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It has been estimated that 10^{11}–10^{12} cells, primarily of haematogenous origin, die in the adult human body daily, and a similar number is regenerated to maintain homeostasis. Despite the presence of an efficient scavenging system for dead cells, considerable amounts of fragmented genetic material enter the circulation in healthy individuals. Elevated blood levels of extracellular nucleic acids have been reported in various disease conditions; such as ageing and age-related degenerative disorders, cancer; acute and chronic inflammatory conditions, severe trauma and autoimmune disorders. In addition to genomic DNA and nucleosomes, mitochondrial DNA is also found in circulation, as are RNA and microRNA. There is extensive literature that suggests that extraneously added nucleic acids have biological actions. They can enter into cells *in vitro* and *in vivo* and induce genetic transformation and cellular and chromosomal damage; and experimentally added nucleic acids are capable of activating both innate and adaptive immune systems and inducing a sterile inflammatory response. The possibility as to whether circulating nucleic acids may, likewise, have biological activities has not been explored. In this review we raise the question as to whether circulating nucleic acids may have damaging effects on the host and be implicated in ageing and diverse acute and chronic human pathologies.

[Mittra I, Nair NK and Mishra PK 2012 Nucleic acids in circulation: Are they harmful to the host? *J. Biosci.* **37** 301–312] **DOI** 10.1007/s12038-012-9192-8

1. Introduction

Nucleic acids that are no longer confined within cells but are dispersed in body fluids or in circulation are termed circulating nucleic acids (CNAs). It is now well established that measurable quantities of nucleic acids circulate in healthy individuals as well as in patients with various disease pathologies. The origin, nature and the precise mechanism(s) as to how nucleic acids end up extracellularly are not fully understood. Accumulating evidence suggests that these molecules are preferentially released in circulation in the form of nucleosomes through apoptosis and necrosis. In addition, other types of nucleic acids have been detected in the circulation that includes DNA, RNA, mitochondrial DNA and microRNA. Although CNAs are shown to have promising diagnostic utility as biochemical and genetic biomarkers for a variety of pathologies especially cancer, there is deficiency in our knowledge about the functional significance of CNAs. In this article, we provide several lines of evidence pointing to potential patho-physiological functions of CNAs that have remained unexplored.

2. Origin and nature of CNAs

CNAs in plasma and serum include various forms of nucleic acids, viz. nucleosomes, DNA, RNA, miRNA and mitochondrial DNA (Peters and Pretorius 2011). Supported by theory and observation, two major sources of CNAs have been postulated: first, fragmented DNA released as a consequence of cell death (apoptotic/necrotic) and, second, active metabolic secretion of DNA from cells (Gahan *et al*. 2008). Several hundred billion cells divide daily in the human body and the same number is lost through apoptosis to maintain cellular homeostasis (Fliedner *et al*. 2002; Nagata *et al*. 2010). Although apoptosis is an evolutionary conserved phenomenon and the apoptotic debris is removed from circulation via efficient clearance mechanisms, a proportion of

Keywords. Circulating nucleic acids; DNA damage; ageing; cancer

Published online: 25 April 2012

CNAs in the form of fragmented DNA and nucleosomes escape complete degradation or scavenging by macrophages (Fleischhacker and Schmidt 2007). Approximately 1–10 g of DNA from nucleated leukocytes, which include lymphocytes, monocytes and granulocytes, is degraded each day in the human body by inter-nucleosomal fragmentation (van der Vaart and Pretorius 2008). Although during necrosis or tissue injury, DNA in the range of few kilo- to megabases of nucleotides is randomly degraded by simultaneous activation of lysosomal proteases and nucleases, a proportion escapes degradation and is released into circulation (van der Vaart and Pretorius 2008). DNA in plasma being primarily double-stranded yields a ladder pattern (180–1000 bp) on electrophoretic separation, suggesting that necrosis is unlikely to be a major source of circulating DNA under normal conditions (Jahr *et al*. 2001). Active release of newly synthesized 'metabolic' DNA has also been proposed as a source of circulating DNA. This form of DNA is usually complexed with glycolipoproteins and has associated RNA, and has been shown to be shed from cells *in vitro* (van der Vaart and Pretorius 2007).

CNAs in plasma are not 'naked' but circulate in the form of complexes bound to proteins and lipids. Since plasma/serum are rich in various endonucleases, most pure forms of DNA are degraded rapidly (Fleischhacker *et al*. 2011). Metabolic DNA secreted by cells, being highly negatively charged molecules, bind to plasma proteins. Specialized kits are now commercially available for direct extraction and estimation of DNA from plasma and serum. The isolation and estimation of DNA require extraction from plasma, and hence the characterization of the protein molecules to which they were bound while in circulation has not been possible. Chromatin, because of its highly organized association with histone proteins, is protected from nuclease digestion (Holdenrieder *et al*. 2001b). Fragments of chromatin are derived from apoptotic cells and are cleaved by endonucleases present in circulation into oligo- and mono-nucleosomes (Holdenrieder and Stieber 2009).

