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Clinical implications and pathological associations of circulating mitochondrial DNA

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1. ABSTRACT

Mitochondria are membrane-enclosed organelles, the energy-producing centers in almost all eukaryotic cells. The evolutionary emergence of mitochondria is a result of the endocytosis of a-proteobacteria. There are several characteristic features which refer to its prokaryotic ancestors including its independent sets of double-stranded mitochondrial DNA, which is uniquely circular in form and contains a significant amount of unmethylated DNA as CpG islands. Resent research has proven that free mitochondrial DNA found in blood was associated with innate immunomodulation in a broad-range of clinical conditions. Upon release, mitochondrial DNA acts as a danger-associated molecular pattern in the circulation, it is recognized by pattern recognition receptors and it facilitates inflammatory responses. Besides its high receptor activation potential, mitochondrial DNA is likely to perform direct crosstalk with activated leukocytes and to be contributed to other anti-microbial activities. Here we highlight the pathological conditions where cell free mtDNA is involved, describe the potential sources and mechanisms of extracellular mtDNA release and explore evidence for its mechanism of action after being excreted and potential therapeutic strategies.

2. INTRODUCTION

Due to its high mutation rate, mitochondrial DNA (mtDNA) has been studied in the context of ageing-related and multifactorial diseases where mitochondrial dysfunction is thought to play a role (1, 2). However, in the last few years, a novel and significant role of mtDNA has emerged, involving its ability to trigger innate immune system responses and drive inflammation when released from mechanically injured cells (3). MtDNA, along with other host molecules released upon cell damage, falls into the category of damage-associated molecular patterns (DAMPs). According to the endosymbiotic theory, the evolutionary emergence of mitochondria is a result of the endocytosis of a-proteobacteria (4, 5). Phylogenetic investigations support this assumption, as mitochondria possess several features characteristic of their prokaryotic ancestors, such as their barrel-shape and diameter of 0.2. to 1.0. μm. Mitochondria have their own independent sets of double-stranded mitochondrial DNA (mtDNA), which is not linear like nuclear DNA, but circular in form and consists of a high number of unmethylated CpG islands, as typically found in bacteria (6). The genome size of mtDNA is significantly smaller than the nuclear (16.5.69 bp vs. 3.2. billion bp) in humans, the number of encoding mitochondrial genes is only 37, which encode no more

than 16 proteins, all belonging to the electron transport-chain (7). The release of mtDNA and its presence in the circulation were described to play a significant role in various clinical conditions; in particular, inflammatory diseases, several types of cancer and in conditions leading to critical illness requiring intensive care unit admission (8-10). Currently, several conditions are being explored where mechanical and chemical stress can lead to cellular necrosis accompanied by mtDNA release. This review will provide a summary of the pathological conditions where cell free mtDNA is involved, describe the potential sources and mechanisms of extracellular mtDNA release and explore evidence for its mechanism of action after being excreted and potential therapeutic strategies.

2.1. Conditions when mitochondrial DNA release is accompanied by mechanical and chemical stress

The release of mtDNA has been described in diverse inflammation and cell necrosis-related clinical conditions, where the loss of cell membrane integrity leads to the release of intracellular content (11). However, it is important to note that in these conditions, the stress destroying the mitochondria can be of either extracellular or intra-mitochondrial origin, with the latter case initiated by oxidative burst inside the mitochondrion, resulting in mitochondrial and cellular disintegration (12). As such, mechanisms of mtDNA release may vary depending on the origin and the type of insult.

2.1.1. Mechanical stress related mtDNA release

Any type of cell death that culminates in lysis and subsequent release of intracellular content into the extracellular environment could conceivably result in mtDNA being released into the circulation (11). MtDNA and other particles are not exposed to the innate immune system following normal apoptosis, but cell death due to mechanical stress and subsequent lysis can mediate their entry into the systemic circulation to provoke immune response (13). Tissue injury is one such example of mechanical stress leading to release of mtDNA (14). In major trauma patients, the initial cell death due to the injury is an important but non-modifiable factor for post-injury inflammation-associated complications (15). The recognition of mtDNA by innate immune cells (primarily neutrophils) plays a pivotal role in the pathophysiology of sterile inflammation after major trauma (16). Our group has hypothesized that mtDNA may have a primary inflammatory source following major trauma rather than as a result of direct tissue injury and subsequent cell necrosis (17). It is supported by our recent findings, where elevated and increasing concentration of mtDNA was shown to be present in the sera of major trauma patients. We also identified sustained high concentrations of cell free mtDNA in the sera of postoperative trauma patients undergoing major orthopedic trauma surgery without association with well-established markers of tissue necrosis (17).

