Neutrophil in diabetic stroke: emerging therapeutic strategies

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Post-ischemic inflammation is a coordinated process, which lasts from hours to days and involves recruitment of inflammatory cells from blood to the brain endothelial cells. Recently, the adhesion of leukocytes at endothelium, especially neutrophils, and its implication in post-stroke neuronal injury have been extensively explored and reported in both experimental and clinical settings (Jian et al., 2019). However its role in diabetic patients following stroke is still elusive. Some significant differences such as risk factors, stroke subtypes and clinical outcomes are different between diabetic and non-diabetic. The higher prevalence of lacunar stroke, higher frequency of hypertension and lower neurological deficit at admission were reported earlier in diabetic patients. We found that early increase of neutrophils plays a prominent role in instigating a larger stroke size and worse clinical outcomes as compared to patients that do not have diabetes. After ischemic stroke, neutrophils are recruited to ischemic brain and can enter into the brain following hypoxia-ischemia (HI) through cerebral vessels, choroid plexus, and subarachnoid space. **Figure 1A** shows the various routes of entry of neutrophils in the db/db mouse brain 24 hours post stroke. Among all immune cells, neutrophils are the first one to appear in the brain at day 1 post HI and remain until 7 days in the perilesional space, and subsequently other cells such as T & B lymphocytes migrate to the lesion (Chu et al., 2014). These neutrophils remain in the vessel, release matrix metalloproteases and other proteases to damage the blood-brain barrier and the secondary damage starts, when neutrophils penetrate the brain parenchyma (Jickling et al., 2015) Previously, we have seen an increased matrix metalloproteinase-9 with graded infarct size and a direct relationship between matrix metalloproteinase-9 and neutrophils, which confirms the role of neutrophils mediating stroke injury (Kumari et al., 2020).

Diabetes augments cerebral ischemic damage: Following reperfusion of ischemic brain, experimental and clinical studies have shown that neutrophils produce reactive oxygen species (ROS), which causes the disruption of neurovascular units, thereby increasing the blood-brain barrier permeability and cerebral edema (Tang et al., 2019). Another neurotoxic effect of neutrophils is the production of neutrophil extracellular traps (NETs), which are decondensed DNA and proteases, in response to various stimuli and cytokines following stroke (**Figure 1B**). In addition, an increase in NETs formation reduces neovascularization, which impairs vascular remodeling during stroke recovery (Kang and Yu, 2020).

Under diabetic conditions, hyperglycemia causes chronic inflammatory conditions in various organs and tissues, which is manifested by expression of proinflammatory cytokines, and activation/expression of other inflammatory mediators. It has been suggested that high glucose can cause ROS production and increased expression of inflammatory markers in a variety of cells including neuronal cells. In addition, hyperglycemia induces nuclear factor kappa B-dependent production of proinflammatory cytokines, TLR expression, and increased oxidative stress. The other factors to be considered in diabetics is activation of hypothalamus-pituitary adrenal (HPA) and the sympathetic nervous system axis. Existing data from studies on diabetes suggest that already impaired HPA axis activation in diabetics is further affected by influx of pro-inflammatory cytokines post stroke. The cytokines-induced HPA axis activation results in excess release of cortisol, which consequently leads to more pronounced insulin resistance. Similarly sympathetic over activity has been linked to the onset of diabetes is more pronounced post HI. Activation of sympathetic nervous system at post HI leads to the release of catecholamine, which in turn stimulate spleen to release additional neutrophils into periphery (Silverman and Sternberg, 2012). These neutrophils later migrate to the central nervous system, worsening the post-stroke outcomes. Altogether these factors aggravate neuronal cell deaths in diabetics and result into increased mortality and morbidity (Bettermann et al., 2020).