In addition to DNA, and nucleosomes, mitochondrial DNA has also been identified in circulation (Chiu *et al*. 2003). The presence of a known mitochondrial DNA mutation in plasma and serum of patients with type 2 diabetes mellitus has been reported (Zhong *et al*., 2000). Data from studies conducted in age-related degenerative diseases and malignancies confirm the existence of both particle-associated and non-particle-associated forms of mitochondrial DNA in plasma (Mehra *et al*. 2007; Zachariah *et al*. 2008; Tsai *et al*. 2011).

Extracellular RNA in serum/plasma has also been described (Wieczorek *et al*. 1987). Given the fact that RNA is a labile molecule, and plasma being an enriched source of RNase, the notion that cell-free RNA could survive in plasma was not easily accepted. Subsequently, Kopreski and co-workers unequivocally demonstrated the presence of tumour-associated RNA in plasma of cancer patients (Kopreski *et al*. 1999). Since then, this observation has been confirmed and it seems clear that the presence of circulating RNA is an ubiquitous phenomenon (Vlassov *et al*. 2010). Recently, microRNAs (miRNAs), a class of 19–23 nucleotides long, post-transcriptional gene expression regulators, have been found in extracellular human body fluids including plasma and serum (Pritchard *et al*. 2011). Most of the miRNAs that circulate in blood of both healthy and diseased subjects are highly stable and withstand the ribonuclease activity of plasma (Mitchell *et al*. 2008). miRNAs are released from cells through a ceramide-dependent secretory mechanism and are entrapped in lipid or lipoprotein complexes such as apoptotic bodies, microvesicles (up to 1 μm) or in small membrane vesicles of endocytic origin called exosomes (50–100 nm) (Iguchi *et al*. 2010; Kosaka *et al*. 2010). It is also likely that large parts of extracellular circulating miRNAs are by-products of dead or dying cells that persist due to the high stability of the miRNA/Argonaute 2 binding complex (Wentz-Hunter and Potashkin 2011; Schöler *et al*. 2011).

There is no consensus as to whether plasma or serum is a better source of CNAs. The amount of DNA in serum can be 2 to 24 times higher than in plasma and most of this is attributed to the release of nucleic acids from destroyed leukocytes during the clotting process (Chan *et al*. 2005). Comparison of DNA yield from serum obtained from fresh (2 h) and stored (24 h) samples also verified that cell lysis during the clotting process contributes markedly towards variations that exist between serum and plasma (Jung *et al*. 2003). On an average, in healthy individuals, a DNA range of between 0 and >1000 ng per mL of blood with a mean of 30 ng/mL have been reported (Board *et al*. 2008). However, it is difficult to draw any firm conclusions about blood levels of DNA from these studies since a variety of different methodologies were used for isolation of DNA by different laboratories. Several novel isolation and quantification strategies have now been developed to determine the nature of DNA present in circulation. With the help of magnetic bead systems, silica-column isolation methods and a variety of fluorescence quantification approaches, it is now possible to detect DNA in plasma and serum of healthy and diseased individuals (van der Vaart and Pretorius 2010).

The most commonly used technique for measuring nucleosomes in serum has been the Cell Death Detection ELISA^{Plus}, which is commercialized by Roche Diagnostics. The kit is a sandwich immunoassay that utilizes simultaneously two monoclonal antibodies, one each against DNA and histones (Salgame *et al*. 1997). The kit was originally designed to measure apoptosis, but was later modified by Holdenreider *et al.*, so that the assay is more applicable to nucleosomes in serum/plasma, and is more reproducible (Holdenrieder *et al*. 2001c). The results are expressed in arbitrary units (AU).