2.1.2. Stress causes oxidative damage and a subsequent DNA release in mitochondria

In addition to its presence in major trauma patients, alterations in mtDNA content of the blood have been measured in a number of diseases relating to oxidative stress. Besides the measurable oxidative damage, the involvement of the mitochondrial genome was described in response to focal or acute myocardial ischemia/reperfusion in animal models and in human cells (18-20). Elevated blood mtDNA content was described to be in association with higher cardiovascular risk or development of coronary heart disease (21, 22). This latter study was conducted on patients with diabetes mellitus and others found hyperglycemia-induced elevation in mtDNA of the peripheral blood in early diabetes (23). Likewise, neurodegenerative diseases such as Alzheimer's or Huntington's disease are also seemed to be associated with alterations in mtDNA concentration (24-26). Also, different types of cancer or critical conditions like sepsis or hemorrhagic shock are considered to be connected with mtDNA copy number changes (16, 27-30). In the context of high-altitude oxygen deprivation, blood mtDNA content was observed to be increased in lowlanders as compared to highlanders, regardless of age and gender, suggesting that the changes in mtDNA concentration might be due to the ROS-stress adaptation mechanisms (31, 32). Both acute and chronic inflammatory diseases have been associated with increased cell-free mtDNA and correlate with elevated free radical production that may have originated from mitochondria (33), but the question of the source of ROS remains open, as the ROS productive capacity of immune cells is also well-known in these conditions. Liu and co-workers published in 2003, that the copy number of mtDNA in human leukocytes was highly affected by alterations in plasma antioxidants/pro-oxidants (34). In addition to its role in diseases itself, mtDNA is also affected by diagnostic and therapeutic tools. Mitochondria are highly susceptible to ionizing radiation at the clinically relevant dosages and oxidative stress resulting from irradiation was found to be accompanied by a rise in extracellular mtDNA release (35, 36). In fact, the natural process of aging is relevant here, since oxidative stress within aging mitochondria can lead to a vicious cycle in which damaged mitochondria produce increased amounts of reactive oxygen species. This could explain the significant increase in the mtDNA mutation rate also found in human clinical studies in healthy older people's plasma, as compared to young volunteers (37, 38). The cut-off for clinically relevant rise in these mutations is likely to be close to the 6^{th} decade of life in humans, highlighting the importance of age-matching in mtDNA concentration-based human studies (39).

Paradoxically, oxidative injury to mitochondria takes place during "reductive stress": when electron acceptors are expected to be mostly reduced, some redox proteins can donate electrons to O_2 instead,

which increases the NADH/NAD+ ratio of mitochondria. Conditions such as high-intensity exercise training, alcohol intake or chronic fatigue syndrome and other forms of hyperglycemia-induced diseases all result in reductive stress and were reported to be accompanied by cellular damage and therefore mtDNA excretion (40-42).

2.1.3. Cell death pathways culminating in lysis

If a cell is directly injured through a physical insult or severely stress it may become necrotic. Necrosis is characterized morphologically by cell rounding, swelling (oncosis), and expansion of organelles and de-condensation of nuclear chromatin (43). This process culminates in cell lysis. Recent evidence suggests that complex signal transduction mechanisms can control necrosis (43). Further cell death essentially occurs as a result of the physiological stress caused by the mass release of cytokines and other cell signaling molecules from injured tissues and the innate immune cells involved in the acute inflammatory response. “Necrosis” in the post-injury state can be triggered through subsequent complex tightly regulated intracellular signaling cascades, not just through the initial mechanical tissue injury. Necrosis can be triggered by exogenous molecules such as TNFα and Fas ligand binding to cell surface receptors (44). The activation of such receptors can lead to a tightly regulated and controlled form of necrosis. This process is mediated through caspase-8 (anti-cell death enzyme) and receptor interacting protein kinases (RIPK family) and the term “necropoptosis” coined for it (11). The role of DAMPs in triggering necropoptosis has also been explored through their activation of pathogen recognition receptors (PRRs) which then trigger intracellular signaling cascades through RIPK1 and RIPK3 (11). Whilst RIPK1 and RIPK3 can play a role, they are not essential in necrosis following ischemia reperfusion (IR) injury (44). This is characterized by exposure to high levels of hydrogen peroxide (H_2O_2) and is dependent on the activity of a different enzyme poly (ADP-ribose) polymerase (44). Free intracellular iron redox reactions with H_2O_2 appear to play a pivotal role in this modality of cell death by inducing lysosomal permeability (43). Intracellular chelation of free iron was demonstrated to be cell-protective in such conditions (43). Regardless of the initiating stimulus, loss of membrane continuity and lysis leads to the extravasation of the intracellular contents, including mtDNA and associated mtDAMPs into the extra-cellular environment.

