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Review paper

Netosis as a bridge between inflammation and liver cell injury

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Abstract

One of programmed cell death types, netosis, discovered in ’96, was first described by Zychlinsky *et al.* and has gained elevating popularity among many researchers. It is a process occurring in order to catch pathogens into a trap and kill them. This trap is a scaffold somewhat consisting of chromatin and particles with antimicrobial properties such as histones. Extracellular histones are capable of proinflammatory cytokines production and platelets aggregation what accounts for inflammatory response. Unfortunately, these proteins have also a toxic potential which leads to cell injury through Toll like receptors presented by

neutrophils. Mentioned neutrophils participate in an oxidative burst which yields to production reactive oxygen species performing a crucial role in the development of hepatocytes injury. Thus, alcohol abuse appears to result in a rapid development of liver cell injury through progression of inflammation induced by netosis. However, controlled NETs forming can be a future therapeutic option.

Introduction and purpose of work

According to WHO, European Region has primacy on alcohol consumption in the world. In Poland by the latest research of WHO from 2010 due to liver cirrhosis ASDR (age-standardized death rates) is estimated at 28.8% among males and 10.4% among females. [1] It shows that men drink more than women, further they are more exposed to alcohol-attributable sequelae. However, alcoholic liver disease (ALD) is not only cirrhosis but also previous stages such as fatty liver, alcoholic hepatitis, processing further with liver cirrhosis or fibrosis, and steatohepatitis, including both hepatitis and steatosis. Although, the pathophysiology of ALD remains not fully researched, it is known that alcohol metabolites including acetaldehyde or reactive oxygen species (ROS) perform the crucial role in the development of liver injury. Notably, the last data shows that recently popular in medical literature, process called 'netosis' can be involved in development of overwhelming amount of diseases such as ALD, other liver injuries, carcinogenesis, brain and lung damage.

Toxicity of ethanol metabolites

Transformation of ethanol into acetaldehyde requires presence of ADH. Among five classes of ADH, the first one which is ADH 1 is the most important in the circulation of ethanol in hepatocytes. Not only ADH but also ALDH, triggers build-up of NADH which is further oxidized to NAD^+ thus leads to creation of ROS including hydrogen peroxide. [2] After that, hydrogen peroxide can be fully reduced into water or partially reduced into hydroxyl radical which is the neutral form of the hydroxide ion (OH^-). This uncertain fate of hydrogen peroxide, mentioned above may be a cause of mutations, inactivation of enzymes, lipid peroxidation or even cell damage which could happen before transformation into water. Toxic aldehydes formed during lipid peroxidation just as acetaldehyde can bind and built-up adducts with lipids, DNA and more. These DNA adducts conduce to mutations in a DNA molecule, damage of nucleotides thus leading to not only toxic but carcinogen results as well. The study of Ceni *et al.* [2] showed that adducts in alcohol-fed rats and in human abusing alcohol appeared to cumulate close to tissues surrounding veins. ALD patients show presence of antibodies against one of the aldehydes which create hybrid adducts during the lifespan of ethanol in hepatocytes. The antibody titer is associated with liver cell injury.

Toll-like receptors in a developing liver injury

Additionally, alcohol abuse occurs to enhance level of lipopolysaccharide (LPS) – gram negative bacterial endotoxin which through Toll-like receptor 4 (TLR4) activates macrophages. The TLR4 receptor complex appears to cause production of chemokines which induce inflammation including activation of tumor necrosis factor alpha (TNFα) or IL-1β. [3] Furthermore, ethanol increases intestine permeability which develops migration of LPS from guts to the hepatic portal system. Afterwards, LPS attaches to TLR4 and it causes production of cytokines (by macrophages) mentioned above such as interleukin 1β which is said to be lymphocyte activating factor. This upgrowth consisting of TLR4 and LPS becomes main factor of ALD development. This connection is also found in hepatocellular cancer to which ALD leads to. [3] However, which of these cells contributes to liver damage linked to alcohol abuse, stays unknown. The studies showed that TLR4 is a receptor that can be expressed in different types of cells including circulating macrophages or Kupffer cells which are stellate macrophages located in liver. According to Jia *et al.* studies [4] that were demonstrated in alcohol-fed mice, it is claimed that TLR4 hepatocytes (especially Kupffer cells) had a high fat content. Removal of Kupffer cells in rats leads to decreasing of advancement of fatty liver, inflammation and other pathological processes happening in liver. [4] Thus, we can contend that Kupffer cells play a main role in progress of ALD. However, Toll like receptors 4 can be presented outside the liver, for example in myeloid cells. The data [4] showed that after deletion of TLR4 in myeloid cells in alcohol-fed mice it was observed that level of TNFα has been decreased. It proves that presence of TLR4 on cells may be a future target for the treatment of ALD. Inflammation inducted by TLR4 in patients abusing alcohol may be situated not only in liver but in brain or intestine as well. Then again, a boost of LPS wasn't observed in brain what demonstrates that TLR4 might be presented on different ligands than LPS. [5] TLR4 creates complex with lymphocyte antigen 96 (MD-2) which can bind with high mobility group box 1 protein (HMGB1) and through this connection macrophages are activated. Further, TNFα and other cytokines are blown out. [6] Research of Yang *et al.* [6] showed that HMGB1 is a part of inflammatory path after being discharged from hepatocytes which had been damaged before. In pathogenesis of acute liver failure (ALF) we can observe necrosis and apoptosis. Via necrosis HMGB1 can be a plasmatic biomarker of progression of liver damage.