3. CNAs in health and disease

CNAs have been detected in healthy individuals and their levels vary from scantly detectable to few micrograms per litre, but in higher concentrations in several disease conditions. Excellent reviews are available on the presence of DNA, RNA and nuclesomes in various pathological states (Rykova et al. 2010; Swarup and Rajeswari 2007; Holdenrieder and Stieber 2009). The presence of DNA in plasma of patients with systemic lupus erythematosus (SLE) was demonstrated for the first time in 1966 and several reports have appeared since then (Tan et al. 1966; Pisetsky and Ullal 2010). Galeazzi *et al*. characterized the pattern of DNA in circulation and demonstrated that DNA has an anomalous pattern in SLE, thus implicating a biological role of DNA in this disease (Galeazzi *et al*. 2003). Practically all published articles are in consensus that the concentrations of DNA and nucelosomes in individuals with cancer are higher than normal (Gal *et al*. 2004; Sozzi *et al*. 2001; Umetani *et al*. 2006; Holdenrieder *et al*. 2001a; Kuroi *et al*. 1999; Trejo-Becerril *et al*. 2003). A considerable increase in nucleosomes levels following radiochemotherapy has been observed (Kremer *et al*. 2005). In patients with systemic inflammation and sepsis, apoptosis resulting from the action of excessive amounts of inflammatory cytokines on cells is directly responsible for the elevated levels of nucleosomes in plasma of these patients (Zeerleder *et al*. 2003). DNA concentrations (of non-mitochondrial and mitochondrial origin) were significantly higher in plasma of patients with severe sepsis or septic shock (Saukkonen *et al*. 2008; Rhodes *et al*. 2006), blunt traumatic injury (Lam *et al*. 2004) and burn injury (Chiu *et al*. 2006). Elevated levels of CNAs have been reported in diabetes, cerebral stroke and myocardial infarction, and in the case of the latter two, the levels of nucleosomes and DNA in plasma correlate with the severity of the damage (Butt *et al*. 2006; Geiger *et al*. 2006; Chang *et al*. 2003). Comparative levels of nucleosomes in sera of healthy volunteers, and patients with diabetes, renal failure, sepsis and cancer, both before and after they received chemo- or radiotherapy, are shown in figure 1. Investigations using real-time quantitative PCR and counter-immunoelectrophoresis have detected increased amounts of DNA in plasma of patients with severe injuries, organ failure, multiple organ dysfunction syndromes, pulmonary embolism, preeclampsia and Whipple's disease (Lam *et al*. 2003; Barada *et al*. 1980; Zhong *et al*. 2005; Benoit *et al*. 2007). Subsequent reports have demonstrated significantly elevated levels of nucleic acids in other disease conditions such as in rheumatoid arthritis, hepatic autoimmune diseases, connective tissue diseases and vasculitis associated antineutrophil cytoplasmic antibodies (ANCA) (Koffler *et al*. 1973; Holdenrieder *et al*. 2006). Elevated levels of DNA have been found in patients who have undergone organ transplantation (Lui *et al*. 2003). Foetal DNA has been detected in maternal plasma and has been used for prenatal diagnosis of foetal abnormalities (Chiu and Lo 2004). mRNAs in significantly higher amounts have been detected in patients with diabetic retinopathy and diabetic nephropathy (Butt *et al*. 2006).

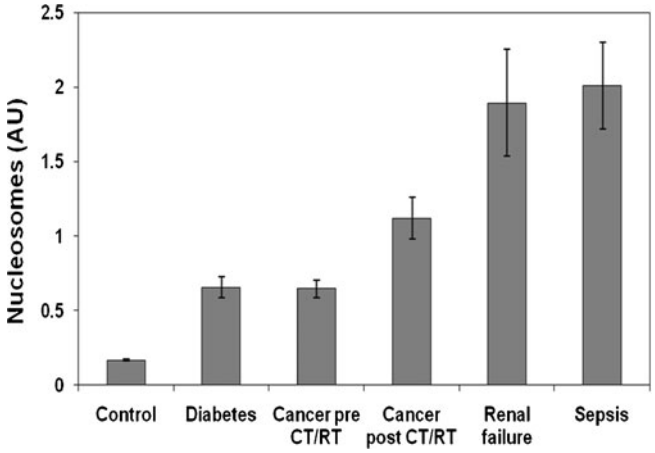

Figure 1. Levels of serum nucleosomes in healthy subjects and diseased states (mean ±SE; 30 subjects in each group). Nucleosome levels were measured using the Cell Death Detection ELISA^{Plus} kit (Roche Apllied Sciences, Mannheim, Germany). Values are expressed as arbitrary units. CT/RT = chemotherapy/radiotherapy.

4. CNAs as biomarkers

Considerable research effort has been expended on the use of CNAs as biomarkers in cancer (For review Schwarzenbach *et al*. 2011; Holdenrieder *et al*. 2008). CNAs from malignant conditions have characteristic changes like mutations, deletions, methylations and microsatellite aberrations which are distinct from those in benign conditions, and thus might be useful in diagnosis of cancer (Shapiro *et al*. 1983; Nawroz *et al*. 1996; Botezatu *et al*. 2000). Recent genomic analysis using Affymetrix SNP 6.0 arrays to determine tumour-specific copy number variation (CNVs) in circulating DNA from patients with breast cancer could achieve a clear separation between patients and healthy controls; and specific CNVs were detected in DNA in circulation up to 12 years follow-up after diagnosis and treatment in asymptomatic patients (Shaw *et al*. 2012). The integrity of circulating DNA, defined as the ratio of longer fragments to total DNA, is linked to stage, tumour size and nodal metastases in breast cancer (Umetani *et al*. 2006). Levels of DNA and nucleosomes have been used as tumour markers, as well as prognostic and predictive biomarkers of cancer therapy (Zimmermann *et al*. 2007; Mancuso *et al*. 2010; Shacter

