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Gene expression profiling in stroke: relevance of blood-brain interaction

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Abstract

Biomarker profiling is utilized to identify diagnostic and prognostic candidates for stroke. Clinical and preclinical biomarker data suggest altered circulating immune responses may illuminate the mechanisms of stroke recovery. However, how peripheral blood biomarker profile(s) relate to brain profiles following stroke is unknown. Data show that neutrophil lymphocyte ratio (NLR) predicts stroke outcome. Neutrophils release Arginase 1 (ARG1) resulting in T lymphocyte suppression in peripheral blood. Interestingly, cellular response to stroke may have implications for known biomarker profiles. Conversely, preclinical evidence suggests that upregulation of ARG1 in microglia is a marker of M2 macrophages and may influence neuroprotection. Comparing clinical and preclinical studies creates opportunities to explore the molecular mechanisms of blood and brain biomarker interactions in stroke.

Keywords

stroke; brain; immune function; vascular system; arginase

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Introduction

Stroke affects 15 million people globally per year and is a leading cause of long-term disability, requiring extensive post-stroke patient care [1]. Given the limited therapeutic options for stroke patients, there remains great interest in identifying novel therapeutic interventions that may prevent stroke or improve stroke recovery.

It is becoming increasingly evident that stroke has systemic immune consequences. Animal studies have confirmed the immune system is associated with stroke severity and outcome [2]. Stroke results in suppression of innate and adaptive immune system pathways that increase the risk of post-stroke infection contributing to increased mortality and poor outcome [3]. Recent preclinical studies suggest that restoring stroke-induced peripheral immunosuppression could be an important therapeutic option [4,5]. However, there is limited clinical research to suggest whether the biomarker profiles are similar between animals and humans and what the best therapeutic approach to augment the peripheral systemic response to stroke should be. Stroke results in a biphasic immune response characterized by initial over-activation [6] followed by immunosuppression over the course of days [7] that eventually alters the pathophysiological cascades associated with stroke recovery. The complexity of this systems level response is clear; however, the methods by which we should study these biomarker profiles is not. An ideal approach would not only assess immune function, but also attempt to link immune function to cerebrovascular, neurological function, and brain damage. Evidently, there are gaps in our understanding of how the alteration of immune function is linked to stroke incidence and outcome. It is beyond the scope of this single review paper to describe the whole scheme of the genome in relationship to the pathophysiology of stroke; however, one cannot argue that factors in the blood are critical initiators as well as mediators of stroke incidence and outcome. Thus, this review will focus on the challenge of stroke research as it relates to gene expression profiling in blood and the possible connection of the immuno-cerebrovascular system in stroke. Furthermore, potential new biomarkers and pharmacological targets are also discussed.

Challenges in gene expression profile in stroke

High throughput gene expression profiling provides a snapshot of a tremendous amount of gene activity to attempt to describe the complex systems level response to disease or a clinical outcome. Cancer research has effectively utilized this powerful tool to enhance clinical diagnosis, and intervention as well as predict clinical response and outcome [8]. The challenge of gene expression profiling in stroke is that the dissection of gene activity at the cellular or systems levels in stroke is often confounded by one or more existing co-morbidities such as hypertension, obesity and aging. [9,10]. Stroke can be classified as ischemic or hemorrhagic with varying etiologies (i.e cardioembolic vs atherosclerosis) and locations (large vessel vs lacunar stroke), which further complicates interpretation of biomarker profiles. As predicted, gene expression profiles in blood vary depending on the type and etiology of stroke [11,12]. Aside from the challenge of interpreting the complexities of human stroke, blood is a simple and clinically relevant alternative to brain tissue (i.e. separating specific cell types, RNA isolation, and PCR). Work by our laboratory

and others has demonstrated that blood genomic profiles are similar to those discovered in animal studies of stroke and, thus, could not only be utilized to identify biomarkers of stroke but may also help to discover novel mechanisms of stroke recovery [13-16].