2.1.4. Oxidative cellular damage pathways initiated by mitochondria

The majority of ROS are products of mitochondrial respiration, as the electrontransport-chain contains several redox centers that may leak electrons to O_2, serving as the primary source of $O_2^{\cdot -}$ production in most tissues (12). A major threat to this controlled equilibrium is hypoxia, since the absence of the electron acceptor O_2 leads to a shift in reducing potential to a higher than normal reducing power, which results in progressive structural and functional cell damage. It is therefore widely accepted that several disease states are linked to “oxidative stress” and a subsequent mtDNA release (45). The effect of abnormal Ca^{2+} is also inevitable in response to stress, but the mechanisms of the harmful effect of Ca^{2+} on mitochondria is not well characterized (46). ROS and Ca^{2+} constitute important mediators of the propagation of the necrotic signal from the mitochondrial matrix towards the outside and can cause damage to all of the major classes of biological macromolecules, including nucleic acids, proteins, carbohydrates, and lipids (47). Mitochondrial calcium has been described to stimulate oxidative phosphorylation, thereby promoting more ROS generation (48). In addition calcium-mediated activation of calpain can lead to cleavage and inactivation of caspases (49) whereas ROS can target the active site of caspases and render them inactive, promoting necrosis (50). Likewise, mitochondrial H_2O_2 can cause the release of cytochrome c from mitochondria into the cytosol and H_2O_2 may also activate nuclear transcription factors, like NF-κB, AP-1, and p53, which may upregulate death proteins or produce inhibitors of survival proteins (51).

3. CIRCULATING MITOCHONDRIAL DNA IN INNATE IMMUNE RESPONSES

Despite their seemingly independent existence within the cell, mitochondrial transcription and replication are co-dependent on nuclear encoded factors transported into mitochondria (52). The aforementioned similarity to bacterial DNA makes mtDNA highly immunostimulatory to cells of the innate immune system (6). The latest evidence suggests that it does not only facilitate antibacterial immune responses, but significantly contributes to further adverse effects and may have important roles in inflammatory diseases and complicated outcomes following cellular damage or oxido-reductive stress (8). MtDNA has been shown to bind PRRs, namely to the Toll-like receptor (TLR) superfamily members or nucleotide oligomerization domain (NOD)-like receptors (NLRs) and more recently it has been shown to be linked with the stimulator of interferon genes (STING) pathway (53, 54).

3.1. MtDNA and TLR interaction

MtDNA has been demonstrated to induce neutrophil activation and facilitates adverse immune reactions through activation of TLR 4 (55) and TLR 9 (56), mediated through MAP kinases p38 (16, 57) and p44/42 (58). MtDNA triggers activation of the nuclear factor kappa B pathway (NFκB) via TLR9, resulting in upregulation of pro-inflammatory cytokine production including TNF-α (59), IL-1β (60) and IL-6 (61). MtDAMPs have been shown to potentiate inflammatory lung injury when introduced into healthy rats in a landmark paper by Zhang and colleagues (9). One possible contributory factor is that mtDNA triggers increased neutrophil

expression of matrix metalloprotease 8 (MM8) through p38 activation, which is a collagen cleavage enzyme that potentiates tissue degradation (57).

3.2. MtDNA-Nod-like receptor 3 relationship

Of the NLR receptors NLR pyrin domain 3 (NLRP3) inflammasome is the most widely studied mainly due to its affinity for a wide variety of ligands (62). Mitochondria have been implicated in the recruitment of NLRP3 in a variety of different ways including through direct activation with mtDNA (63). The assembly of the NLRP3 inflammasome in complexes containing caspase-1 has now been directly implicated in triggering a novel form of cell death termed “pyroptosis” (64). Interestingly when cells lack mtDNA (induced by treatment with ethidium bromide) NLRP3 inflammasome formation was completely inhibited (65). Conversely, NLRP3 inflammasome formation releases mtDNA (65). This indicates a possible positive feedback loop where mtDNA potentiates its own release by stimulating further NLRP3 inflammasome formation.

