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Can Immunotherapy be used to Treat Ischemic Stroke?

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Abstract—There are two kinds of stroke: ischemic stroke and haemorrhagic stroke. Ischemic stroke constitutes about 85% of all stroke cases. In the UK, stroke is the 4th leading cause of death. The available treatment for stroke is intravenous tissular plasminogen activator (ivtPA), but this is not a sufficient therapeutic approach. It needs to be administered 3 hours following stroke, in order to be effective. Moreover, recent findings have implicated tPA to interact with NMDA receptors resulting in noxious effects. As the population ages, more people are likely going to suffer from a stroke. Therefore, it is imperative to find novel therapies for its treatment. This study, through reviewing existing literature on stroke research, explores the possibility of treating ischemic stroke with immunotherapy. This study communicates that there are numerous immunotherapeutic targets in ischemic stroke. But, while animal studies have been very successful, most of this success has failed to translate into clinical success. Immunotherapy remains an exciting option to treat ischemic stroke, however, the best therapeutic approach still remains the administration of ivtPA.

Keywords—*Stroke, immunotherapy, ischemia, ischemic injury, inflammation, leukocytes, antibodies*

I. INTRODUCTION

Stroke is a neurological condition affecting mostly people over 65 years of age, however it can also affect people of any age. There are two broad types of stroke, ischemic and haemorrhagic, of which ischemic stroke is more prevalent, and constitutes about 85% of all stroke incidences. Ischemic strokes are due to a blockage of blood supply to the brain, and this consequently deprives the brain of oxygen and glucose, which is essential for its normal function [1]. Haemorrhagic stroke on the other hand, involves bleeding within and around the brain. About 1.2 million people in the UK experience and/or live with stroke, and in 2010, stroke was the 4th leading cause of death in the UK [2]. With the ageing population, these figures are predicted to rise.

While brain damage occurring after stroke pose a serious threat to the survival of the affected individual, it is infection that most patients are likely to die from [3]. The contribution of the immune system during stroke appears to be biphasic. The first phase involves the activation of immune cells, which go on to initiate and mediate the inflammatory response leading to brain tissue damage, while the second phase is a global immunosuppression state [4].

Immunotherapy involves the stimulation or suppression of the immune system in either a passive or active manner using vaccines. Active vaccines trigger the immune system into action, while passive immunity involves the transfer of mostly antibodies or other components of the immune system to the host to provide immunity against a disease. There are numerous molecules implicated in stroke, and these provide targets for immunotherapy [2]. Following ischemic stroke, immunotherapy could delay brain tissue damage and death, before revascularisation therapy is done. Immunotherapy could also be used to reverse the immunosuppressed state after stroke.

Vaccines have been very useful in recent years to prevent numerous infectious diseases. More recently, the idea of treating diseases such as cancer, autoimmune diseases, and neurodegenerative diseases using immunotherapy have been excitingly interesting. Understandably so, the specificity of the immune response and the ability to harness this is an attractive medium to treat diseases. However, immunotherapy can come with fatal side effects as observed in the Amyloid-beta vaccines in Alzheimer's disease (AD) [5].

The author discusses stroke biology, the current stroke treatment and were it falls short, and finally, communicates how some of the inflammatory processes, molecules, and secondary infections in stroke pathology could be targets for immunotherapy. This paper reviews the work done in the field of stroke immunotherapy, including the trialling of both animal and clinical active and passive vaccines as better candidates for stroke treatment.

II. FACTORS THAT PREDISPOSE AN INDIVIDUAL TO STROKE

The risk factors for both ischemic and haemorrhagic stroke could either be non-modifiable or modifiable.

The non-modifiable factors are factors that cannot be improved and include: age, gender, ethnicity, race and genetics. Age has been identified as the greatest contributing factor to stroke occurrence [6]. Individuals over the age of 65 are generally at a higher risk of having a stroke, and although men are more likely to get stroke than women, more women die of stroke, as women live for longer. Several studies have also implicated genetics in stroke. These studies showed that individuals whose parents died of stroke, face an increased risk of stroke [7]. Also, several genes such as Apolipoprotein-E, endothelial nitric oxide synthase and phosphodiesterase 4D have been implicated in stroke [8].

Modifiable risk factors for stroke include transient ischemic attacks (TIAs), hypertension, cardiac disease, smoking, high alcohol consumption, drug abuse, and lifestyle factors such as diet and exercise. TIAs are caused by blood vessel injury which although do not lead to a permanent brain damage, serves as a warning sign for stroke. TIA patients do need medical monitoring as they may later develop a full-blown stroke [9].

III. BASIC ANATOMY OF THE BRAIN AND ITS VASCULAR SUPPLY

A. Brain lobes

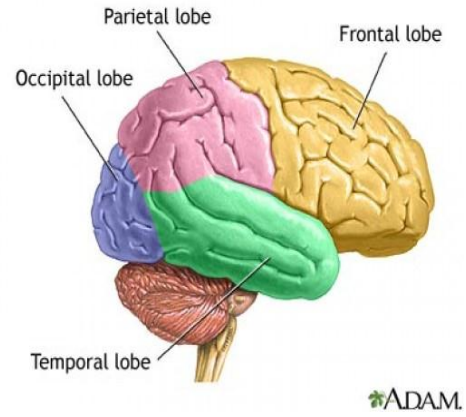

Figure 1. The different brain lobes. This diagram is taken from [10].

There are 3 main brain components: cerebrum, cerebellum and brainstem. The cerebrum mediates higher functions such as speech, control of movement, emotions, and an integration of sensation. The cerebellum on the other hand, serves to maintain balance, and controls coordination. The brain stem is responsible for the control of heart rate, sleep, arousal, blood pressure, and respiration [11].

The cerebrum consists of four brain lobes (shown in figure 1): frontal, parietal, temporal and occipital, and can be divided into the left and right hemispheres. The left hemisphere controls the activities on the right side of the body, while the right hemisphere controls the activities of the left side. Consequently, injuries to the left hemisphere, would lead to the impairment of activities of the right side and vice versa. A stroke to the cerebellum results in impaired motor functions and a deficient coordination, and due to the automatic functions mediated by the brainstem, a stroke to the brainstem can be very fatal, and may even lead to the individual going into a vegetative state [11].