Although there is an on-going controversy regarding the models that allow for true translation between clinical and preclinical research [17], a well-designed preclinical study possesses several advantages over human clinical trials, such as the ability to conduct invasive procedures, setting a cross-sectional design and controlling confounding variables. Thus, preclinical studies and animal models provide an excellent tool to assess each piece of information and elucidate the mechanism(s) of stroke to bridge the gap among types of strokes, interconnections of different systems and preexisting co-morbidities. Translational research is a two way road or “bench to bedside and back to bench” approach that keeps the end goal of improving clinical practice in mind [18]. An example of translational stroke work that could improve the clinical treatment of stroke is the discovery of a causal link between matrix metalloproteinase (MMP) 9 and cerebrovascular barrier dysfunction following cerebral ischemia, confirmed in both MMP9 knock out (KO) mice [19] and clinical studies [20]. These studies demonstrate the association between blood brain barrier (BBB) dysfunction and elevated MMP9 levels in ischemic patients. However, this model for translation is not without limitations, such as surgically inducing stroke in animals, and the translation of rodent immunity to the human immune response which is highly debated. A recent study confirms that transcriptional profiles of human and mouse immune cells are largely conserved; however, specific genes show divergent expression [21]. Care must be taken when comparing the immune systems of rodents and humans because experimental rodents are inbred and the colonies are routinely tested to ensure a specific pathogen free environment, whereas human samples are heterogeneous and exposed to a number of pathogens that experimental rodents never have the chance to encounter. Details regarding the translation of preclinical stroke models to clinical stroke studies have been elaborated in a recent excellent review [22]. Figure 1 summarizes this challenge using gene expression profiling in translational stroke research and emphasizes the need for well-designed simultaneous preclinical and clinical research studies.

An important question for the field is whether peripheral blood profiles actually reflect the brain injury response to stroke. The substantial integrative work of clinical and preclinical expression profiling studies by Sharp and colleagues suggest that peripheral blood profiles are associated with brain profiles [23-26]. They demonstrated distinct blood gene expression profiles from different types (ischemic or hemorrhagic) of experimental stroke and other brain insults (seizure, hypoxia) using rats [23]. With the same experimental design, these different types of brain insults were associated with specific gene expression in the brain, including inflammation, BBB disruption, cytoskeletal alteration, DNA damages/metal chelating, and heat shock proteins [24]. Similarly, altered peripheral blood profiles were related to brain injury and increased heat shock protein expression was reported after different duration of the ischemic insult (5, 10, 120 min) using a rat middle cerebral artery occlusion (MCAO) model [25,26]. A subsequent study further confirmed these initial rodent studies using a nonhuman primate ischemia model with a combination of peripheral blood mononuclear cell expression profiles, MRI imaging and neurobehavioral assessments [27]. Collectively, although the exact causal relationships between peripheral blood and brain

profiling data are not yet clear, preclinical evidence suggests that blood biomarkers are associated with brain structural damage and neurological function. Thus, peripheral blood profiles have potential to be useful for diagnosis, monitoring treatment and intervention response, and predicting stroke outcome. To provide further evidence for a scientifically valid overlap between brain and blood expression profiles, a recent study has identified the presence of central nervous system lymphatic vessels in mouse and human [28]. This surprising finding suggests that the cerebrovascular route may not be the only route for immune cells to enter and exit the brain, adding a whole new avenue to explore neuroinflammation compared to the traditional neurovascular route.

Circulating micro RNA (miRNA)

Recent reviews on RNA expression profiles in human total leukocytes have been published [29,30]. Circulating miRNA, small noncoding RNA, is released into the peripheral blood as a message describing the cellular insult, likely playing a role in cellular adaptation [31]. Since miRNA is not easily degraded and is more stable than RNA, recent studies are focusing on circulating miRNAs as biomarkers for stroke [32-35]. One of the earliest miRNA reports in a stroke study was published in 2008; using a rat MCAO model, the authors demonstrated the alterations of blood and brain miRNAs related to the genes for stroke-associated pathology including brain edema and BBB disruption [32]. A recent animal study using a rat thrombotic MCAO model identified differences in circulating miRNA gene expression between gender and age [34]. Gender and aging are well-known contributing factors for stroke severity, thus, this miRNA profile study demonstrated that gender and aging differences in stroke could be detected at circulating miRNA levels. Interestingly, aging differences in circulating miRNA profiles were noticeable at 2 days post-stroke whereas gender-related miRNA regulation was evident at 5 days post stroke. This suggests an interesting phenomenon in stroke recovery that may vary by gender. A more recent study reported that endothelial cells communicate with monocytes via miRNA to regulate inflammatory responses [36]. Specifically, *in vitro* experiments demonstrated that miR-10a from endothelial cells negatively regulates NF- κ B signaling in monocytes. These studies suggest that miRNA are new biomarkers for stroke and may be promising targets for intervention. For instance, a study using miRNA-based intervention showed that amelioration of stroke damage can be achieved by immune-modulating mechanisms [37]. In this study, decreased miRNA 424 levels in plasma were confirmed in stroke patients and mouse permanent MCAO model and overexpression of miRNA 424 by intracerebroventricular (ICV) administration was associated with decreased ischemic injury through suppressing microglia activation. Taken together, the application of miRNA gene expression profiles in stroke research is attempting to shed new light on some of the possible mechanisms of stroke as well as on the identification of potential intervention targets. However, the precise roles of miRNA (i.e., types, target tissues, origin) in stroke remain uncertain.

