Non-coding RNAs and other determinants of neuroinflammation and endothelial dysfunction: regulation of gene expression in the acute phase of ischemic stroke and possible therapeutic applications

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Abstract

Ischemic stroke occurs under a variety of clinical conditions and has different pathogeneses, resulting in necrosis of brain parenchyma. Stroke pathogenesis is characterized by neuroinflammation and endothelial dysfunction. Some of the main processes triggered in the early stages of ischemic damage are the rapid activation of resident inflammatory cells (microglia, astrocytes and endothelial cells), inflammatory cytokines, and translocation of intercellular nuclear factors. Inflammation in stroke includes all the processes mentioned above, and it consists of either protective or detrimental effects concerning the "polarization" of these processes. This polarization comes out from the interaction of all the molecular pathways that regulate genome expression: the epigenetic factors. In recent years, new regulation mechanisms have been cleared, and these include non-coding RNAs, adenosine receptors, and the activity of mesenchymal stem/stromal cells and microglia. We reviewed how long non-coding RNA and microRNA have emerged as an essential mediator of some neurological diseases. We also clarified that their roles in cerebral ischemic injury may provide novel targets for the treatment of ischemic stroke. To date, we do not have adequate tools to control pathophysiological processes associated with stroke. Our goal is to review the role of non-coding RNAs and innate immune cells (such as microglia and mesenchymal stem/stromal cells) and the possible therapeutic effects of their modulation in patients with acute ischemic stroke. A better understanding of the mechanisms that influence the "polarization" of the inflammatory response after the acute event seems to be the way to change the natural history of the disease.

Key Words: acute phase; cerebrovascular disease; endothelial dysfunction; epigenetics; genetics; neuroiflammation; non-coding RNAs; stroke

Introduction

Ischemic stroke encompasses a broad spectrum of clinical conditions, with different pathogenesis, leading to necrosis of brain parenchyma. The first hours after the vascular occlusion represent a crucial point that could significantly influence stroke patients' prognosis by involving almost the entire repertoire of innate and adaptative immunity cells. These cells regulate the mechanisms of neural and glial cell apoptosis and regulate brain restoration processes (Anrather and Iadecola, 2016).

Inflammation and endothelial dysfunction play a vital role in ischemic stroke's pathogenesis, especially in the first hours after ischemic injury (Tuttolomondo et al., 2020). Some of the main processes triggered in the early stages of ischemic damage are the rapid activation of resident inflammatory cells (microglia, astrocytes and endothelial cells), production of inflammatory mediators, and translocation of intercellular nuclear factors (Garcia-Bonilla et al., 2015; Gülke et al., 2018). These processes can lead to brain damage and amplification of cerebral inflammation, and they can also result in neuroplasticity, brain regeneration, and neurovascular remodelling. In recent years, several epigenetic factors have been related to the polarization of inflammatory processes. Neuroinflammation includes all the processes mentioned above. It exhibits either protective or detrimental effect on the "polarization" of these processes. This polarization comes out from the interaction of all the molecular pathways that regulate genome expression: the epigenetic factors.

One of the questions that researchers have been trying to answer in recent years is whether it would be possible to change the natural history of this disabling disease by identifying the genetic factors behind it.

In recent years, new gene regulation mechanisms have been cleared, and these include non-coding RNAs (ncRNAs). Non-coding RNAs are RNA molecules transcribed from the genome that do not encode proteins that play a big part in epigenetic regulation of gene expression in addition to their roles at the transcriptional and post-transcriptional level (Ning and Li, 2018). This family includes microRNA (miRNA), intronic RNA, repetitive RNA and long non-coding RNA (lncRNA) (**Table 1**).

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(AMP) degradation. High extracellular concentrations of adenosine can be considered a general harm signal that contributes to the recruitment of damage-associated molecular effectors. Adenosine action is mediated by four metabotropic receptors (G-protein coupled) called: A1, A2A, A2B, and A3 adenosine receptors (A1Rs, A2Ars, A2BRs, and A3Rs, respectively).

A2BR needs much higher adenosine concentrations (in the μM range) than A1Rs, A2Ars and A3Rs, for being activated, the clue is that these levels are reached only under conditions of tissue damage or injury (Coppi et al., 2020).