B. Vascular supply to the brain

Blood supply to the brain is via two major blood vessels, the carotid and vertebral arteries, of which there is a left and right for each. The carotid arteries are further divided into two: the external and internal carotid arteries. The external carotid arteries supply blood to the scalp and face, and the internal carotid arteries function to supply the anterior cerebrum with blood. The vertebral arteries on the other hand, supply blood to the posterior cerebrum, some regions of the cerebellum, and the brainstem. Any decrease in blood flow, would lead to deficits in these brain regions [12].

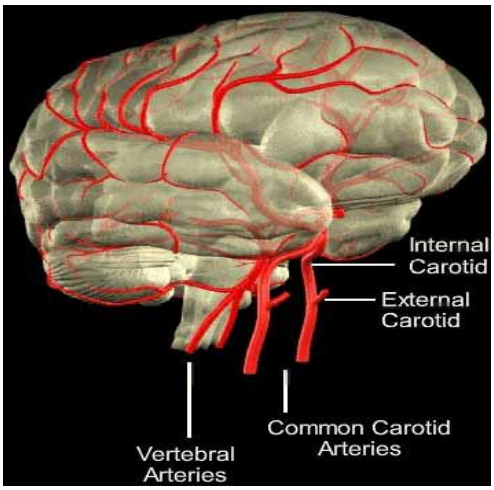

Figure 2. A schematic of the major blood vessels supplying blood to the head. The face and scalp are supplied with blood by the external carotid arteries. The internal carotid arteries supply the anterior part of the cerebrum with blood. The vertebral arteries as shown are supplying blood to the posterior end of the cerebrum. This diagram is taken from [12].

The carotid and vertebral arteries go on to form a circle at the base of the brain, and this circle is known as the Circle of Willis. The Circle of Willis is a circle of communicating arteries from where other arteries such as the middle cerebral artery (MCA), anterior cerebral artery (ACA), posterior cerebral artery (PCA) protrudes and project to different brain parts. The circle formed by the carotid and vertebral arteries mean that following an occlusion in one of the main arteries, distal smaller arteries that these main arteries supply blood to, can obtain their blood supply from the other arteries. This phenomenon is known as collateral circulation [12].

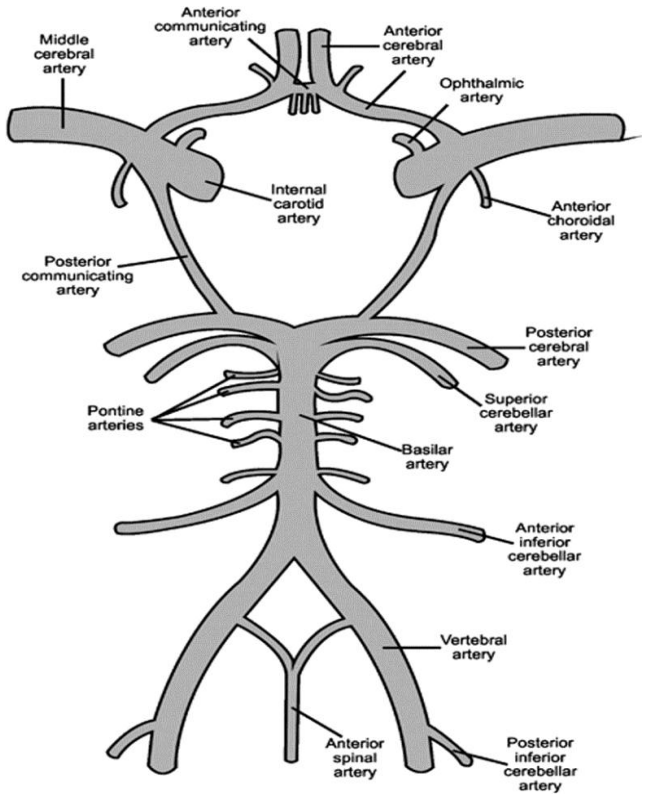

Figure 3. Branching off of arteries from the circle of Willis. These arteries all communicate with one another and can substitute to enable blood supply following an occlusion of any. This diagram is taken from [12].

The anterior cerebral artery (ACA) supplies blood to the frontal lobes that control voluntary motor functions, thought, and personality. A stroke in the ACA usually leads to weakness in the opposite leg. The middle cerebral artery (MCA) supplies blood to the frontal, parietal, temporal lobes. The parietal lobe mediates the integration of sensory information and spatial visualisation while the temporal lobe mediates the ability to understand language, have visual memories, and form new memories. Therefore a stroke in the MCA usually leads to sensory and spatial visualisation deficits, and an inability to remember visual memories and form new memories. In stroke, the MCA is the artery that is most frequently occluded. The posterior cerebral artery (PCA) supplies blood to the temporal and occipital lobes of both the left and right hemispheres. A common consequence of blocking the PCA is occipital lobe infarction which in turn results in an opposite vision deficit. Another important group of arteries are the lenticulostriate arteries, which are a group of small but deeply penetrating arteries that protrudes from the MCA. An occlusion of these arteries is referred to as a lacunar stroke. Approximately 20% of all stroke cases are lacunar strokes, and hypertensive patients are at an increased risk of developing these strokes [12].

IV. ISCHEMIC SROKE PATHOLOGY

There are two key mechanisms that cause ischemic strokes: thrombosis and embolism. An embolus is a material that causes a blockage in the blood flow, and this blockage could go on to affect a body part that is at a distance from the site of the actual embolism [13]. This is distinct from thrombosis, which is a blockage that affects the body part at which it occurs [14].

Ischemic injury can be to a selective region or involve an entire brain damage. Global ischemia involves a reduction in the cerebral blood flow (CBF) in the entire brain. Focal ischemia on the other hand, involves an ischemic core and penumbra concept. There is reduced cerebral blood flow to the infarct core, while the penumbra region still receives an optimal blood supply [15].

A. Ischemic Stroke Pathogenesis

Following an ischemia, there is a dynamic sequence of events leading to damage and/or death to surrounding cells [16].