Inflammation induced by NLRs

During inflammation proinflammatory cytokines are released, influenced by inflammasomes. Inflammasome is a multipolymer intracytoplasmic protein complex that is activated by macrophages. Very common in literature, Nod like receptor 3 (NLRP3) inflammasome becomes active only in the presence of ROS. This complex can bind with ROS, nitrogen species (RNS) or potassium. However, it is unknown how they recognize each other. Inflammasomes take part in apoptosis by releasing of caspase 1 and further, they influence on elevation of IL-1β. [7] According to Deigendesch *et al.* [8] there are many pathologies such as infections or inflammation, depending on increased activity of NLRP3 which is associated to copper elevation. Copper is claimed to be a part of NLRP3 inflammasome essential to its function. It pertains to macrophages, excluding monocytes. [8] What is more, inflammasomes can be found not only in hepatocytes but also in the brain and intestine what was confirmed in the data [5] in alcohol-fed mice.

Signally, pro-inflammatory cytokines including IL-17 and IL-22 correlate with development of ALD. However, Capparos *et al.* [9] showed that elevation of IL-22 is claimed to be a positive marker for depletion of liver damage. In ALD patients IL-22 is hepatoprotective and anti-steatotic what can be used in the nearest future for treatment of this disease. [9] Elevated level of IL-10 in alcohol-induced liver injury generates M2 macrophages

in correlation with microRNA expression which plays a key role in inflammation process. [10]

NET as a trap

There are different types of programmed cell death, including apoptosis and non-apoptotic ones. We can group non-apoptotic cell death into three: netosis, pyroptosis and necroptosis. [11] Netosis was discovered in 1996 by Takei *et al.* and first described by Zychlinsky and others in 2004. [12] It is a phenomenon of creating a neutrophil extracellular trap (NET) by neutrophils. NET is composed of DNA of neutrophils and antimicrobials, such as histones. [13] Histones are able to trigger production of cytokines and aggregation of platelets which leads to inflammation processes. Their increased level can be a result of damage of hepatocytes while histones are released or precisely as an effect of NET existence. [14] Furthermore, the development of ALF is linked with toxicity of histones. In study of Wen *et al.* [14] it was revealed that histones could be inflammatory biomarkers in ALF patients. What is more, they proposed noncoagulant heparin as a future therapy in development of ALF. It comes from the fact that heparin can connect with histones because of their opposite charges. In mice heparin treatment caused elevation of survival rate by its anti-inflammatory influence. However, this kind of therapy must be applied carefully because heparin can contribute to increased risk of hemorrhage which is already elevated in ALF patients. [14] NETs are claimed to be a perfect procoagulant backbone and be a part of intrinsic pathway of coagulation. [13] The raised extracellular histones and NETs levels can cause thrombosis during sepsis through platelets activation. Their neutralization leads to limitation of progression of ALF. Patomechanism of liver, brain and vascular damage through histones consists of toll like receptors 2,4 and 9. [6]

