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

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; neuroinflammation; 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 adaptive 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 (IncRNA) (Table 1).
been documented for several years now, several significant body of evidences that miRNA-based therapies hold great target for future stroke therapies, and there is a growing the microRNAs have to be considered as a very promising To date, we do not have adequate tools to control patients' outcome? knowledge into therapeutic approaches that affect our understanding these mechanisms and transforming this implication of these findings? Are we really close to event represents a new direction for stroke research, inflammation in the first minutes/hours after the ischemic 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).

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 antagonim, and their usage could be a useful approach to inhibit miRNA function. Therefore, an antagonim may be another therapeutic option when upregulated miRNAs are pathogenic (Zhang et al., 2013a).

Antagonims’ 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 antagonims 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 antagonim’s application as therapeutic reagents in humans are the need for high doses and their possible side effects (Krützfeldt et al., 2005).

Contrariwise antagonims, 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 RNA, 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
was significantly upregulated in patients with ischemic stroke, pathway. Ni et al. (2020) showed that the expression of NEAT1 (Delrio Ortega cells) through the regulation of AKT/STAT3 abundant transcript 1 (NEAT1) and demonstrated NEAT1 is an severe brain injury caused by uncontrolled reperfusion. Ni et al. (2020) investigated the role of lncRNA nuclear enriched 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 FOXD3-AS1: FOXD3 Antisense RNA 1; IL-10: interleukin 10; IL13Rα1: interleukin 13 receptor alpha 1; MHC-II: major histocompatibility complex 2; NEAT1: 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.

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 IncRNA 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 IncRNA NEAT1 reduced apoptosis and increased neuronal viability. Additionally, the IncRNA 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 IncRNA as CC2dat and AK038897 and the potential benefits of modulating some IncRNAs 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 IncRNAs in the endothelium following ischemic stroke are still unclear. Identification of new IncRNA 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 IncRNA-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

Review

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

| ncRNA                  | Principal functions                                                                 | References                      |
|------------------------|--------------------------------------------------------------------------------------|---------------------------------|
| 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)                |
| mir-15S                | 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               | Astroglisosis 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); Veremeenko 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.
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-kB (NF-kB) 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-kB 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 C/EBP-β (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-kB, exerts its anti-inflammatory action by targeting PDCD4, leading to downregulation of NF-kB 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 tissue. 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 derived from several 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 (Musial-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 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 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.

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