Recently, Colotta et al. (2012) showed that the selective block of A2BRs by the antagonist PSB-603 and by MRS1754 prevents irreversible synaptic failure after 7-minute oxygen and glucose deprivation event in the hippocampus and that anoxic depolarization is wholly abolished in A2BR antagonist-treated hippocampal slices exposed to 7-minute oxygen and glucose deprivation and is significantly delayed in slices undergoing a 30-minute oxygen and glucose deprivation insult. Some studies also tried to investigate the effects of modulating A2bR in endothelial damage.

Eckle et al. (2008) demonstrated that there was an attenuate vascular leakage in mice ablated of A2BRs on bone marrow cells during hypoxia. However, other studies demonstrated that the same receptor's activation is also neuroprotective in a different phase of brain ischemia. Li et al. (2017) showed that treatment with intravenous A2bR agonist (BAY60-6583) reduced lesion volume in the absence or presence of tPA and attenuated brain oedema, blood-brain barrier disruption, and tPA-exacerbated hemorrhagic transformation at 24 hours after ischemia induced by transient middle cerebral artery occlusion (tMCAo).

These data suggest that stimulation of A2BR plays a dual time-related role after brain ischemia. In the early hours after ischemia, an increase in adenosine's cerebral extracellular levels able to activate low-affinity A2BRs may contribute to expanding excitotoxicity. On the contrary, in the hours following ischemia, when neuroinflammation develops, A2BRs located on glial, vascular endothelial, and blood cells exert a principal immunomodulatory role attenuating the neuroinflammation (Coppi et al., 2020).

Possible Therapeutic Applications of Epigenetic Factors

Although stroke has represented and still represents a field of enormous scientific and clinical interest due to its significant prevalence and mortality, therapeutic strategies are still limited to date. The only drugs significantly associated with a reduction in morbidity, mortality, and sequelae, and therefore recommended in the specific scientific society's principal guidelines, are thrombolytic factors (with a strict therapeutic window) and some antiplatelet drugs (Powers et al., 2019).

Full understanding of the regulatory networks linked to inflammation in the first minutes/hours after the ischemic event represents a new direction for stroke research, but which is the state-of-art and the clinical therapeutic implication of these findings? Are we really close to understanding these mechanisms and transforming this knowledge into therapeutic approaches that affect our patients' outcome?

To date, we do not have adequate tools to control pathophysiological processes associated with stroke. Thus, the microRNAs have to be considered as a very promising target for future stroke therapies, and there is a growing body of evidences that miRNA-based therapies hold great promise. Nevertheless, despite their potential, which has been documented for several years now, several significant limitations are related to finding factors that can bind their miRNA targets. Other related limitations include *in vivo* stability, limited tissue distribution, and untoward side effects. Administration of miRNAs in the absence of a specific and stable carrier presents limited tissue distribution, and these miRNAs are fast metabolized by the liver and kidney and rapidly excreted in the urine. Besides, the lethal dosage (LD50) of specific miRNAs has yet to be recognized.

As shown before, in early stages following ischemic damage, there is an increase in the expression of a large number of microRNAs. These miRNAs can lead on one hand to an expression of different protective genes, or on the other hand to an increase in the transcription of genes that stimulate inflammatory processes. Thereby, inhibition of these miRNAs may be a therapeutic target for ischemic stroke (Li et al., 2020).

One of the first-studied methods to decrease miRNA level is the utilization of complementary sequences of nucleotide (anti-sense oligonucleotide) binding to the mature miRNA and blocking miRNA activity. These molecules are called antagomir, and their usage could be a useful approach to inhibit miRNA function. Therefore, an antagomir may be another therapeutic option when upregulated miRNAs are pathogenic (Zhang et al., 2013a).

Antagomirs' advantage is that they can be delivered into cells directly without any vector assistant, considering that they are nuclease resistant, which avoids the complication of using delivery vehicles. However, although antagomirs could easily be delivered intravenously, there is poor brain distribution due to the blood-brain barrier, which prevents most exogenous substances from entering the CNS. The drawbacks that limit antagomirs application as therapeutic reagents in humans are the need for high doses and their possible side effects (Krützfeldt et al., 2005).