3.3. MtDNA-STING pathway relationship

MtDNA has the ability to stimulate the innate immune system through stimulation of interferon genes (STING) pathway, resulting in interferon release. The STING pathway was recently mechanistically dissected to reveal an intricate relationship demonstrating how mtDNA triggers interferon release (54). The study showed that through depletion of mitochondrial transcription factor A (TFAM) during a herpes viral infection, mtDNA stability was disturbed, causing enlargement of the mitochondrial nucleoid. Subsequently, fragmented mtDNA was released, activating peri-mitochondrial cyclic GMP-AMP synthase (cGAS) causing increased cGAMP formation. The second messenger cGAMP then activates the endoplasmic reticulum bound STING pathway which ultimately upregulates type I interferon (IFN I) expression which inhibits viral propagation. Interestingly, pro-apoptotic caspase activation inhibits this response and suppresses downstream interferon production (66).

3.4. MtDNA-neutrophil extracellular trap formation

Neutrophil extracellular trap (NET) formation or “NETosis” was first described by Brinkmann and colleagues in 2004 (67). It is characterized by smooth extracellular filaments-17nm in diameter- which are composed of stacked and probably modified nucleosomes (68). This filamentous chromatin backbone is adorned with globular domains of approximately 50nm diameter containing neutrophilic granular proteins. The principle function of the NET is believed to be to entrap and kill circulating pathogens and this function has been directly shown in both Gram- and Gram+ bacteria, viruses and fungi (68, 69).

The composition of NETs was initially widely believed to be predominantly nuclear DNA (nDNA), however under specific stimulatory conditions NETs composed exclusively of mtDNA were demonstrated (70). Our group described that NETs formed after trauma and subsequent surgery were predominantly composed of mtDNA (71). More recently NETs rich in oxidized mtDNA have been discovered in systemic lupus erythematous (72). The emerging body of evidence suggesting NETs can indeed be composed exclusively or predominantly of mtDNA means NETosis may represent a significant source of circulating mtDNA in certain inflammatory conditions.

In addition to the role of intracellular mtDNA in NET composition, mtDNA may also trigger NET formation as a DAMP. NETosis has widely been considered as a NADPH oxidase (PHOX) dependent process, reliant on mitochondrial release of reactive oxygen species (73). However mtDNA as a trigger for NETosis is a much more recent concept and there is growing evidence that extracellular trap formation takes place independently from pro-oxidant activity (74, 75). MtDNA has been demonstrated to be a trigger for NETosis after major trauma and with signaling mediated through a TLR9-dependent pathway, independent of PHOX (38). The concept of mtDNA as a signaling molecule involved in NETosis suggests it may have a more diverse role in regulating certain inflammatory processes in a novel and previously unstudied way.

4. CLINICAL IMPLICATIONS OF CELL-FREE mtDNA

4.1. Diagnostic application of mtDNA

The number of studies investigating the concentration of mtDNA as a potential biomarker in different human body fluids has grown significantly in recent years. Real-time PCR allows simultaneous detection and quantification of mtDNA using a small amount of sample and a downstream real-time PCR analysis give an accurate reproducible result within 2 hrs. The detection from blood, saliva, urine or sperm is a minimally or non- invasive process for diagnosis and was proven to be valuable for the prognosis of various clinical conditions, such as different types of cancer, type 2 diabetes, sepsis, multiple organ failure, fertility impairment or neurodegenerative disorders (28, 76-81). However, the exact cellular mechanisms and cell-type of origin which cause mtDNA concentration to fluctuate in many conditions remain unclear.

Elevated mtDNA content in peripheral blood has been demonstrated as a diagnostic factor in various types of cancer, including non-Hodgkin lymphoma, lung cancer, pancreatic cancer, breast cancer, colorectal cancer, or glioma (81-86). In contrast, an increased risk of renal cancer or hepatocellular carcinoma was observed to be associated with decreased circulating mtDNA concentrations within the tumor tissues of

cancer patients (87, 88). In other human studies, mtDNA quantity measured in the blood of patients with sepsis, pulmonary embolism or out-of-hospital cardiac arrest was proven to be a more powerful prognostic marker than those conventionally used, including nuclear DNA or other existing semiquantitative score systems (89, 90).