and Weitzman 2002). DNA levels decreased after surgery in breast cancer patients (Huang *et al*. 2006). The levels of CNAs vary during the course of follow-up of patients after chemotherapy/radiotherapy. After chemotherapy, peak levels of CNAs were seen at 24–72 h followed by a decline in levels (Holdenrieder *et al.* 2001a; Umetani *et al*. 2006). Declining levels of nucleosomes after chemotherapy suggests remission, while consistently increasing levels suggest progression (Holdenrieder *et al.* 2001a; Kuroi *et al*. 2001). Furthermore, the rise in nucleosomes values are more pronounced in patients who are responsive to chemotherapy compared with non-responsive patients (Mueller *et al*. 2006). Apart from cancer, Chiu *et al*. demonstrated that elevated levels of DNA after burn injury significantly correlated with some of the outcome measures and severity of the injury (Chiu *et al*. 2006). It has been reported that elevated levels of CNAs found in sepsis correlate with mortality from this condition (Rhodes *et al*. 2006).

5. Biological effects of nucleic acids

5.1 *Cellular uptake and genomic integration*

Horizontal transfer of DNA is widespread in bacteria and plays an important role in the development of antibiotic resistance and adaptation to new environments (Lake *et al*. 1999; Ochman *et al*. 2000). Exchange of genetic material between cells in plant tissue grafts is also known to occur (Stegemann and Bock 2009). There is extensive literature published in the 1960s and 1970s to indicate that eukaryotic cells can, under experimental conditions, take up extraneously added DNA and RNA (for review: Bhargava and Shanmugam 1971; Gahan and Stroun 2010). There seems to be no source specificity of the donor DNA, and every type of DNA tested has been found to be taken up by recipient cells (Bhargava and Shanmugam 1971). Heterologous DNA after cellular entry is extensively degraded, but a small proportion has been shown to be integrated into the recipient cell genome (Gartler and Pavlovskis 1960; Gartler 1959; Ayad and Fox 1968). Exogenously added DNA can infrequently induce genetic transformation of the recipient cells. For example, it has been observed that inosinic acid pyrophosphorylase −ve (IMPPase −ve) D98S human cells can be genetically transformed by DNA from IMPPase +ve cells so that the treated cells survive under highly selective conditions (Szybalska and Szybalski 1962). Evidence is also available that strongly suggests DNA taken up by mammalian cells can be replicated, transcribed and translated into proteins (Szybalska and Szybalski 1962; Szybalski *et al*. 1962, cited by Bhargava and Shanmugam 1971). Radioactively labelled DNA when injected *in vivo* is taken up by tissue cells as demonstrated by autoradiography or by monitoring radioactive counts (Yoon 1964; Yoon and Sabo 1964). Bacterial DNA metabolically labelled with ^{3}H-thymidine injected intra-peritoneally has been shown to cross the blood–brain barrier to be incorporated in to the nuclei of brain cells as detected by auto-radiography (Anker and Stroun 1972). When mice were intra-peritoneally injected with live bacteria together with ^{3}H-uridine followed by injection of an antibiotic to kill the organisms, radioactive uridine could be recovered from the brains of the injected animals (Anker and Stroun 1972). Taken together, these experiments suggest that DNA is capable of being incorporated and transcribed in the brain cells of experimental animals. This phenomenon forms the basis of DNA vaccines that are currently being widely experimented with (Kutzler and Weiner 2008). SW 480 colon carcinoma cells containing *K-ras* mutation in both alleles are known to release DNA containing the mutated oncogene into the culture medium. When the latter was added to mouse fibroblast cells, the presence of the mutated *K-ras* gene was confirmed in the recipient cells, which also showed foci of transformation (Anker *et al*. 1994). Exogenously added DNA has been reported to induce chromosomal damage (Woll 1953; Karpfel *et al*. 1963). The karyotype of chick embryo cells was grossly altered by the addition of bovine DNA (Kok 1959, cited by Bhargava and Shanmugam 1971). Abnormal anaphase cells were observed in bone marrow cells treated with heterologous DNA from spleen or thymus (Karpfel *et al*. 1963). More recently, it was reported that when mouse fibroblasts cells were cultured in the presence of plasma from patients with colon cancer carrying *K-ras* mutation, the oncogene sequence was detectable in the recipient cells upon PCR analysis. Moreover, the treated mouse fibroblasts when injected into immune deficient mice were capable of inducing tumours that also showed the presence of *K-ras* sequences (García-Olmo *et al*. 2010).