Our experimental and clinical studies suggest that increased inflammatory reaction, high cortisol level and suppressed immune system due to diabetes are further exaggerated after stroke, leading to increased ischemic damage and poor clinical outcomes (Kumari et al., 2020). The underlying mechanisms are still unknown; however we and others observed that early rise of neutrophils plays an important role in expanding the neuronal injury in type 2 diabetic rodent models and diabetic patients post HI. This *db/db* mouse model has spontaneous mutation of leptin receptor ($Lepr^{db}$) and exhibit morbid obesity, chronic hyperglycemia, elevated plasma insulin at 4 to 8 weeks. The uncontrolled rise in blood sugar leads to peripheral neuropathy, increased myocardial disease and delayed wound healing. We observed an acute rise in peripheral neutrophil concentration in diabetic *db/db* mice at 4 hours post stroke. Those neutrophils invaded the ischemic tissue within first 24 hours post stroke. In our studies the depletion of neutrophils prior to stroke reduced the infarct size in diabetic mice, but not in heterozygous *db*/+ non-diabetic controls, strengthening our hypothesis regarding the important influence of neutrophils on the stroke outcomes and prognosis (Chu et al., 2014; Kumari et al., 2020). Similar observations were reported in diabetic patients in both prospective and retrospective studies (Bettermann et al., 2020).

Current status of neutrophils as a therapeutic target: The rapidly growing data to date strongly suggests that neutrophils have a detrimental effect on stroke, and making the neutrophils promising therapeutic targets for stroke treatment. The leukocytes infiltration exacerbates ischemic tissue damage, but, in the LeukARREST human stroke trial, antibodies directed against various cell adhesion molecules such as anti-ICAM-1 or anti-CD18 failed to show any improvement. A treatment targeted to block the neutrophils selection and activity was shown to have a variable outcomes in rodent models of stroke (Jickling et al., 2015). On the other hand, in Enlimomab clinical trial, blocking or inhibiting the activity of neutrophils resulted in increased infections and hemorrhagic transformation. Loss of neutrophils as a result of disease or therapies leads to severe infections and chronic inflammation, thereby causing tissue damage not repair. Interestingly, this phenomenon may be more relevant in diabetic patients because infection rates increase significantly due to an acute immunosuppression following stroke. In contrast, earlier studies have shown that there is abundance of neutrophils at the wound sites despites the impaired wound healing in diabetic patients (Segel et al., 2011). This suggests potential impairment in phagocytic activity of the recruited neutrophils. Another phenomenon, namely the polarization of neutrophils to the anti-inflammatory N2 subtype rather than the proinflammatory N1 subtype, has been seen in rodents models of stroke, when neutrophils accumulate in the

