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Thesis abstract

The rôle of mitochondrial DNA in the post-injury inflammatory response following major trauma

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Trauma is the leading cause of death in the developed world in those aged under 45 years. The main potentially modifiable cause of late death after injury is post-injury multiple organ failure (MOF). Early MOF is characterised by a lethal combination of systemic inflammatory response syndrome (SIRS) which is underpinned by neutrophil proliferation and "priming" as a result of the initial injury and haemorrhagic shock. If primed neutrophils are then exposed to a "second hit" then dysregulated neutrophil-driven inflammation can occur, resulting in end organ sequestration, parenchymal damage, MOF and ultimately death. Interest has increased in endogenous drivers of the innate immune system that exert a potent pro-inflammatory effect by activating pathogen recognition receptors (PRRs), which are designed to respond to pathogen associated molecular patterns (PAMPs) found in bacteria. Endogenous factors that can trigger this response in the absence of sepsis have been termed "alarmins" or damage associated molecular patterns (DAMPs). Mitochondrial DNA (mtDNA) is a potently pro-inflammatory DAMP, which has been found to be highly elevated in the post-injury state. Mitochondrial DAMPs have also been associated with neutrophil-mediated end organ injury.

The primary aim of this thesis was to characterise the effect of post-injury non-life saving orthopaedic surgery on circulating mtDNA levels. No study had looked at the effect of surgical intervention on levels of mtDNA after initial injury and possible sources of mtDNA release. Initially a pilot study of 35 trauma patients who subsequently underwent orthopaedic surgery was performed, primarily measuring cell-free mtDNA and nuclear DNA (nDNA) with sequential plasma measurements over a 5-day perioperative period with comparison to 20 healthy control subjects. mtDNA levels continued to rise over the 5-day observation period following surgery and had no correlation to markers of cell-necrosis either in the form of direct musculoskeletal injury, or secondary inflammatory end organ injury. Whilst nDNA levels were elevated when compared to healthy controls, no increase was observed in the 5-day observation period. Elevated mtDNA perioperative levels were directly correlated with the magnitude and early timing of surgical intervention. mtDNA levels were inversely proportional to the volume of crystalloid infused, indicating a possible rôle for adequate resuscitation in modulating circulating mtDNA levels. A positive trend between mtDNA levels and incidence of post-injury SIRS and MOF was observed, but this failed

to reach statistical significance. This led to the genesis of the hypothesis that the persistently elevated mtDNA levels may have a primary inflammatory source.

The secondary aims of this thesis were threefold. Firstly, to determine whether there was a primary inflammatory source of mtDNA, namely focusing on possible neutrophil extracellular trap (NET) formation or "NETosis". Secondly, to determine what factors may propagate and influence mtDNA release. Finally, to investigate mechanisms for modulating circulating mtDNA levels following injury and subsequent surgery by looking at DNase activity. NETosis is characterised by the release of chromatin in conformational net-like structures in response to sepsis, however some authors had shown that under certain conditions NETs could be composed of mtDNA (mtDNA-NETs). The next study performed focused on demonstrating whether NETs were formed after injury and subsequent surgery and what type of DNA they were composed of. The presence of NETs had been postulated after traumatic injury by one group based on observed high concentrations of cell-free DNA but they failed to define any microscopic evidence of NET formation. In our next paper we definitively demonstrated that NETs were formed after injury and subsequent surgery and also in response to elective orthopaedic hip replacement surgery. This was achieved microscopically using fluorescent DNA avid dyes to demonstrate the presence of conformational DNA-NET structures. Molecular genetic analysis of the NETs formed in response to injury and subsequent surgery or in response to elective surgery alone revealed that the NETs were mtDNA-NETs. Due to molecular similarities between mtDNA and bacterial DNA (bDNA) we hypothesised that mtDNA might trigger NETosis through a PRR mediated pathway. In the next paper we studied the effect of exposing healthy neutrophils and post-injury perioperative neutrophils to physiological concentrations of mtDNA we had measured in our initial pilot over the 5-day observation period. We then conducted a series of positive control experiments using phorbol myrisate acetate (PMA), a known potent stimulator of NETosis. NETs were triggered after trauma and healthy neutrophils were exposed to mtDNA. Notably the NETs formed in response to mtDNA were mtDNA-NETs in both trauma and healthy neutrophils, however trauma neutrophils were less responsive compared to healthy control neutrophils. This observation was thought to be possibly due to the exposure of trauma neutrophils to high levels of mtDNA after injury and surgery causing prior mtDNA-NET production. NETs formed in response to PMA exposure were composed almost exclusively of nDNA (nDNA-NETs). Finally, we studied the plasma activity of DNase alongside mtDNA and nDNA concentrations in a much larger cohort of trauma patients (n=103) compared to our initial pilot (n=35). Circulating DNase isotypes are responsible for the digestion of extracellular DNA whether mtDNA or nDNA and also digest NETs. DNase activity was significantly reduced compared to that measured in healthy controls. This greater powered study did reveal a statistically significant positive correlation between perioperative mtDNA levels and SIRS but not MOF, despite a strong trend.

Our data suggest that after traumatic injury, the timing/magnitude of surgery and adequacy of resuscitation influence the levels of circulating mtDNA. Neutro-

phils contribute a significant amount of mtDNA through mtDNA-NET formation in the post-injury and perioperative period. mtDNA can essentially drive its own release through a positive feedback loop. This occurs through circulating mtDNA triggering further mtDNA-NET release, resulting in a vicious cycle of dysregulated inflammation and associated SIRS with a likely link to post-injury MOF. Most excitingly the finding of reduced DNase levels in the face of high mtDNA levels. This may offer up a novel therapeutic target for modulation of aggressive post-injury SIRS, through the potential administration of exogenous DNase in the post-injury and peri-operative recovery period.

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