Histones have been shown to be present in circulation, and extracellular histones are cyctotoxic to endothelial cells *in vitro* and are lethal when injected into mice (Xu *et al*. 2009). Histones can directly translocate across cell membranes by a process that does not involve endocytosis in a non-energy-dependent manner (Hariton-Gazal *et al*. 2003). Penetration of DNA associated with histones (as nucleosomal units) into intact cells involves a ‘non-specific’ form of non-covalent ionic interaction with plasma membrane. The ability of histones to carry DNA inside the cell is an energy-dependent event but the potential varies among different histones. Given the electrostatic nature of DNA, the level of positive charge of histones imparts stability and ability to transport DNA as a mobile unit (Hariton-Gazal *et al*. 2003). It has been shown that cell surface proteoglycans can bind nucleosomes, while DNA has been shown to enter into cell through the toll-receptor system (Watson *et al*. 1999; Barton *et al*. 2006; Dalpke *et al*. 2006).

It was recently shown that when isolated genes are reconstituted into chromatin *in vitro*, they readily entered into host cells and could be localized in their nuclei. It was suggested that reconstituted nucleosomes could be used as a vehicle for gene therapy (Wagstaff *et al*. 2008). Nucleosomes, purified from calf thymus, when added to isolated lymphocytes from healthy individuals and patients suffering from lupus erythematosus, induced cell death which could be abolished by prior treatment with DNase I, proteinase K or nucleosome-specific antibody (Decker *et al*. 2003). Figure 2 shows that nucleosomes recovered from serum that have been fluorescently labelled in their DNA are readily taken up by isolated lymphocytes and they are localized within the nuclei within 6 h. The lymphocytes are seen to be undergoing apoptotic changes. A markedly greater increase in the induction of apoptosis in isolated lymphocytes is observed following addition of plasma from patients suffering from sepsis and diabetes compared with plasma from healthy controls (figure 3). The specific involvement of nucleosomes in inducing apoptosis is indicated by the fact that the apoptotic activity is markedly reduced when plasma is immune-adsorbed in an affinity column containing biotinylated anti-histone antibodies bound to streptavidin beads. Much of the activity is recovered when the bound nucleosomes are eluted and added to lymphocytes in culture (figure 3).

It would appear from earlier studies that chromosomes or chromosomal fragments behave in a fashion similar to nucleosomes. When radioactively labelled isolated homo- or heterologous chromosomes were added to different cultured cells, they got readily incorporated into the host cell chromosomes after undergoing extensive fragmentation (Chorazy *et al*. 1963; Burkholder and Mukherjee 1970; Ittensohn and Hutchison 1969; Yosida and Sekiguchi 1968). The recipient cells often showed pronounced cytotoxic effects and cytological changes such as vacuolated cells, micro-nuclei and nucleosomes and chromosome-like inclusion bodies (Ittensohn and Hutchison 1969). Chromosome-mediated gene transfer techniques are well established in which a free functional chromosome fragment containing the relevant gene can be readily taken up and retained in the genomes of the progeny for many generations (for a review, see McBride and Peterson 1980).

RNA from heterologous cells that are radioactively labelled are also taken up by host cells, and radioactivity could be detected in the RNA isolated from the treated cells (Shanmugam and Bhargava 1966; Shanmugam and Bhargava 1969; Niu *et al*. 1968). It has been recently shown that exogenously added RNA can be translated into proteins. When naked luciferase-encoding mRNA were added to a variety of cells in culture, not only were the mRNA actively taken up by the cells, but they were also expressed in to translated fluorescent proteins (Lorenz *et al*. 2011). Finally, although little information is available on the effects of mitochondrial DNA, isolated mitochondria can cause damage to cells, leading to inflammation (Zhang *et al*. 2010).

5.2 *Immunological effects and inflammation*

The recognition of a role for CNAs (possibly in the form of nucleosomes) in immune activity dates to its discovery as a target antigen in SLE, and the recent identification of pattern recognition receptors has suggested a potential role for endogenous CNAs as activators of the innate immune system (Tan 1989; Kawashima *et al*. 2011). Tissue damage leads to the release of CNAs when cells undergo apoptosis or necrosis (Pisetsky 2007). It has been demonstrated that DNA derived from host cells, or chemically synthesized double-stranded DNA, can activate both immune and non-immune cells when introduced into the cytosol of recipient cells. The cellular response does not depend upon the nucleotide sequence but on the double-stranded helical nature of the molecule (Suzuki *et al*. 1999). It has been experimentally

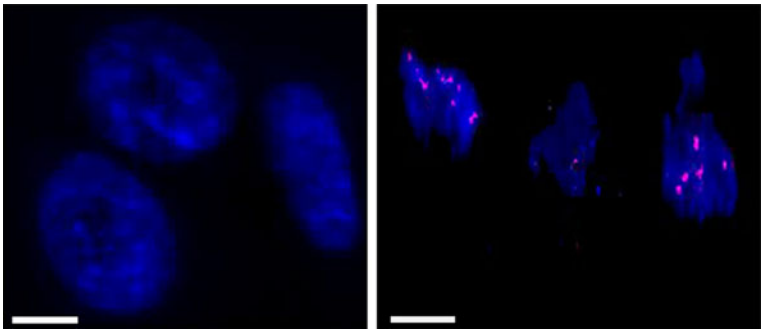

Figure 2. Nucleosomes isolated from serum are taken up by lymphocytes in culture and are localized in their nuclei. The treated cells exhibit apoptotic changes. The left photomicrograph is of untreated cells while the right one is of treated cells. Nucleosomes were labelled in their DNA using 564 Alexa-dUTP and added to lymphocytes isolated from healthy subjects and examined after 6 h treatment. Scale bar= 5 μm.