1. Cerebral blood flow regulation and haemodynamism

Following the obstruction of an artery in the brain, a collateral circulation ensues which serves to provide blood supply to peripheral parts of the obstructed artery [17]. When the collateral circulation is not able to maintain a normal perfusion pressure, flow regulation – which involves autoregulatory dilation of vessels - steps in to briefly compensate the decrease in blood supply. Following the full dilation of blood vessels, auto-regulation stops, and blood flows passively following systemic blood pressure fluctuations [18].

2. Tissue viability thresholds of ischemia

The occlusion of a vessel during stroke brings about impairment in brain functions at different rates [19]. Normal cerebral blood flow is about 0.6mL.g.min−1, which is about 14% of the cardiac output. There is an approximately 50%

inhibition of protein synthesis at about 0.55mL.g−1.min−1. Tissue acidosis also become prominent at below the rates of 0.26mL.g−1.min−1. Interstitial dialysis techniques, which involves measurement of the constituents of the extracellular compartment based on their ability to cross the interstitium, have shown that excitatory and inhibitory neurotransmitters are also released into the extracellular compartment at about 0.2mL.g−1.min−1 [16].

It is noteworthy that these threshold values vary slightly depending on the time point measured following vascular occlusion. For example, the ATP depletion threshold rises from 0.13mL.g−1.min−1 thirty minutes after occlusion to 0.19mL.g−1.min−1 two hours after occlusion [16].

3. Peri-infarct spreading

Imaging techniques have shown that following ischemic injury, the brain infarct actually grows, and this progression can be categorised into 3 phases: the acute phase, the subacute phase, and the delayed injury phase.

The acute phase occurs just a few minutes following ischemia and is due to the energy failure and anoxic depolarisations. The subacute phase on the other hand, presents 4-6 hours after ischemia, and involves the infarct core expansion into the penumbra, and it is at this phase that the greatest increase in infarct volume occurs. Finally, the delayed injury phase presents, and this can go on for several days to weeks [16]. This phase is characterised by inflammation, vasogenic oedema (which involves the breakdown of the blood brain barrier to allow fluid permeate the brain parenchymal extracellular space), and apoptosis, and these events all contribute to further aggravate the infarct injury [20]

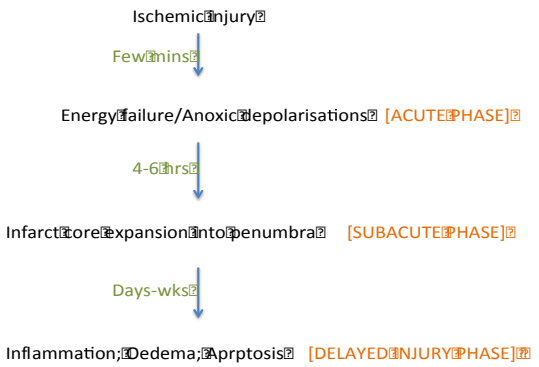

Figure 4. A simple schematic depicting the different phases of brain infarct progression

4. The ischemic penumbra

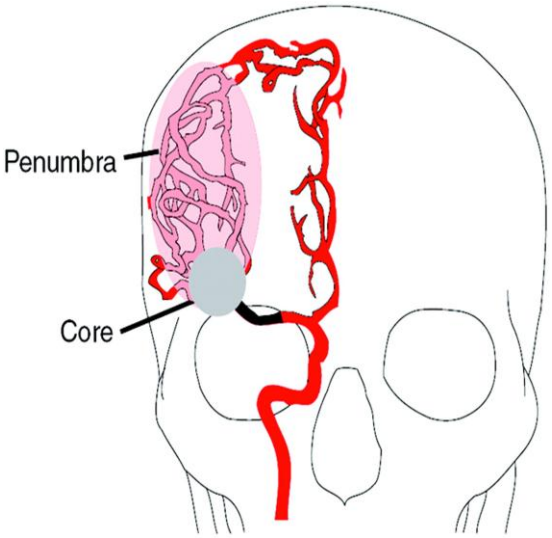

Figure 5. A schema showing the infarct core and the penumbra region. The infarct core is the region where the CBF decreases below threshold for viable energy supply. The penumbra is the region of reduced blood supply but of which the energy state is preserved. This diagram has been adapted from [21].

Two major regions have been classified following an ischemia: the infarct core and the penumbra regions. As depicted in Figure 5, the infarct core corresponds to the area where the vascular occlusion has occurred and more precisely refers to the region where the cerebral blood flow has reduced to levels insufficient to maintain a viable energy supply. The penumbra region on the other hand, refers to the region surrounding the infarct core where although there is a reduced blood supply to it, the energy state is still preserved [16]. Therefore; the penumbra region is one that is fundamentally reversible.

5. Molecular mechanisms of injury

During ischemia, several cellular disruptions develop that cannot be thoroughly explained by a disrupted blood flow, and are termed molecular injuries.

After ischemia, there is the release of both excitatory and inhibitory neurotransmitters that go on to activate their specific receptors of which glutamate receptors are particularly important. Glutamate receptors are subdivided into ionotropic and metabotropic glutamate receptors. The abnormal activation of the ionotropic receptors, lead to excessive calcium influx, mitochondrial calcium overload, and an activation of deleterious calcium-mediating catabolic events. The metabotropic receptors on the other hand, do not conduct ions but are coupled to G-proteins and their activation during ischemia can bring about endoplasmic

reticulum (ER) stress [16],[22]. These process and events are termed excitotoxicity, and due to its importance in stroke pathology, the benefits of glutamate antagonists to prevent further peri-infarct depolarisations and therefore treat focal ischemia has been explored in this paper.

The low blood perfusion leads to the formation of reactive oxygen species (ROS) which causes a disruption of plasma membranes and damage of intracellular organelles [23]. Nitric oxide and zinc toxicity are also molecular injuries that result following vascular occlusion. The role of zinc in an ischemic injury is not fully known, but, it is believed to facilitate the formation of free radicals [24].