Proteomic expression profiling

Genomic studies including RNA and miRNA provide a tremendous amount of information at the transcriptional and translational levels; however, to uncover novel mechanisms for

treatment it is important to clarify how the identified gene products play a role at the protein level. In order to elucidate the gene messages, the protein level information such as the expression time-course, site(s), expression levels, post-transcriptional modification, and three-dimensional structural analysis, are necessary to understand the function of proteins. One of the earliest stroke-related proteomic reports was a study using 2D gel electrophoresis for serum and urine samples from spontaneously hypertensive stroke prone rats [38]. In this study, markers of an inflammatory response such as thiostatin were detected in the serum at least 4 weeks before the stroke occurrence. Another interesting clinical study focused on proteomic substrate profiling which indicated not only did the stroke patient plasma exhibit distinct MMP profiles but there was an alteration of MMP profiles between stroke patients who did and did not receive rtPA treatment [39]. More recently, proteomic profiling of *in vitro* human cerebral endothelial cells in response to oxidative stress showed increased inflammatory proteins and a marker for barrier dysfunction (thrombospondin-1) that was also confirmed in the plasma of stroke patients [40]. Similar observations such as elevated inflammation have been reported for the experimental ischemic response in both mouse and rat brain endothelial cells [41,42]. One limitation for proteomic profiling may consist in the fact that, similar to the genomic studies, intra- and inter-laboratory comparisons appear to be difficult due to dynamic protein expression response against stroke. Although in an experimental setting subject variability and species can influence the magnitude of increase in a particular marker for inflammation, the general trends of elevated inflammatory response and endothelial dysfunction appear to be consistent. Collectively, evidence at the proteomic level also supports circulating inflammation as a key mediator after stroke from both clinical and preclinical studies, similar to that seen in genomic profiling studies.

Systemic immune response and neuroinflammation after stroke

Based on multiple levels of “omic” studies in animals and humans following stroke, there is consensus that disruption of immune function after stroke represents an important contributor to the progression of poor stroke outcomes. In addition, both clinical and preclinical data show that post stroke immunosuppression often contributes to mortality [43,44]. Neutrophil lymphocyte ratio (NLR) is a readily available clinical marker of immune status. The NLR measurement is routinely performed in clinical practice because it is a simple reflection of the general inflammatory index. NLR is also prognostic in a variety of cancers and cardiovascular diseases [45,46]. Several studies have shown that the NLR is strongly associated with stroke severity and outcome [47-49]. A recent study by our laboratory confirms that elevated NLR is associated with increased stroke severity, mortality and poor outcome [50]. These studies suggest that NLR may be a useful biomarker to not only predict short-term mortality in stroke, but also to reflect post-stroke outcome. The mechanism by which NLR is increased after stroke is unclear, however it has been suggested that arginase 1 (ARG1) from neutrophils may play a role in immunosuppression via T cell dysfunction [51]. ARG1 is an essential enzyme that catalyzes the urea cycle by converting L-arginine to L-ornithine. ARG1 is most traditionally known for its role in cardiovascular disease; however, more recently it has been associated with regulation of the immune system [52]. ARG1 inhibition dramatically improves host immune response to tuberculosis infection [53] demonstrating its role in immune suppression. A recent study