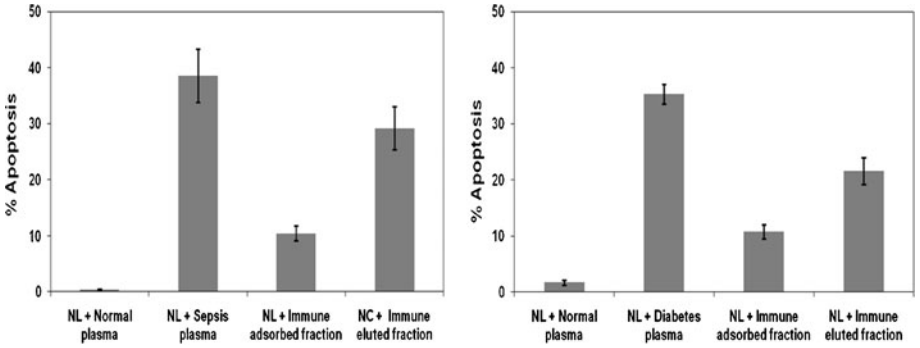

Figure 3. Critical role of nucleosomes in enhanced lymphocyte apoptosis induced by plasma from patients suffering from sepsis and diabetes. Plasma from 30 patients suffering from sepsis and diabetes and an equal number of age- and sex-matched healthy volunteers was used for this study. Lymphocytes isolated from healthy subjects were treated in individual experiments with plasma (100 μL) and the apoptotic index was measured by flow cytometry after labeling with annexin v following 24 h treatment. The figures show marked increase in apoptosis in sepsis and diabetes. The apoptotic index is greatly reduced following immune adsorption of plasma in an affinity column containing biotinylated anti-histone antibodies bound to streptavidin. The apoptotic activity could be largely restored when the bound nucleosomes were eluted (0.25 M Nacl) and used for treatment.

demonstrated that exposure of immune cells to double-stranded DNA activates a set of genes, including those encoding major histo-compatibilty complex, co-stimulatory molecules and interferon regulating factors (Ishii *et al*. 2001). Signalling through these receptors triggers the activation of kinases such as TBK1 and Ikki, and the downstream phosphorylation of transcription factors IRF3 and NFκβ. Numerous studies have noted a robust pro-inflammatory cytokine response upon stimulation of macrophages and innate immune cells with double-stranded DNA (Hefeneider *et al*. 1992; Tanner 2004; Choi *et al*. 2005). Recently, several proteins have been identified that sense extracellular nucleic acids and act as inducers of interferon (IFN) (Rock *et al*. 2011; Kawasaki *et al*. 2011). Normally, DNAse participates in degradation of inefficiently cleared CNAs released from dying cells (Gaipl *et al*. 2006). Deficiency of DNAse I, and the consequent inadequate removal of DNA from nuclear antigens, promotes susceptibility towards autoimmune disorders (Kawane *et al*. 2001, Kawane *et al*. 2006). DNAse I-deficient mice exhibit classical symptoms of lupus. Hepatic macrophages from DNase-II-deficient mice fail to digest DNA from engulfed apoptotic cells and secrete type I IFN, resulting in severe anaemia and chronic arthritis. Mice deficient in DNase III develop inflammatory myocarditis and premature mortality accompanied by cells that accumulate extra-nuclear DNA. Defective phagocytosis of apoptotic macrophages in diabetes-prone mice result in accumulation of extracellular nucleic acids that are capable of promoting autoimmunity (O'Brien *et al*. 2006).

Nucleosome is the main lupus auto-antigen and is believed to play a key role in disease development since it is found as a circulating complex and since both auto-reactive nucleosome-specific B and Th lymphocytes are detected in patients' sera (Williams *et al*. 2001). Moreover, the levels of both anti-nucleosome auto-antibodies and circulating nucleosomes have been shown to be associated with disease activity (Decker 2006). Purified nucleosomes induce cell death of normal and lupus lymphocytes *ex vivo* in a dose- and time-dependent manner, and this activity could be abolished when nucleosomes were first treated with DNase I, proteinase K or with a specific monoclonal antibody. Intravenous injection of purified nucleosomes resulted in apoptosis and a reduction in spleen cell count compared with that in control mice (Decker *et al*. 2003). Nucleosomes have been shown to activate several types of immune cells as well as the complement system, resulting in inflammation (Hefeneider *et al*. 1992). Nucleosomes released from dying cells have been posited to act as pro-inflammatory mediators, although mechanistic insights into the inflammatory stimulus are not well understood. Nonetheless, collateral damage that occurs during sterile inflammation can be significant. Unresolved and uncontrolled inflammation for a sustained period activates an 'injury loop' in which inflammation-derived injury leads to additional inflammation. Inflammation-induced damage to important cellular components (e.g. DNA, proteins and lipids) through release of pro-inflammatory signalling mediators can directly or indirectly contribute to tissue injury. A strong correlation exists between the level of circulating nucleosomes and inflammatory cytokines in serum of healthy individuals, as shown in figure 4. This raises the possibility that CNAs may have a patho-physiological role to play *in vivo* under normal conditions.