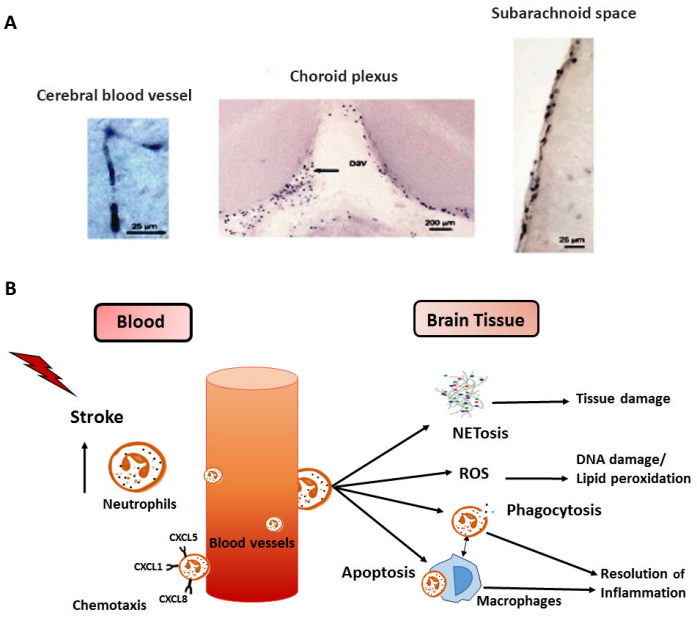

Figure 1 | **Neutrophils mediated neuronal injury in acute ischemic stroke.**
(A) The immunohistochemistry of neutrophils Ly6b.2 antibody, enhanced by Nickel DAB. Immunohistochemistry of neutrophils outlining several routes of entry into the diabetic mouse (*db/db*) brain 24 hours post hypoxia-ischemia (HI). Neutrophils can enter the brain through cerebral vessels, choroid plexus, and subarachnoid space. Arrow is pointing to the choroid plexus of the dorsal third ventricle (D3V). (B) Enhanced chemokines receptor's expression on the surface of neutrophils, helps in adhesion and migration of the neutrophils in the brain. The activated neutrophils cause tissue injury by increased reactive oxygen species (ROS) production, enhanced NETosis in the inflammatory phase. The deleterious activity of neutrophil is controlled by apoptosis and phagocytosis by neutrophils and macrophages, contributing to the healing phase. CXCL: Chemokine (C-X-C motif) ligand.

brain tissue beyond certain levels (Jickling et al., 2015). The N2 subtype is important for the resolution of inflammation and for the healing of the infarcted area in the late phase after stroke. The modulation of the reprogramming of neutrophils towards the N2 phenotype has been found effective and beneficial for the alleviating the inflammatory responses in the brain after stroke (Cuartero et al., 2013). Therefore, complete depletion of neutrophils could have detrimental effects, which could be the reason for inconsistent results in neutrophils block /depletion studies in rodent stroke models and no beneficial effects in aforementioned clinical trial (Jickling et al., 2015). It is important to promote the favorable phenotype N2 in the late phase after stroke and control the N1 neutrophils in the early phase. The imbalance of neutrophil phenotypes with increased production of ROS along with the compromise in phagocytic activity of neutrophils in diabetic patient population, results in delayed wound healing and worse clinical outcomes after stroke. Previously, we have observed a delayed and diminished immune response of the central nervous system following stroke in a type 2 diabetic mouse model of stroke and a similar phenomenon was observed in diabetic patients. We observed an acute peripheral immunosuppression, a reduced T & B lymphocytes, monocytes and NK cells up to 96 hours post stroke along with increased neutrophil numbers up to 48 hours post stroke, associated with unfavorable stroke outcomes up to 90 days post stroke. However, in non-diabetic patients with acute ischemic stroke had a significantly higher peripheral frequency of $CD4^+$ and CD28 null-T cells compared to control without stroke. These observations clearly indicate that the imbalanced immune response in the diabetic population, results in severe stroke and poorer recovery compared to non-diabetic cohorts.