Contrariwise antagomirs, gain-of-function for a specific miRNA can be achieved by overexpressing a specific protective miRNA's mature sequence. miRNA mimics are molecules that can be chemically synthesized as oligonucleotides according to sequences of the endogenous miRNA. Double-stranded miRNA mimics, with the sequence of one strand identical to the endogenous mature miRNA, are usually used to increase the efficiency of augmenting miRNA expression (Wang, 2011). However, the effectiveness of miRNA mimics is low, especially in neurons, and the transfection is usually transient, which has limited its application.

Other studied approaches that could overcome these challenges are related to the utilization of either viral vectors or non-viral delivery systems such as liposomes. However, both liposomes and viral vectors may be toxic or immunogenic, restricting their clinical application. Liposomes are utilized to deliver small interference RNAs, a family of double-stranded RNA molecules that plays a role in post-transcriptional gene silencing. However, synthetic systems such as liposomes have relatively lower yield compared to viral vectors (Zhang et al, 2013b).

miRNAs administration has also been evaluated by the utilization of mechanical methods such as high-pressure injection and electroporation. Still, these methods are linked to significant damage to the tissues (Kishida et al., 2004).

Although MSC-derived extracellular vesicle therapeutics is still at an early stage, research is rapidly increasing and is demonstrating a promising approach for patients with severe stroke (Bang and Kim, 2019).

Despite their recent introduction, MSC therapies have already been tested in preclinical studies and clinical trials, and extracellular vesicle-mediated therapy has shown advantages over other cell therapies in stroke patients, in terms of biodistribution, because of the ability to cross the blood-brain

Table 1 | **Principal non-coding RNA and their functions on the regulation of neuroinflammation**

	ncRNA	Principal functions	References
Long Non-coding RNAs	XLOC_035088	The silencing reduced brain infarct size and improved neurological function.	Chen et al. (2021)
	DLX6-AS1	The silencing reduced acute injury, ameliorated long-term neurological impairments, and reduced neuronal apoptosis in vivo and in vitro.	Hu et al. (2020)
	NEAT1	Regulation of AKT/STAT3 pathway. Silencing of NEAT1 reduced apoptosis and increased neuronal viability.	Ni et al., (2020)
	FOXD3-AS1	The silencing attenuated neurological dysfunction and brain damages.	Lu et al., (2020)
Micro-RNAs	miR-155	Targeting factors such as SOCS1, SHIP1, C/EBP-β and IL13Rα1, contributes to the induction of neuroinflammation.	Cardoso et al., (2012); Wang et al., (2020)
	miR-125b	Astrogliosis increases the expression of glial cell markers glial fibrillary acidic protein (GFAP) and vimentin.	Pogue et al. (2010)
	miR-146a	Regulates inflammation negatively, promoting M2 mononuclear phagocytes.	Wu et al., (2015); Huang et al., (2016)
	miR-21	Exerts its anti-inflammatory action by targeting PDCD4 leading to downregulation of NF-κB and induction of the anti-inflammatory cytokine IL-10. Decreases TNF-α secretion by macrophages.	Sheedy et al., (2010); Wang et al. (2015)
	miR-124	Anti-inflammatory functions, regulation of neuronal differentiation, monocyte polarization towards an M2 phenotype. Increased expression of miR-124, in microglial cells, reduces inflammation through downregulation of TNF-α and MHC-II and reduces the production of reactive oxygen species (ROS).	Makeyev et al., (2007); Veremeyko et al., (2013); Louw et al., 2016
	let-7 miRNAs	Promotes the polarization of macrophages and microglial cells towards the anti-inflammatory M2 phenotype. Serves as a damage-associated molecular pattern for TLR7 and promotes the activation of microglia and macrophages.	Cho et al., (2015); Lehmann et al., (2012)

AKT/STAT3: Protein kinase B/signal transducer and activator of transcription 3; C/EBP-β: CCAAT enhancer binding protein beta; DLX6-AS1: DLX6 antisense RNA 1; FOXD3-AS1: FOXD3 Antisense RNA 1; IL-10: interleukin 10; IL13Rα1: interleukin 13 receptor alpha 1; MHC-II: major histocompatibility complex 2; NEAT1: nuclear paraspeckle assembly transcript 1; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; PDCD4: programmed cell death 4; SHIP1: SH-2 containing inositol 5' polyphosphatase 1; SOCS1: suppressor of cytokine signaling 1; TLR7: toll-like receptor 7; TNF-α: tumor necrosis factor alpha.