There are two types of netosis which are dependent on the presence of reactive oxygen species: NOX-dependent (which consists of ROS in its pathway) or NOX-independent (which doesn't consist of ROS). It should be noted that NOX-independent pathway can be triggered by ionomycin or calcium which are researched together in many different studies. In the NOX-independent netosis mitochondrial ROS (mROS) occurs instead of ROS. In this pathway it is worth to mention about peptidylargininedeiminase 4 (PAD4) which is a main mediator of chromatin decondensation and takes part in citrullination of histones. [12] Furthermore, PAD4 is essential to form NETs – in mice deprived of PAD4 netosis was impossible to happen. However, the same mice showed the immunological response to sepsis as mice which were in the possession of PAD4. This leads us to the last revelation that NETs can be developed in many possible mechanisms such as 'suicidal netosis' in which neutrophils perish. Contrarily, there is a mechanism called 'vital netosis' that is mostly unknown but it is said that neutrophils can exist. [15] Recently, many researchers commit their time to pH influence on NET creating. Naffah *et al.* study [12] showed that increased alkaline pH (7.4-9.0) causes higher levels of calcium, mROS, histone H4 fracture and PAD4 activity that contribute to new NETs. However, changes in histone H4 amount involves calcium presence so it is not confirmed if pH has any influence on histone H4 with neutrophils not stimulated by calcium ionophores. [12] There are five types of core histone proteins (we need to remember that there are also extracellular histones) which take part in chromatin condensation and build nucleosomes: H1, H2A, H2B, H3 and H4. [15] Histone H4 is the smallest one that can be disparted by neutrophil elastase (NE), further it leads to chromatin decondensation and helps to intensify netosis. [12] NE is essential for NET development. [16] Histone 1 is claimed to be the linker histone. However, opinions are divided – H1 can appear in NETs or be damaged in netosis. Certainly, histones H2A and H2B took control on NET formation. [15] Extracellular histones demonstrate high toxicity, activate Toll like receptors, inflammasome NLRP3 and even creating of thrombus. They are

concerned with many pathological processes such as inflammation, sepsis, acute liver or brain injury. On the other hand, histones can be some kind of security to extracellular DNA and protect it from getting destroyed. [17]

NETs are considered to take part in bacteria or virus killing by immobilization of pathogens. However, because of the presence of cytotoxic histones in their scaffold, they also participate in development of pathological processes such as inflammation, sepsis or even lupus. There are also two types of NETs depending on presence of ROS. In the first one, ROS independent NET, there is leukotoxic hypercitrullination. This process is dependent on activation of PAD4 through increased level of calcium inside cells and the freeing of chromatine starts. The second one, which is ROS dependent NET, is known for its antimicrobial features and is associated with neutrophil elastase. [18] Clark *et al.* [18] gathered research which proclaims that this division into two NETs isn't as clear as it seems to be. They added β-glucan to ROS dependent NET related to PAD4 acivation and received the new one, third type of NET which denies previous findings.

The crucial role of neutophils

Neutrophils have their own role to act in netosis. They are responsible for wrapping chromatin and transforming it from nucleus to extracellular space in a dangerous situation such as inflammation or cell injury. Extracellular chromatin is created to kill bacteria, participates in immune defense and starts clotting. [19] Notably, neutrophils are arranged into many processes in which they play pro-inflammatory role, as well as anti-inflammatory role. Neutrophil cells structure an oxidative burst which produces cytotoxic ROS . [20] What is more, hydrogen peroxide which is produced during the oxidative burst, causes releasing of NE from a complex called the 'azurosome' to the nucleus and NET forming. [21, 22] The azurosome owes its name to azurophilic (primary) granules from which it comes from. [21, 23] Neutrophils via ROS can recognize microbial pathogens and present them to other first defense cells through activation of mitogen-activated protein kinase (MAPK) pathway. [2, 20] MAPK pathways are necessary to signal a necessity of NET formation. [22] However, the recent study claims that ROS can have different roots than the oxidative burst – they can be produced by the mitochondria in the presence of calcium ionophores what proves that there are different pathways leading to netosis. [24]

Neutrophils present TLRs (in human excluding TLR3 or TLR7) what sparks their relocating and creating interleukin-1 receptor associated kinase complex (IRAK complex) which consists of kinase IRAK1 and kinase IRAK4 and further, it leads to releasing of cytokines. This processes of neutrophil activation go ahead through MyD88-dependent pathway (MyD88 – myeloid differentiation primary response 88 – a human protein), adversely to other cells which use only TLR expression. [23] TLR4 activates interferon regulatory factor (IRF3) which is capable of interferons production and induction of apoptosis in liver cells. [25] The research [26] indicates that elevated level of IRF3 during apoptosis can start secondary necrosis. Secondary necrotic cells have already undergone the apoptosis. [27]

Conclusions

Uncontrolled netosis can be harmful to cells and induce pathological processes. [28] However, controlled netosis is a future therapeutic option for many diseases such as any kind of liver injury, especially alcohol-induced. The developing problem in treatment of ALD is hepatic encephalopathy which can be observed in many ALD patients. Sanyal *et al.* [29] showed that pentoxyfylline is no more effective, while steroids are not allowed to be used on patients with any infections. It generates problem in finding the right therapy for patients with alcohol-damaged liver.

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