6. Brain oedema

Brain oedema also results from focal ischemic injury and can be in two phases: early cytotoxic oedema, followed by a later vasogenic oedema. Cytotoxic oedema is initiated at reduced flow values of about 20% below normal, and involves the osmotic movement of water along with sodium down its concentration gradient into the cells, resulting in swelling. Following a middle cerebral artery occlusion (MCAO), the net tissue water content rises in a few minutes and that of the cerebral cortex could rise by 17% and the white matter by about 24%, within 4 hours. Vasogenic oedema on the other hand, is iso-osmotic and occurs in the extracellular compartment to further increase the water content [25].

7. The inflammatory cascade

The contribution of the immune system during stroke appears to be biphasic. The first phase involves the activation of leukocytes and lymphocytes. The second phase on the other hand is a global immunosuppression state involving the apoptosis of the spleen and an immune cell depletion [4].

A strong inflammatory response is initiated to brain infarcts, and this significantly contributes to ischemic injury progression. Numerous pro-inflammatory cytokines such as IL1-β, IL-6, and IL-10 are all hugely upregulated in both permanent and focal transient ischemia [26]. These cytokines are released by leukocytes and lymphocytes recruited to the site of injury. This recruitment process is a dynamic process, involving the recruitment of different inflammatory cell types during acute or chronic inflammation. In addition to the cell types, several different adhesion molecules are also responsible for mediating both types of inflammation [27] and have increasingly become targets of therapies for stroke treatment. Therefore, it is important to describe this recruitment process and the molecules involved, to set the tone as to why and how they can be targets for immunotherapy.

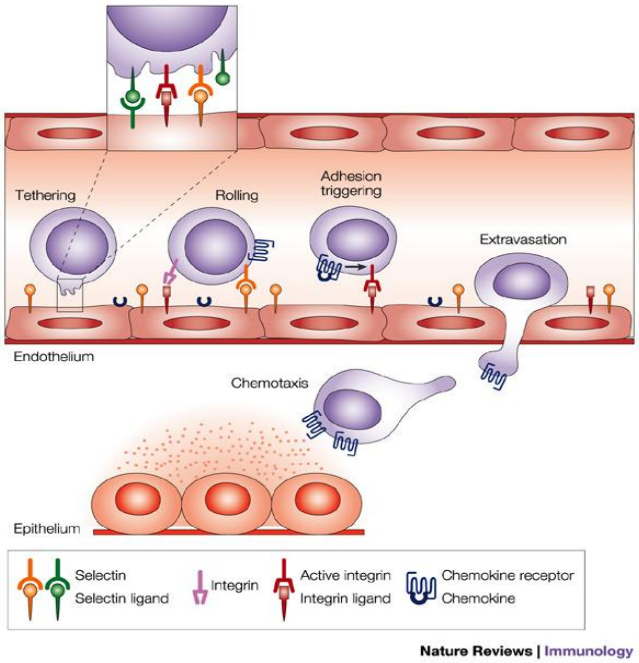

Figure 6. The inflammatory recruitment cascade. Following inflammation, several adhesion molecules become expressed on both the surfaces of the endothelium and the infiltrating immune cell. This diagram is taken from [28].

Blood is a non-Newtonian viscous fluid, and this ensures an effective and sufficient recruitment of immune cells. The generalised immune cell recruitment cascade involves rolling, adhesion, crawling and transmigration [27]. Following an infarct injury, although the free circulating leukocytes are at a state ready to go, it is their interaction with the endothelium that switches them on. There are multiple mechanisms and adhesion molecules, which mediate the attachment of leukocytes to the endothelium. They include: the Immunoglobulin (Ig) superfamily, integrins, and selectins. Free circulating neutrophils flow at a speed of ~2000μm/sec, and are slowed down to 3-5μm/sec by tethering to the endothelium via selectins. There are 3 kinds of selectins, P-, E-, and L-selectins. P-selectins interact with P-selectin glycoprotein ligand-1 (PSGL-1) which are constitutively expressed on the surface of leukocytes. P-selectins are an acute phase selectin, which are only expressed immediately following inflammation and as leukocytes already have PSGL-1 attached to their surfaces they bind within about 17 seconds. P-selectins are only transiently expressed on endothelial surfaces (10-15 mins) before they get internalised. E-selectins on the other hand, though they bind a similar ligand, E-selectin ligand-1 (ESGL-1), they need to be made ‘de novo’ (from scratch), therefore, they appear in chronic inflammation [27],[29]. In addition, hours following inflammation, eotaxin and IL-8 are released and they recruit other cell types such as eosinophils and T-lymphocytes respectively.

Following the tethering of the immune cells to the endothelium, they undergo slow rolling predominantly mediated by the Ig-like cell adhesion molecules (IgCAM) superfamily of adhesion molecules, which include ICAM-1. ICAM-1 is constitutively expressed on endothelial cells and binds its integrin ligand, lymphocyte functional antigen-1 (LFA-1). Leukocytes also express other integrins such as Very late antigen-4 (VLA-4), which bind the IgCAM, VCAM-1, present on the endothelium. Once the leukocyte is firmly adhered, it needs to follow the chemotactic gradient, which causes it to alter its polarity. This stage in the recruitment cascade is known as crawling and involves the integrin macrophage antigen-1, Mac-1. Following this, the immune cell is then extravasated into the infarct site [29].

Several of these adhesion molecules have become the targets of immunotherapy to treat stroke. In inflammatory lung conditions such as asthma and cystic fibrosis (CF), natalizumab, a humanised monoclonal antibody directed against the α4β1 integrin molecule is used to prevent the recruitment of eosinophils [30].

8. Immunosuppression

After the initial inflammatory cascade, the immune system goes into an immunosuppressed state. Damaged inflammatory products such as myelin basic protein (MBP) could leak through the blood brain barrier (BBB) to result in a reciprocal activation of the systemic circulation. In the periphery, isolation of mononuclear cells after MCAO has shown that at a later time of 22 hours, the anti-inflammatory cytokine, IL-10, was increasingly secreted. Also, after focal stroke there was a more than 80% reduction in B-cell levels in the blood [4].