Search Strategy and Selection Criteria

Authors retrieved PubMed. No limits on the dates were established for revising the bibliography.

Non-coding RNAs

lncRNAs, with lengths exceeding 200 nucleotides, have critical functions in various disorders. The lncRNAs are shown to develop angiogenesis, cell apoptosis, inflammation, and also cell death. Recently, many abnormally expressed lncRNAs have been detected in ischemic stroke patients or animal models of ischemia using modern technologies, including deep sequencing, RNA-sequencing, and microarrays. Using RNA sequencing (RNA-seq) technology, Zhang et al. (2021) profiled long lncRNA expressional signatures in primary brain microvascular endothelial cells after oxygen-glucose deprivation, an *in vitro* mimic of ischemic stroke conditions. These authors showed that the most highly upregulated lncRNAs were Snhg12, Malat1, and lnc-OGD 1006, whereas the most highly down-regulated lncRNAs were 281008D09Rik, Peg13, and lnc-OGD 3916.

To date, lncRNAs have emerged as an essential mediator of some neurological diseases, and clarifying their roles in cerebral ischemic injury may provide novel targets for the treatment of ischemic stroke. Chen et al. (2021) showed how silencing the lncRNA-XLOC_035088 in middle cerebral artery occlusion (MCAO)-induced rats reduced brain infarct size, and improved neurological function through inhibiting NOTCH1 following depression of presenilin 2. Hu et al. (2020) performed a study involving mice with cerebral ischemia. They concluded that downregulation of DLX6-AS1 reduced acute injury and ameliorated long-term neurological impairments. These authors also showed that silencing of DLX6-AS1 reduced neuronal apoptosis *in vivo* and *in vitro*.

It is well known that acute cerebral ischemia may lead to severe brain injury caused by uncontrolled reperfusion. Ni et al. (2020) investigated the role of lncRNA nuclear enriched abundant transcript 1 (NEAT1) and demonstrated NEAT1 is an essential modulator of macrophages, particularly microglia (Delrio Ortega cells) through the regulation of AKT/STAT3 pathway. Ni et al. (2020) showed that the expression of NEAT1 was significantly upregulated in patients with ischemic stroke, and knockdown of the lncRNA NEAT1 reduced apoptosis and increased neuronal viability. Additionally, the lncRNA NEAT1 may inhibit microglial polarization towards the M1 phenotype to reduce the activity of the AKT/STAT3 pathway.

In a recent study, Lu et al. (2020) investigated the role of FOXD3-AS1 in cerebral ischemia/reperfusion injury and demonstrated that FOXD3-AS1 knockdown promoted the inhibitory impact of miR-765 on the expression of BCL2L13 and the apoptosis, leading to attenuated neurological dysfunction and brain damages.

In a comprehensive review, Wolska et al. (2020) mentioned the potential of early treatment with lncRNA as CC2dat and AK038897 and the potential benefits of modulating some lncRNAs as GAS5, N1LR and Rian. These can reduce the severity of neurological impairment after ischemic stroke and should be further investigated in preclinical research.