Although nucleic acids are generally not considered as signals of damage-associated molecular patterns (DAMPs), their release during cellular stress or tissue injury and their role in mediating a sterile inflammatory response has been

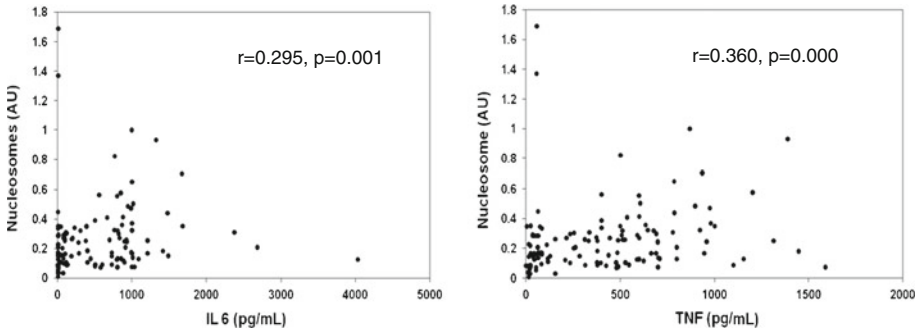

Figure 4. Levels of nucleosomes in serum correlate with those of inflammatory cytokines. Serum was separated from blood taken from 140 healthy subjects (age 15–-70 years) and nucleosome levels were measured using the Cell Death Detection ELISA^{Plus} kit. Flow cytometric evaluation of interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α) levels in the serum were performed using cytometric bead array assay kit. Serum nuclesome values are expressed as arbitrary units.

recorded (Lotze *et al*. 2007). Sterile inflammation has been implicated in several disease processes including gout, chemically induced pulmonary interstitial fibrosis, trauma, ischaemia-reperfusion injury, atherosclerosis, Alzheimer's and cancer (Chen and Nuñez 2010). Although inflammation is important in tissue repair, unresolved, chronic inflammation that occurs when the offending agent is not removed, or present in the circulation, can prove detrimental to host immunity. Inefficient clearance of apoptotic cell remnants can result in the accumulation of nucleic acids that can insinuate a self-cascade cycle responsible for the initiation of systemic inflammation (Nagata and Kawane 2011). Subclinical levels of inflammation may contribute little, but when the damage is substantial or repetitive, inflammation can be an important etiological factor that underlies the pathogenesis of a number of diseases.

Mitochondria can also damage tissue cells. Mitochondria contain several copies of a circular genome (mtDNA) that code for key proteins of the oxidative phosphorylation system. When enzymes of the latter system are degraded, they give rise to formyl peptides. When cells are coping with an insult that is potentially harmful, mtDNA and degraded formyl peptides can be released into the surrounding milieu and can trigger inflammation (Zhang *et al*. 2010). The conditions in which these mitochondrial alarmins are generated and their possible role in chronic inflammatory state is only just beginning to be appreciated.

5.3 *Role in ageing*

The accumulation of somatic DNA mutation and damage increases with age as a result of exposure to a variety of toxic or damaging substances, such as free radicals (Nusbaum 1998). DNA damage contributes to aging by inducing cellular senescence, apoptosis and cell dysfunction (Best 2009). The fragility of lymphocytes is known to increase with age, and the variety of cellular damage with increasing age is accompanied by a chronic low-grade inflammation (Esposito *et al*. 1989; Franceschi 2007). Correspondingly, various biomarkers of inflammation have also been shown to increase with age (Bandeen-Roche *et al*. 2009; Hsu *et al*. 2009). Increasing levels of cell-free DNA also accompany advancing age. In a study of 12 nonagerian women and 11 young people aged 22–37 years, it was observed that the concentration of cell-free DNA was significantly higher in the former group. Furthermore, the DNA differed qualitatively between the two groups, in that in the nonagenarians a fragmented pattern of low-molecular weight DNA was observed in a majority of the women (Jylhävä *et al*. 2011). A strong correlation exists between the levels of circulating nucleosomes and age of healthy individuals, and this is shown in figure 5. Thus, there seems to be a tantalizing