showed that arginase released from neutrophils acts as a mediator for suppression of T-cells following MCAO in mouse [54]. It would be interesting to evaluate whether *in vitro* splenic mononuclear cells from mouse MCAO with L-arginine supplementation could rescue reduced T- cell proliferation post-stroke. Elevated ARG1 expression in brain has been reported in other pathologies such as Alzheimer disease and traumatic brain injury associated with immunosuppression [55,56]. On the other hand, ARG1 has also been recognized as a marker of the M2 macrophage phenotypic shift that is associated with neuroprotection. Preclinical evidence suggests that increased expression of ARG1 in microglia following ischemic stroke is associated with improved tissue remodeling as well as behavioral recovery [54,57-61]. Therefore, a balanced response is likely necessary to achieve the maximal effect of ARG1. Because of these collective data, inhibition of arginase could be a novel intervention for immunosuppression in stroke patients; however, the potential negative impacts on post-stroke neurogenesis must be considered. Identifying a critical therapeutic window, dose, and drug delivery system to specifically target cells (i.e. neutrophils) is therefore important. Table 1 summarizes the studies related to arginase in stroke.

Taken together, these clinical and experimental observations reinforce the idea that altered NLR could be an “immuno-cellular-omic” marker for stroke severity and outcome. Furthermore, neutrophil function may be involved in stroke-associated immunosuppression and the elucidation of its function may lead to the identification of a novel intervention to improve stroke outcome.

Conclusion

Clearly, a tremendous amount of information can be obtained from blood profiling experiments at the genomic, proteomic and cellular levels. Since stroke involves a complex dynamic blood inflammatory response, the expression profile approach will likely be different from cancer, aging, and diabetic studies. It will require a more stratified time course analysis of the specific “inflammatory phase” of stroke. Although preclinical studies possess limitations, the close relationships between preclinical and clinical studies are essential for successful translation and real impact. If a preclinical study could identify a causal relationship between the biomarker profiles discovered to date and the mechanism of stroke-induced immune suppression, these peripheral blood profiles could guide clinical decisions at the bedside. Much work is needed to confirm the clinical relevance of preclinical data and begin to move the use of biomarker profiling into stroke clinical practice.

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Highlights section

- Expression profile data suggest stroke involves a complex inflammatory response.
- Neutrophil and lymphocyte ratio may be a useful biomarker for stroke outcome.
- Arginase from neutrophil may play a role in stroke-associated immunosuppression.

Figure 1.

Stroke not only induces alterations of peripheral blood profiling, but also produces changes in the immune system. A) Blood samples can be collected from stroke patients to obtain peripheral blood profiles. Dynamic inflammatory response requires multiple time course sampling to identify subcategories of stroke events, to predict patients' outcome and to develop new biomarkers in stratification and diagnosis of stroke. Samples from multiple organs can be collected from preclinical stroke models to describe stroke phenotype, obtain the gene expression changes and their associated with brain damage. B) Stroke induces rapid activation of the peripheral immune system and subsequent immunosuppressive phase.

Table 1

Arginase 1 expression in stroke.

Model	Time Course	Tissue	Type of Measure	Expression levels/Physiological role	Ref.
Photothrombotic stroke model male Wistar rats	8 and 15 days post ischemic stroke	Brain	WB IHC Activity	Increased/neuroprotective (M2 marker)	[58]
MCAO 60 minutes male C57Bl/6 mice	6 weeks post stroke	Brain	WB IHC	Increased/neuroprotective (M2 marker)	[60]
MCAO 30 minutes male C57Bl/6 mice	4 weeks post stroke	Brain	WB	Increased/neuroprotective (M2 marker) *an intervention increased ARG1 expression	[59]
MCAO 60 min Male ICR mice	24 h post stroke	Brain	qPCR	Increased/neuroprotective (M2 marker) *an intervention increased ARG1 expression	[61]
Model	Time Course	Tissue	Type of Measure	Expression levels/Physiological role	Ref.
MCAO 60 minutes male C57Bl/6 mice	4 days post stroke	Plasma	ELISA	Increased/Immunosuppressive	[54]
Stroke patients	3, 5, and 24 h ischemic stroke	Whole blood	Microarray	Increased/biomarkers	[62]
Stroke patients	24 h from symptom onset	Whole blood	Microarray	Increased/biomarkers	[13]
Stroke patients	3, 5, and 24 h ischemic stroke	Whole blood	Microarray	Increased/biomarkers	[63]

Middle cerebral artery occlusion (MCAO), Western blot (WB), immunohistochemistry (IHC), quantitative polymerase chain reaction (qPCR)