The endothelial dysfunction and the alteration of the brain-blood barrier are essential steps for the beginning of ischemic damage (Di Raimondo et al., 2013; Tuttolomondo et al., 2015; Petta et al., 2017; Tuttolomondo et al., 2020). In this field, functional importance and molecular regulatory mechanisms of lncRNAs in the endothelium following ischemic stroke are still unclear. Identification of new lncRNA transcripts will provide a novel window of opportunity to study RNA-directed epigenetic regulators in cerebral endothelial biology, and their essential role in stroke-induced vascular endothelium-dependent cerebrovascular pathologies, as well as likely reveal novel targets for a promising translational future of lncRNA-based diagnostics and therapeutics in ischemic stroke.

miRNAs are small sequences of non-coding RNAs (ncRNAs; from 18 to 22 nucleotides) that bind specific regions of messenger RNAs regulating their expression and represent one of the subtler mechanisms of regulation of the gene expression (Correia de Sousa et al., 2019). miRNAs have a crucial function in a significant number of cellular and molecular pathways. Substantial evidence showed that following stroke, miRNAs would affect various physiological and pathological mechanisms, such as neurogenesis, hematopoiesis, proliferation, metabolism, immunity cells activation or depression (Jolana and Kamil, 2017; Mirzaei et al., 2018). Several studies have shown that miRNAs can be

used as prognostic, diagnostic, and therapeutic biomarkers of stroke (Wang et al., 2017).

It has been well documented that the inflammatory processes underlying neuroinflammation may be promoted or suppressed by different miRNAs.

miR-155 is a central pro-inflammatory mediator of the central nervous system (CNS) which is induced within macrophages and microglia in response to nuclear factor-κB (NF-κB) dependent toll-like receptor (TLR) signalling, and targeting factors such as SOCS1, SHIP1, C/EBP-β and IL13Rα1, contributes to the induction of neuroinflammation (Wang et al., 2010; Cardoso et al., 2012). Pogue et al. (2010) showed that miR-125b played a role in the process of astrogliosis, decreasing the expression of CDKN2A, a negative regulator of cell growth that inhibits the growth of astrocytes by binding to the 3′UTR of cyclin-dependent kinase inhibitor 2A (CDKN2A). The authors also showed a strong positive correlation between miRNA-125b abundance and the glial cell markers glial fibrillary acidic protein and vimentin.

Contrary to other miRNAs, miR-146a regulates inflammation negatively. miR-146a is expressed in different cytotypes such as neurons, microglia and astrocytes, and it is induced by TLR signaling (Cui et al., 2010). miR-146a acts as a negative feedback regulator of NF-κB signalling by targeting components of the MyD88 signalling complex, including IRAK1 and TRAF6, it targets other pro-inflammatory mediators including STAT-1, IRF-5 and CFH (Wu et al., 2015). miR-146a performs a function in the intracerebral macrophage polarization through the Notch1 pathway, promoting M2 mononuclear phagocytes which play functions in tissue repair processes through phagocytosis and immune tolerance (Huang et al., 2016).

miR-21 is another highly expressed miRNA in a variety of cytotypes such as active immune cells (macrophages, mast cells, neutrophils and T-cells) (Sheedy, 2015) and various cell types of the CNS including microglia, astrocytes, neurons and oligodendrocytes (Zhang et al., 2012; Li et al., 2018). miR-21, induced by TLR signalling through MyD88 and NF-κB, exerts its anti-inflammatory action by targeting PDCD4, leading to downregulation of NF-κB and expression of anti-inflammatory cytokine IL-10 (Sheedy et al., 2010). Additionally, miR-21 decreases tumor necrosis factor-alpha (TNF-α) secretion by macrophages (Wang et al., 2012).

miR-124 is another brain-specific miRNA with anti-inflammatory functions that is involved in the regulation of neuronal differentiation (Makeyev et al., 2007) and the monocyte polarization towards an M2 phenotype. The expression of miR-124 may be induced in monocytes and macrophages in response to the Th2 cytokines IL-4 and IL-10 (Veremeyko et al., 2013). Increased expression of miR-124, in microglial cells, reduces inflammation through downregulation of TNF-α and MHC-II and reduces the production of reactive oxygen species (Louw et al., 2016). miR-124 is a crucial negative regulator of neuroinflammation by reducing inflammatory mediators and restricting microglia to an inactive state.

The let-7 miRNAs, a family of evolutionarily conserved miRNA, serve as essential modulators of neuroinflammatory processes. Let-7 miRNAs, targeting the C/EBP-δ transcription factor, promote the polarization of macrophages and microglial cells towards the anti-inflammatory M2 phenotype (Cho et al., 2015). A peculiar action carried out by let-7 miRNAs is to serve as a damage-associated molecular pattern for TLR7 and to promote activation of microglia and macrophages (Lehmann et al., 2012).