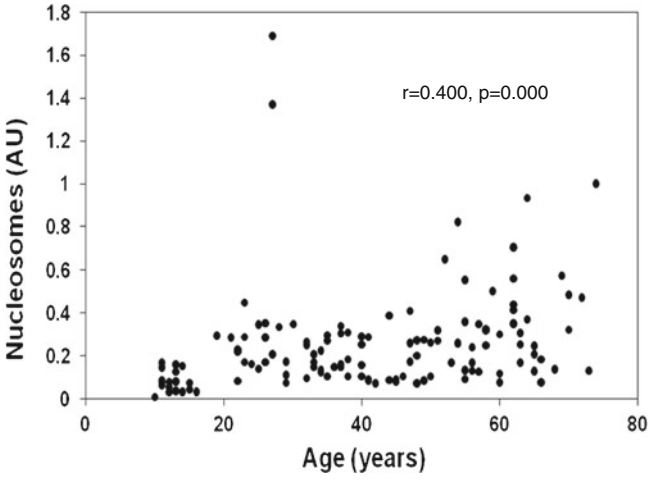

Figure 5. Levels of nucleosomes in serum increase with age. Serum was separated from blood taken from 140 healthy subjects (age 15–70 years) and nucleosomes levels were measured using the Cell Death Detection ELISA^{Plus} kit. Values are expressed as arbitrary units.

biological connection between CNAs and the ageing process.

6. Are CNAs harmful to the host?

Currently, the biological effects of CNAs are unknown and this area has remained largely unaddressed. We have summarized in our review the body of evidence that suggests that DNA, nucleosomes, RNA and mitochondria, that have been derived from sources other than plasma or serum, can have diverse biological and pathological activities *in vitro* and *in vivo*. Although nucleic acids in circulation are highly fragmented, and to that extent are physically distinct from nucleic acids that have been generally used under experimental conditions, the tantalizing question remains as to whether CNAs can have biological activities similar to nucleic acids derived from other sources and be of pathophysiological relevance to the host.

From our review, the biological actions of nucleic acids can be summarized as follows: (1) Eukaryotic cells can take up exogenously added nucleic acids *in vitro* and *in vivo*; (2) exogenously added nucleic acids can get incorporated into the nuclei of host cells *in vitro* and *in vivo* and can be transcribed; (3) exogenously added nucleic acids can cause genetic transformation of the recipient cells, albeit rarely; (4) exogenously added nucleic acids can cause chromosomal damage and cytotoxic changes in the recipient cells; (5) DNA that contain oncogenes can transform recipient cells in culture; (6) exogenously added DNA and nucleosomes can trigger induction of pro-inflammatory cytokines; (7) nucleosomes can induce the production of auto-antibodies; and (8) exogenously added RNA can be taken up by the cells and are capable of being transcribed and translated into proteins.

CNAs are elevated in several disease conditions, which can be broadly categorized as follows: (1) ageing and age-related degenerative disorders including cancer; (2) acute and chronic inflammatory conditions; (3) severe trauma and (4) auto-immune disorders. Our review of the literature suggests the possibility that CNAs, like exogenous nucleic acids, can be taken up by tissue cells. Once inside the cells, CNAs may induce a DNA-damage-repair response that could facilitate their integration into the host cell genomes by homologous recombination. By acting as potential DNA-damaging agents, CNAs could continually damage DNA of healthy cells of the body throughout life to promote progressive cellular ageing *in vivo* (Campisi and Vijg 2009). CNAs-induced DNA damage may also be implicated in multiple ageing-related disorders such as cancer, diabetes, atherovascular conditions and Alzheimer's disease, all of which are known to exhibit increased cellular DNA damage (Stephens *et al*. 2009; Blasiak *et al*. 2004; Mahmoudi *et al*. 2006; Shackelford 2006). It had been observed that infectious DNA from tumour-forming polyoma virus and pneumococcal-transforming DNA could be recovered from blood of mice in biologically active form after intraperitoneal injection. It was proposed that metastatic spread of cancer may possibly involve circulating tumourigenic DNA (Bendich *et al*. 1965). This proposal is similar to the hypothesis of 'geno-metastasis', which is based on the observation that sera from colon cancer patients carrying K-ras mutation can transform mouse fibroblast cells, and that K-ras sequences could be detected in the latter by PCR (García-Olmo *et al*. 2010). Inflammation produced by CNAs, or by nucleic acids liberated from dying cells, may induce sterile inflammation. A strong association between inflammation and cancer is well recorded (Coussens and Werb 2002). Sterile inflammation has also been implicated in arthrosclerosis, diabetes, Alzheimer's disease, ischaemia-perfussion injury and trauma (Chen and Nuñez 2010). Clearly, further research is warranted to study the biological and pathological roles of CNAs which may help to elucidate the mechanisms underlying various common disorders that have remained elusive thus far.

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MS received 27 February 2012; accepted 06 March 2012

Corresponding editor: DURGADAS P KASBEKAR