CD4+CD25+ Treg cells are also induced 96 hours after MCAO in mice. Treg cells normally function to dampen down the inflammatory response and also prevent autoimmune diseases, and they do so via the upregulation of their transcription factor gene, Foxp3. It is the overexpression of these Treg cells that function to further suppress the immune system following stroke [4].

V. CURRENT AVAILABLE OPTION TO TREAT ISCHEMIC STROKE AND ITS INADEQUACIES

Over the years, intravenous tissue plasminogen activator (ivtPA) has been used to treat stroke. It has been shown to ameliorate the clinical outcome following stroke, and also improve permanent disability [31]. ivtPA was licensed in North America in 1996 and a restricted license in Europe was not granted until 2002. This license allowed ivtPA to be administered 3 hours after ischemic stroke [32].

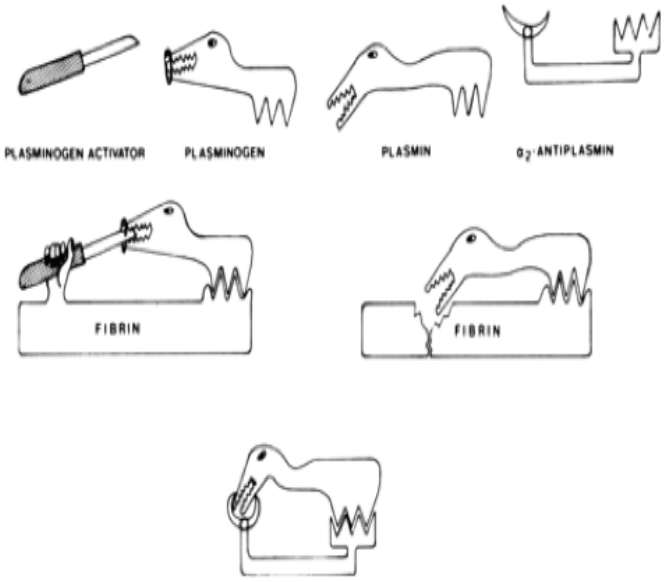

Figure 7. A schematic depicting the mechanism of action of tPA. This diagram is from [33].

Tissue plasminogen activator (tPA) is an endogenous protein, precisely a serine protease that functions to break down clots. Plasminogen is the inactive form of the proteolytic enzyme plasmin. It is converted to plasmin by tissue-type plasminogen activator, and this conversion only occurs efficiently on the fibrin surface where both the plasminogen and activator are assembled. In the blood, free plasmin is rapidly inactivated but plasmin on fibrin surfaces are subtly protected from inactivation [33]. It is this property of tPA as an upstream molecule in the natural fibrinolytic pathway of blood clot breakdown that has enabled it to be used as a thrombolytic therapy in ischemic stroke.

Much recently, ivtPA has been found to interact with the amino-terminal domain (NTD) of NMDA receptors NR1 subunit, leading to noxious effects [34]. This interaction is shown in figure 8 below.

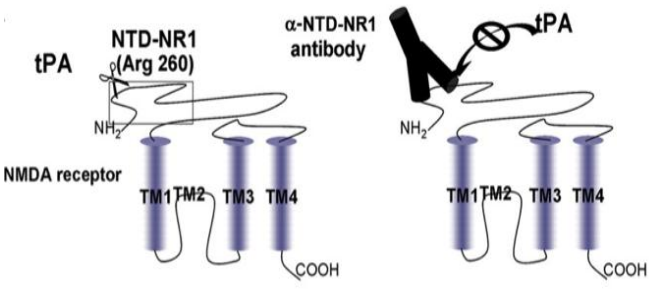

Figure 8. The interaction of tPA with the NTD of NMDA receptors. This diagram is from [34].

VI. STROKE IMMUNOTHERAPY

Numerous molecules implicated in stroke have been the target of both active and passive immunotherapy.

A. Active immunotherapy - animal models

1. NMDA receptors

Benchanane and collaborators have shown that immunisation does significantly reduce excitotoxicity in the mouse brain tissue [34]. They actively immunised mice against the N-terminal domain (NTD) of NR1, and observed that this prevented the interaction of tPA with NR1 and its subsequent cleavage. It is noteworthy that while female animals were mostly used in the experiments, they also demonstrated that the deleterious effects of tPA occur in male animals. Females were used as they mount a much better antibody response to antigens and generally possess higher immunoglobulin titre levels.

2. Tolerisation to e-selectin

Takeda and colleagues [35] attempted a novel active immunisation approach in the treatment of stroke. They, tolerised circulating lymphocytes by intranasally instilling E-selectin into spontaneously hypertensive stroke-prone (SHR-SP) rats. Rats that received the nasal instillation of E-selectin did not go on to develop either ischemic or haemorrhagic stroke. To measure E-selectin tolerisation, the levels of transforming growth factor (TGF) -β-1-positive splenocytes, anti-E-selectin antibodies, and plasma IFN-γ levels were measured. Treated rats, had a significant rise in TGF-β-positive cells but while there were detectable levels of IFN-γ in E-selectin tolerised rats, it was no different to controls. E-selectin tolerisation was not related to anti-E-selectin accumulation, as only rats that received E-selectin followed two weeks later by an intravenous LPS injection, had elevated serum anti-E-selectin antibody levels [35]. Therefore, it is unlikely that the neutralisation of the anti-E-selectin antibodies was what was preventing stroke. These findings imply that the inflammatory response can be prevented or dampened by lymphocyte tolerisation. However, since the inflammatory response is a complex process that does not only involve lymphocytes, but also leukocytes, the tolerisation of lymphocyte may not be enough.