As to inflammatory cytokines, miRNAs could also act by modulating their expression in the ischemic stroke brain tissues. An explicative example is represented by interferon β, an anti-inflammatory cytokine that can prevent the neuron against ischemic injury. Some miRNAs such as miR-34a, let-7b, miR-26a, miR-145, have their targeting site in the 3′UTR of interferon β and they regulate the expression of interferon β to influence the outcome of ischemic stroke (Wanve et al., 2019).

Mesenchymal Stem/Stromal Cells

Today, a promising field of research is represented by the utilization of mesenchymal stem/stromal cells (MSCs). MSCs are a subset of cells with a robust immunomodulatory function that can be readily obtainable and easily expandable *in vitro*. These cells can be reached from many different tissues (peripheral blood, bone marrow, adipose tissue, umbilical cord blood), and these cells are being studied for several pathological conditions because of their ability to differentiate into various cell types, to migrate to multiple tissues, and to exert potent immunomodulatory functions (Hass et al., 2011). MSCs can influence target cell function through the secretion of large amounts of exosomes. MSC-derived exosomes (Msc-exosomes) expressing miRNA have been used in the treatment of various diseases such as stroke, which can be associated with the implementation of processes of neurogenesis, angiogenesis, and neuroprotection (Musiał-Wysocka et al., 2019).

Voltage-Gated Potassium Channel KV1.3 Role in Microglia-Mediated Neuroinflammation

The voltage-gated potassium channels KV1.3 were initially described as a target for treating T-cell mediated immune diseases such as multiple sclerosis and psoriasis (Beeton et al., 2006; Tarcha et al., 2012; Chandy and Norton, 2017). Recently, KV1.3 blockers were studied as a pharmacological target to reduce neuroinflammation by modulating microglia activation. In both mouse and rat models of ischemic stroke, KV1.3 blockers showed a potential to reduce ischemic area and improve neurological damage (Ma et al., 2020).

KV1.3 blockers could be useful, as shown in mouse models, in Alzheimer's disease, white matter pathology after traumatic brain injury and radiation-induced brain damage. KV1.3 blockers are overexpressed in mRNA, and protein levels in lipopolysaccharide stimulated microglia, which simulate neuroinflammation and amyloid-β stimulated microglia, that are used for simulating Alzheimer's disease microenvironment.

In a recent study, Nguyen et al. (2017) showed the effects of KV1.3 blockers (microglia inhibitor minocycline on differentially polarized neonatal mouse microglia) reduced expression and production of the pro-inflammatory cytokines interleukin (IL)-1β and TNF-α at 24 and 28 hours and prevented upregulation of the inflammation associated enzymes cyclooxygenase-2 and inducible nitric oxide synthase more than KCa3.1.

In another study, Sarkar et al. (2020) showed as both pharmacological KV1.3 inhibition with PAP-1 and genetic deletion of the channel resulted in reductions of IL-1β, IL-6, IL-12 and TNF-α production and prevented NLRP3 protein upregulation at 24 hours after microglia stimulation.

To date, Nguyen et al. (2017) reported that microglia cells express KV1.3 and that KV1.3 inhibition suppresses the functions of pro-inflammatory, neurotoxic microglia and might even shift microglia toward a more neuroprotective phenotype (M2). Further studies are needed to understand the importance of their role in ischemic stroke.

Adenosine 2B Receptor

Adenosine is a nucleoside belonging to the purinergic system, involved in various physiological and pathological processes. In the CNS, adenosine derives from adenosine monophosphate

barrier, and in terms of other cell therapy related problems such as tumor formation and infarcts caused by vascular occlusion (Bang and Kim, 2019).

Conclusions

The data emerging from the literature continues to fuel hopes that these molecules can significantly improve the outcome of patients with ischemic stroke. A better understanding of the mechanisms that influence the "polarization" of the inflammatory response after the acute event seems to be the way to change the natural history of the disease. Nevertheless, it is probable that an increasing number of these molecules will progress and will eventually be developed to become an approved treatment for ischemic stroke in the coming years.

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