Neutrophils as a possible future therapeutic approach: Overall, our observations and other studies suggest that neutrophils mediate post-stroke neuronal injury, and play an important role in stroke recovery in diabetic patients. However, balancing the neutrophils number in the early phase of stroke and promoting the anti-inflammatory N2 phenotype of neutrophils in late phase of stroke is challenging. A strategy attempting to induce apoptosis of pro-inflammatory neutrophils at the first phase of stroke could be one of the viable approaches. Recently, the study by Zhang et al. (2019) showed that doxorubicin-conjugated nanoparticles selectively induced apoptosis of neutrophils and maintained the immune homeostasis, which resulting in reduced neurological severity following stroke and increased survival in mice following sepsis. The apoptosis of neutrophils was associated with reduced cytokines levels, which potentially diminish further recruitment of neutrophils. This study shows encouraging findings regarding an approach to control the acute inflammation. However, the impact of such apoptosis is still unknown in the late phase of inflammation or on the anti-inflammatory N2 neutrophils. Beyond the neutrophils phenotypes, it is also crucial to consider the movement of neutrophil based on the phases of infection and tissue repair. The forward and reverse migrations of neutrophils occur based on the phases of inflammation, being guided by different mediators. Initially, acute forward migration of neutrophils is initiated by signaling from damaged or necrotic tissues and is mostly mediated by damage-associated molecular patterns, ROS, chemokines and leukotrienes followed by amplification of the number of neutrophils that are newly recruited by tissue- residents cells such as macrophages (de Oliveira et al., 2016). The reverse migration of neutrophils, which mainly occurs via apoptosis and phagocytosis by macrophages, is required to prevent the tissue damage and resolve the inflammation. However, the other evidence shows that hypoxia inducible factor-1 and other chemoattractant such as CXCL8 act as mediator for the neutrophils' return to the vasculature. Neutrophils return from the site of inflammation or the distal sites of neural injury towards the blood circulation through reverse trans-endothelial migration (de Oliveira et al., 2016). These studies suggest that promoting reverse migration of neutrophils may be another potential target for the prevention and repair of neural tissue necrosis following stroke. This will be crucial in case of diabetes, when neutrophils counts are high and an abundance of neutrophils linger at the injury site. Recently, Stamatovic et al. (2020) redirected neutrophil migration through a peripherally implanted sponge soaked in CXCL1, improving neurological outcomes, reducing brain inflammation and increasing survival at day 1 and day 5 in the thromboembolic stroke model in mice. This study indicates that CXCL1 could be another target to redirect neutrophils to prevent the progression of ischemic cell death. However, other chemokines such as CXCL5, CCL2 and CCL9 are upregulated during the second phase of inflammation and their role in neuronal injury post-stroke is yet to be elucidated. Hence maintaining the optimal concentration of peripheral neutrophils during recovery is still a challenge. All of these approaches, it is difficult to simultaneously control neutrophil activity and maintain immune cell homeostasis in

diabetics, something essential for preventing infections and improving survival/ mortality rates. Another target is to control the neutrophils-mediated ROS activity for the prevention of inflammation and tissue injury (**Figure 1B**). A previous study has shown that oxidant signaling mediated by NADPH oxidase-2 in neutrophils, regulates regeneration of tissues in post ischemic lesion sites. The study by Weisenburger-Lile et al. (2019) differentiated three subsets of neutrophils in the blood samples from acute ischemic stroke patients and it reported that an increased ratio of the senescent/ immunosuppressive neutrophils is primarily responsible for inducing ROS production, and for releasing different elastases and proteases following neutrophils 'degranulation up to day 7 post stoke. The enzymes produced by activated neutrophils induce vascular damage and penetrate into brain parenchyma to aggravate the ischemic lesion. Therefore, identifying and targeting the mediators which can control each particular subset of neutrophils would be the best approach to control the deleterious activity of neutrophils following acute stroke. This approach may subsequently balance the neutrophil subsets, thereby enabling the repair or regeneration of neural tissues. Similarly, Kang and Yu (2020) showed that increased NETs formation or impaired NETs clearance is responsible for impairing neovascularization and vascular remodeling of the peri infarct area in the late phase of stroke. The enzyme peptidylarginine deiminase 4 (PAD4) has been found to be crucial for the neutrophils' NETs formation and PAD4-deficient mice displayed the reduced number of NET-positive neutrophils, which facilitated stroke recovery (Kang and Yu, 2020). This phenomenon could be more pertinent in neovascularization of infarcted area in diabetic stroke patients, for whom wound healing is delayed and compromised.

In conclusion, based on the discussed data and available literature neutrophils play an important role both in neuroinflammation and in the healing of injured brain tissue following stroke. The differential role of neutrophils depends on the phase of stroke and is guided by various mechanisms such as production of ROS, activation of cytokines/ chemokines and release of various enzymes during the inflammatory phase. At the healing phase, the phagocytic activity is involved in remodeling and repairing of the infarcted tissues, but increased NETosis primed by chronic pro-inflammatory responses linked to diabetes and hinder those reparative processes. Thus, preclinical studies suggest four possible targets: (1) control of the excess neutrophil infiltration into the brain; (2) neutralization of the oxidative stress; (3) inhibition of NETosis; and (4) balance of the existing neutrophil subtypes. They are all important targets for consideration during development of future novel therapeutics and hold promise for the enabling neuronal recovery after diabetic stroke.

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