6.2 Active immunotherapy - clinical trials

Pneumococcal vaccination

Recently, the effects of a 23-valent polysaccharide pneumococcal vaccine (PPV23) in the treatment of ischemic stroke patients over the age of 60 years have been trialled [36]. Following reports of an increased risk of thrombotic events in pneumonia patients, there were suggestions that a pneumococcal vaccination could offer protection to patients against ischemia. A closed population-based study involving 27,204 patients in a 3-year follow-up period was done. In this study, an ischemic stroke was recorded as when a patient developed a focal or global impairment to cerebral brain function that lasted more than a day. This was also consolidated by neuroimaging of an ischemic lesion in the patient's brain. The PPV23 vaccine conferred protective effects against stroke, with a 35% reduction in stroke risk. The results of this study remain an interim one, however, they raise a few questions. Although the kind of strokes the individuals suffered was not specifically stated, it would be interesting to know if all the individuals had the same kind of stroke. Could it be that unvaccinated individuals could go on to develop a peculiar stroke a few years down? In addition, could it be that individuals responding positively to the vaccine are presenting mild stroke forms? It would be great to elucidate some of these questions, and this perhaps poses need for a longer follow-up.

B. Passive immunotherapy - animal models

1. Myelin-associated proteins

Myelin is a key component of neurons essential for neuronal insulation and is made of the Nogo-A molecule which functions to prevent neurite outgrowth via its interaction with the Nogo-66 receptor, following a CNS injury [37]. Due to its role in CNS injury, Nogo-A and its Nogo-66 receptor has been the target of immunotherapy with the aim of improving neuroplasticity after CNS injury.

Anti-Nogo-A antibody introduced through intrathecal routes showed a recovery from brain damage at one week following operation [38]. Testing anti-Nogo-A antibodies in hypertensive or aged animals, showed an enhanced neuroplasticity post-stroke. Similarly, antibodies against the Nogo-66 receptor, improved brain damage induced by stroke [39]. However, when anti-Nogo-A antibodies were administered immediately following an MCAO stroke, an exacerbation of neurological deficits was observed. This suggests that during the hyperacute phase of stroke, Nogo-A plays an opposite function in cerebral damage. Tissue damage is more prominent than tissue repair during the hyperacute phase of stroke. Therefore, the benefit of anti-Nogo-A antibody is time-dependent [38],[39].

2. NMDA receptors

Glutamate is an excitatory amino acid neurotransmitter, which binds the NMDA receptor, and also the kainate and AMPA receptors [40]. In ischemic stroke, the disruption of blood flow lead to a release of vast amounts of glutamate into the extracellular space. Consequently, this causes an enormous activation of the NMDA and other glutamate receptors, leading to cell death [41].

A group using an oral genetic vaccine approach have shown that neuroprotective effects could be conferred to a middle cerebral artery occlusion (MCAO) rat stroke model following vaccination. The cDNA of a mouse NR1 was subcloned into an adeno-associated virus (AAV) plasmid, and purified to a give a recombinant AAVNR1 with a high-titre. A complete blockage of the MCA, and the generation of an immense infarct was initiated via endothelin-1 injection. The AAVNR1-treated rats had a substantial 70% reduction of infarct size. However, behaviour tests including line-crossing mobility and circular track tests did not show any changes between groups [42].

Another group isolated and designed a polyclonal antibody, rATD-NR1, against the amino-terminal domain of the NR1 subunit. Mice were then immunised with 30μg rATD-NR1 and complete and incomplete Freund's adjuvants once a week for a total of 3 weeks. Two weeks after the last injection, sera were collected, and the αATD-NR1 antibodies or control antibodies were purified and obtained. α-thrombin was used to induce focal cerebral ischemia in male Swiss mice, and rtPA was used to initiate thrombolysis. Following this, the purified αATD-NR1 antibodies were injected into the tail veins of the animals, randomly. A reduction in neuronal death implied that αTD-NR1 prevented rtPA interaction with NMDARs. Also, NMDA-induced Ca2+ influx that was increased by rtPA was prevented by αATD-NR1 treatment. As endogenous tPA does contribute to the pathological evolution of ischemia, αATD-NR1 was tested as a standalone therapy. They showed that both early (20 minutes) and late (4 hours) administrations of αATD-NR1 were neuroprotective. They also showed that the protective effects of αATD-NR1 translated into improved neurological function, and that this beneficial effect lasted for up to 3 months [43]. Importantly, αATD-NR1 antibodies did not alter NMDA-stimulated neurotransmission.

3. Anti-neutrophil monoclonal antibody

During ischemia-reperfusion, neutrophils release oxygen radicals that go on to mediate brain injury. Therefore, the effect of the administration of a monoclonal antibody, RP3, raised against neutrophil, on rats with MCAO has been evaluated [44]. Concentration levels of endogenous ascorbyl radical (AR) was used as an oxygen-radical marker. During reperfusion, an observed increase in extracellular AR was completely inhibited following RP3 treatment.

Similarly, the effect of RP3 treatment on brain infarct size, neutrophil infiltration, and oedema formation has been evaluated [45]. Neutrophil infiltration was quantified by myeloperoxidase (MPO) levels. MPO is an enzyme stored in neutrophils that actively mediate the inflammatory response. Following reperfusion, MPO levels increased, and this was simultaneous to the appearance of neutrophils. Following RP3 treatment there was a complete inhibition of this increase in MPO levels. In addition, RP3 treatment led to a significant reduction in brain oedema 24 hours following reperfusion. Also, there was a significant decrease in infarct size following RP3 treatment [45]. While these studies established that during cerebral ischemia, neutrophils are a major source of the oxygen radicals and that there were no changes in tissue PO2, the plausibility of depleting neutrophils as a treatment approach for stroke may not be desirable as opportunistic infections may arise following their depletion. A better approach may be to selectively block the activity of the oxygen radicals in the ischemic tissue. However, how this would be carried out is subject to some extensive pharmaceutical investigation.

4. Anti-selectin monoclonal antibody

Suzuki and colleagues [46] have subjected male Wistar rats to 24 hour permanent MCAO and investigated the effect of a monoclonal P-selectin antibody, ARP 2-4, on brain injury. Treatment with ARP 2-4, improved cerebral blood flow after MCAO. Treatment with this antibody reduced infarct size and this was associated with significantly decreased levels of leukocytes. The anti-P antibody was administered 5 minutes before MCAO, and this would be difficult to replicate in humans as it remains impossible to know who is going to have stroke before they do, in order to administer antibody to prevent leukocyte infiltration. Even the idea of administering the antibody to patients who have previously had a transient ischemic attack is a flawed one, as P-selectins are only transiently expressed on endothelial cells during an inflammatory response. Maybe, if the antibody was against P-selectin glycoprotein ligand-1 (PSGL-1), the P-selectin ligand constitutively expressed on leukocytes, this could be a better approach. However, PSGL-1 is expressed on all leukocytes, and its blockage, could lead to susceptibility to infections.