The rapidly elevated concentrations of circulating mtDNA levels that are observed in trauma patients with severe injury suggests that extracellular DNA originates from direct tissue injury and subsequent necrosis. It was described to be a trustworthy prognostic marker either in blood or in cerebrospinal fluid with good prediction for unfavorable outcome, or even mortality (65, 91-93). Although, in some studies, nuclear DNA concentration and well-established markers of tissue necrosis were not found to correlate with mtDNA levels, or mtDNA concentration was observed to have no contribution in the pathophysiology of critical illness (17, 29, 94). These findings together with the above-mentioned dichotomies, raise some concerns regarding the nucleic acid-based diagnosis. The reduced clearance of DNA over time caused by impaired organ function during systemic inflammation may also be a contributing factor (92) and similarly, the limited capacity of inflammatory cells for taking up dying cells, thereby DNA (95). In the near future, investigation of the mtDNA methylation pattern rather than its concentration might be used for the diagnostic purpose for identifying tissue specific origin, as it was successfully performed to predict cardiovascular problems or amyotrophic lateral sclerosis (96, 97) and suggests a promising approach to diagnose health problems caused by environmental pollution exposure, aging, drug treatment, and oxidative stress (98). Moreover, since mitochondria do not contain histones, it is likely that the mtDNA methylation/hydroxymethylation ratio rather than histone modification is important for mitochondrial genome-based diagnostics.

4.2. Therapeutical implications of mtDNA

Major trauma patients often require lifesaving allogenic blood products in which cellular remnants, such as mitochondria and extracellular mtDNA are described to be present and to mediate adverse inflammatory processes, as neutrophil, eosinophil and basophil leukocyte activation (99, 100). It is important to take into account, that platelet units represent a potential reservoir of mtDNA, since unlike leukodepleted red blood cell units, stored platelets contain mitochondria.

Moreover, other therapies might also cause cytolysis and may be accompanied by circulatory mtDNA release. Plasma mtDNA content was proven to be elevated and observed to mediate pro-inflammatory effects in maintained haemodialysis patients (101) and in another study, increased mtDNA amount in the plasma of patients was considered to be related to the overall procedure of artificial kidney therapy and probably was due to the death of leukocytes (102).

The fact that mtDNA has such potent immunostimulatory effects makes it an exciting target for immunomodulation therapy attenuating some of the potentially deleterious effects of excessive innate immune activation. Whether mtDNA is free or conjugated in NETs it is readily digestible with DNAse. There is certainly good evidence to suggest that focally targeting NETs with DNAse has yielded a reduction in associated inflammatory lung damage in a mouse model of transfusion related acute lung injury (TRALI) (103). Human recombinant DNAse therapy has been used to good effect when nebulized in cystic fibrosis patients by enhancing sputum solubilisation (104), however no studies have been performed in humans to treat acute inflammatory conditions. With such an emergent role of mtDNA in NETs associated with trauma (71) and more recently in SLE (105) the investigation of DNAse therapy in different inflammatory conditions would be very reasonable.

Targeting mtDNA receptors may also yield ways to modify its proinflammatory properties. It has a diverse role as a signalling molecule in various inflammatory pathways as a ligand of multiple receptors including mtDNA stimulation of the NLRP3 inflammasome (63), cGAS in mtDNA-STING pathway (54) and TLR9 in mtDNA mediated NETosis (38). Modulation of these receptors may convey benefit in a variety of clinical conditions and attenuate the immunostimulatory effects of mtDNA.

5. FUTURE DIRECTIONS

It is essential to understand the tissue specific origin of circulating mtDNA for both diagnostic and therapeutic considerations. The natural history of free polynucleotides in the circulation in inflammatory conditions is largely unknown. The available active DNAse concentration in physiological and pathological conditions could indicate the potential need for enzyme supplementation as a therapeutic strategy. We believe that our current knowledge on cell free circulating mtDNA is in a rather exploratory phase with a potential for the future to rewrite the pathology of the leading causes of morbidity and mortality such as inflammatory conditions, autoimmune disorders, cancer, heart disease, stroke and injury.

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