An anti-E-selectin monoclonal antibody has also been administered immediately before MCAO. Four hours after MCAO, there was an upregulation of E-selectin in the ischemic vasculature, and anti-E-selectin antibody treatment decreased the recruitment of neutrophils measured by MPO activity levels. Even when treatment was delayed 3 hours after MCAO, neuroprotective effects of anti-E-selectin was maintained [47].

Also, the humanised anti-L-selectin monoclonal antibody, DREG200, has been trialled in rabbits following clot embolisation, and evaluated alongside tPA administration. Animals that received tPA and DREG200 had an improved brain infarct size, and a restored cerebral blood flow. The animals that received only DREG200, had no brain infarct or CBF improvements. However, there was no statistical significance to these observations [48]. While the exact mechanism of this combinatorial treatment is not

completely known, one would expect DREG200 to have some positive effects of its own, if it is to have any in combination with tPA.

5. Anti-ICAM-1 antibodies

In similar manner to the investigations already discussed on exploiting the recruitment of leukocytes during the inflammatory process, Zhang and colleagues [49] have investigated the effects of blocking ICAM-1 with an antibody. They intravenously administered an anti-ICAM-1 antibody, 1A29, to a Wistar rat following a 2-hour transient or permanent MCAO. Histological evaluations showed that there was a significant reduction in the infarct size following the 1A29-treatment of the transient MCAO. Desirably, data from this study also indicate that the treatment with 1A29 does not reduce peripheral neutrophil levels below normal physiological levels [49]. However, there was no improvement after 1A29-treatment of the permanent MCAO. Explanations for this include that permanent MCAO is a severe insult that may have impaired any beneficial effect of the anti-ICAM-1 antibody. Also the drug was administered long before reperfusion set in.

Chopp and collaborators [50] have recorded that in addition to reducing infarct volume, the treatment of transient MCAO upon reperfusion with a monoclonal antibody against ICAM-1 or integrin CD11b/CD18, reduced the number of apoptotic cells. The treatment group also had reduced levels of MPO cells, which as already mentioned, serves as a marker for neutrophil levels. Inflammatory cells can stimulate apoptosis [51], and inhibiting the recruitment of these inflammatory cells prevents the induction of apoptosis. While the exact mechanism of how leukocytes induce apoptosis is not known, it is proposed that the decrease in the number of neutrophils, may be indirectly causing the reduction of apoptotic cells. Treatment with anti-ICAM-1 antibodies gave a more pronounced reduction in the number of apoptotic cells than treatment with anti-CD11b antibodies. This observation has been partly explained by the fact that cell adhesion molecules (CAMs) enhance the axon growth and migration of neurons during development, although, they can also inhibit these actions by maintaining a stable adhesion [52]. During inflammation the expression of ICAM-1 on neurons facilitate their targeting by neutrophils. A limitation of this study was that MPO levels from neutrophils was not substantiated, hence the measurement of MPO levels as a marker of neutrophil levels might be a flawed one as monocytes also possess MPO, albeit < 1%.

Another important finding has shown that preventing the adhesion of leukocyte does increase the therapeutic time window at which tPA remains effective [53]. In this study tPA, anti-CD18, and anti-ICAM-1 were administered to New Zealand white rabbits at different times, and in different combinations, and the neurological outcome of these administrations evaluated. There was a significant improvement in neurological outcome when animals were administered anti-CD18 antibody 5-minutes post-ischemia or tPA 30-minutes post-ischemia. However, when anti-CD18 and tPA were administered in combination at both 5 minutes and 30 minutes post-ischemia, there was no significant improvement in neurological outcome when compared to anti-CD18 or tPA alone. A delayed administration of anti-CD18 or anti-ICAM-1 at 15 or 30 minutes post-ischemia yielded no improvement in neurological outcome. However, an administration of anti-ICAM-1 15-minutes post-ischemia and tPA 2-hours post-ischemia, improved neurological outcome. This study highlights a potential of treating stroke with a combination of anti-ICAM-1 antibody and tPA, as anti-ICAM-1 seems to be improving the effect of tPA treatment [53]. However, if drug administration times from this study is anything to go by, in the clinical setting, it would be difficult to detect stroke as early as 15-minutes post-ischemia and administer anti-ICAM-1 within this time to obtain the improved neurological outcome highlighted herein this study.

6. Anti-integrin antibodies

An anti-Mac-1 antibody, 1B6, has been administered to rats after 2 hours of MCAO. The administration of the anti-Mac-1 antibody significantly reduced the lesion volume, almost by 50%, in comparison to the controls. Also, there was a significant reduction in the levels of neutrophils in the cortex of treated rats [54]. The benefits of 1B6 was dose-dependent as rats treated with 2mg/kg of the antibody had much beneficial results compared to those treated with 1mg/kg. Evidence supports that Mac-1 is important for oxidative bursts [55] and the administration of an anti-Mac-1 antibody may be beneficial to prevent the free radical damage mediated by neutrophils.

7. Anti-TNF antibodies/anti-cytokine antibodies

As the importance of leukocyte recruitment in ischemic injury, has already been established, attention is beginning to shift towards blocking the actions of cytokines.

Yamasaki and collaborators [56] have investigated the effects of blocking cytokine-induced neutrophil chemoattractant (CINC) activity, using an anti-CINC antibody. The right middle cerebral artery of adult male Wistar rats was intraluminally occluded for 60 minutes to produce focal transient ischemia. There was a reduction in oedema in the ischemic areas 24 hours after reperfusion, and also a reduction in infarct size 7 days after reperfusion. Importantly, the administration of the anti-CINC antibody, did not reduce peripheral serum neutrophil levels.

Tumor necrosis factor-alpha (TNF-α) has also been implicated to be deleterious in brain ischemic injury, and therefore has become the target of stroke therapies. However,

TNF-α has also been reported to be neuroprotective, and neuronal damage caused by focal ischemia is exacerbated in the TNF-receptor–knock out mice [57]. Lavine and colleagues [58] set out to investigate the effects of an anti-TNF-α antibody in treating rats with MCAO. Their data showed that anti-TNF-α administration improved neurological outcomes and also significantly reduced volume of ischemic injury. This suggests that while TNF-α is deleterious in stroke, antibodies in circulation raised against TNF-α may offer protection from reperfusion injury.

C. Passive immunotherapy - clinical trials

1. NMDA receptors

Clinically, little success has been recorded in trials in which the NMDA receptor was antagonised. In fact, non-desirable effects have been linked to clinical NMDA treatment. Iuzuka and colleagues have reported a characteristic syndrome, anti-NMDAR encephalitis, associated with treating the NR1 subunit of the NMDA receptor with antibodies [59],[60]. Most patients with this syndrome experience headache, nausea and within less than two weeks, these patients present psychiatric symptoms such as insomnia, delusions, and sometimes, social withdrawal. While the exact molecular mechanism underlying the NMDAR encephalitis pathology, is still poorly understood, what is quite clear is that the autoantibody recognition of the NR1 subunit of the NMDA receptors has dire consequences. Hughes and colleagues [61] have demonstrated that the activity of these autoantibodies does impair the synaptic function mediated by NMDA receptors, resulting in learning and memory deficits. Using in vivo anti-NR1 immunostaining, they were able to show that the antibodies crosslink NMDARs leading to their internalisation, both in mice and human hippocampus.

2. Anti-Nogo-A antibodies

Phase I clinical trials of 50 patients for the human monoclonal anti-Nogo-A antibody, AT1355, has just been completed with no fatal side effects. Therefore, it is safe to say that the anti-Nogo-A antibody holds future promise to be used as an ischemic stroke treatment [38].

3. Anti-ICAM-1

A double-blind clinical trial involving 625 ischemic stroke patients of which 317 received enlimomab, a murine anti-ICAM-1 antibody, and 308 received a placebo over 5 days has been conducted. The effect of treatment was evaluated 5 and 90 days after treatment, and long-term assessments were also conducted 6- and 12-months after treatment. At 90 days after treatment, patients treated wiith enlimomab recorded a worse neurological score when compared to patients that received placebo. Also, there were more adverse effects (infections and fever) associated with enlimomab administration [62]. This study gives evidence that an anti-ICAM-1 antibody as a treatment approach for ischemic stroke may significantly worsen the outcome of stroke and as such, would not an effective therapy.

4. Autologous activated immune cells (ACT)

An interesting study on an 82-year-old Caucasian woman who went into a MCAO stroke with hemiplegia, which is a paralysis of arms, trunk, and legs on one side of he body, has been injected with immune cells and the effect(s) evaluated. The woman had initially gone into a seven months coma state, after which she remained in an unremitting vegetative state.

Immune cells were sampled from her blood and activated in vitro using OKT3 anti-CD3. She was treated once a week for eight months. Treatment with these immune cells known precisely as autologous activated immune cells (ACTs), produced a return in strength to the arms of the patient, and improved motor functions in the hemiplegia side of the body, and after 3 months, swallow reflexes of the patient returned. In the study, it was suggested that these immune cells induce the release of brain-derived neurotrophic factor, and neurotrophin-3, which go on to mediate the improved neurological functions [63]. Findings from this study has led to a fair conclusion that the chronic vegetative state of some stroke patients can be improved via treatment with activated immune cells. Although not fully explored in this study, could it be that it is the treatment and prevention of further secondary infections that is improving the patient's neurological deficits? But then again, how can this be since explicit neurological outcomes were recorded to have improved.

VI. CONCLUSIONS

Reasons for trying to inhibit the inflammatory recruitment cascade are valid, however, the complexity of the process means that several molecules need to be inhibited at the same time to obtain clinically beneficial outcomes. This approach also raises the question of how the body would deal with opportunistic infections that are likely to arise following stroke. Also, activating the immunosuppressed state in stroke seems reasonable, but as already mentioned, could it be that this is an adaptive response to protect the CNS from autoimmune diseases? or maybe protection of penumbra areas?

A lot of the therapeutics discussed above, appear to have potential in the treatment of stroke, but the lack of clinical success implies that a lot of research still needs to be carried out to substantiate findings. It is important to know when to inhibit leukocyte infiltration, and when to supply or treat patients with activated lymphocytes and as our knowledge of molecular mimicry improves, advances in immunotherapy would also improve.

From the review of the literature, the best approach to treating stroke, still remains the administration of ivtPA. Clinical trials encouraging the combinatorial treatment of stroke patients with ivtPA and anti-NR1 antibody, has an immense potential to improve the therapeutic window, and also eliminate the noxious effects associated with tPA treatment.

V. FUTURE SCOPE

Advances in technology has helped in the development of high quality antibodies, that have shown promise, but, there are a still a few general issues to be considered. One of them is animal preclinical studies, which has to be extensively done before any human trials are carried out as animals and humans have different lifespans and a proper comparison of both pathologies would invariably enhance the outcome of stroke immunotherapy. Secondly, the dynamicity of stroke pathophysiology needs to be continually considered in the quest to find a clinically beneficial immunotherapy. It is important to know when to target a particular molecule. Thirdly, target molecules for antibodies should be chosen carefully. It may not be ideal to target a molecule that is ubiquitously expressed in brain tissues. However, the targeting of a deleterious molecule that is exclusively expressed during stroke might be perfect [64].

Alongside the quest for a better stroke therapy, future stroke research should also focus to enhance knowledge on stroke pathogenesis, and the role of the immune system in stroke. Another focus would be to elucidate the reason for the immunosuppressed state in stroke. Can the immune system be successfully activated in stroke to protect patients from infection, without inducing an attack of auto-specific T lymphocytes on the CNS? It would also be interesting to explore the correlation between the implicated genes that predispose an individual to stroke risk factors and the probability to develop stroke. What would be the effects of mildly actively immunising these individuals against molecules of the inflammatory recruitment cascade?

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