RUNNING HEAD: Acute Phase Response in TIA

Evidence of the Acute Phase Response in Transient Ischemic Attack Patients

By

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ABSTRACT

Evidence of the Peripheral Acute Phase Response in Transient Ischemic Attack Patients

A retrospective chart review was done to determine whether the peripheral acute phase response (i.e., elevated WBCs, neutrophils and monocytes) exists in transient ischemic attack patients as it does in stroke patients. 1041 medical records were investigated for inclusion in this study. Strict exclusion criteria where cases must be first time TIA or stroke, and have no evidence of concomitant infection, cancer, NSAID or steroid use, recent surgery or procedure in the month prior to admission, resulted in 12 first time TIA and 43 first time stroke cases. TIA was defined by symptoms lasting less than 1 hour and no evidence of stroke on CT or MRI. In both groups, neutrophil and monocyte percentages were significantly higher than the laboratory means (in TIA, neutrophils, $p=.001$, monocytes, $p=.020$; in stroke neutrophils and monocytes, $p<.001$). Lymphocyte percentages and absolute lymphocyte count in both groups were significantly and abnormally lower than the laboratory mean (in TIA $p=.001$, $p<.001$, respectively; in stroke both $p<.001$). Additionally, in the stroke group absolute neutrophil counts were significantly higher than the laboratory mean, $p=.022$. When the two groups were compared on these variables, all percentages and counts were not significantly different from each other, except the absolute lymphocytes count which was significantly lower in the TIA group than the stroke group, $p=.039$. These findings suggest that the peripheral acute phase response exists in transient ischemia, which hypothetically does not damage brain tissue, as well as in stroke, or permanent ischemia, which is known to produce brain tissue damage.

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CHAPTER 1

Introduction

A stroke occurs every 45 seconds in America. People presenting with symptoms of stroke may have any combination of the following: blurred vision, slurred speech, numbness or weakness on one side of the body, headache, and dizziness. In ischemic stroke, both prior to and during the presentation of symptoms, an innate immune response, specifically the acute phase response occurs. There is evidence that the acute phase response (APR) may have conflicting effects on the evolution of cerebral ischemic infarction, exacerbating brain injury early and then contributing to brain tissue repair later. Leukocytosis, neutrophilia, and lymphocytopenia, are evidence of the mobilization of peripheral immune cells from the marginal pool. Emerging evidence suggests that the peripheral mobilization of immune cells is due to neural-immune signaling tied with the APR.

Patients with transient ischemic attack (TIA) experience stroke-like symptoms of short duration, which leave no objective evidence of permanent tissue damage. TIA is thought to be neuro-protective for subsequent cerebral infarction by conditioning the brain tissue to low blood flow or hypoxia, a phenomenon known as ischemic preconditioning (IP). The APR is a candidate mechanism in experimental animal models of ischemic preconditioning. Yet, it is not known if an APR occurs in humans with transient cerebral ischemia (i.e., TIA). Evidence of the APR in humans with TIA could provide support for the neuroprotective role of transient cerebral ischemia in humans. The existence of the APR in TIA may demonstrate the neuroprotective mechanisms of

the APR in TIA, as opposed to the neural tissue destructive effects associated with the APR in acute stroke.

Purpose

The purpose of this study was to explore whether there is an APR in TIA patients by examining the white blood cell profile of TIA patients, and making comparisons to the acute phase response in stroke patients. The rationale for the study was that the detection and description of the APR in TIA would provide preliminary data on which to base a prospective study of pro-inflammatory and anti-inflammatory cytokines in transient ischemia patients.

Specific Aims

Aim 1: To determine if there is sufficient evidence to conclude that the peripheral APR exists in transient ischemic attack, and stroke patients on admission.

1. On admission, is the WBC count of TIA patients significantly different from the mean of the laboratory normals of 7.8 K/UL?
2. On admission, is the neutrophil % of TIA patients significantly different from the mean of the laboratory normals of 52.5%?
3. On admission, is the lymphocyte % of TIA patients significantly different from the mean of the laboratory normals of 35%?
4. On admission, is the monocyte % of TIA patients significantly different from the mean of the laboratory normals of 6%?
5. On admission, is the absolute neutrophil count of TIA patients significantly different from the mean of the laboratory normals of 4.45 K/UL?

6. On admission, is the absolute lymphocyte count of TIA patients significantly different from the mean of the laboratory normals of 2.7 K/UL?

7. On admission, is the absolute monocyte count of TIA patients significantly different from the mean of the laboratory normals of 0.6 K/UL?

8. On admission, is the WBC count of Stroke patients significantly different from the mean of the laboratory normals of 7.8 K/UL?

9. On admission, is the neutrophil % of Stroke patients significantly different from the mean of the laboratory normals of 52.5%?

10. On admission, is the lymphocyte % of Stroke patients significantly different from the mean of the laboratory normals of 35%?

11. On admission, is the monocyte % of Stroke patients significantly different from the mean of the laboratory normals of 6%?

12. On admission, is the absolute neutrophil count of Stroke patients significantly different from the mean of the laboratory normals of 4.45 K/UL?

13. On admission, is the absolute lymphocyte count of Stroke patients significantly different from the mean of the laboratory normals of 2.7 K/UL?

14. On admission, is the absolute monocyte count of Stroke patients significantly different from the mean of the laboratory normals of 0.6 K/UL?

Additionally, the following exploratory aims were investigated.

Exploratory Aim 2: To determine whether patients with TIA have the same peripheral APR as the patients with stroke.

Research Question. On admission, what is the main effect of group (TIA versus Stroke) on the 7 dependent variables related to the acute phase response, the WBC count, neutrophil %, lymphocyte %, monocyte %, absolute neutrophil count, absolute lymphocyte count, and absolute monocyte count?

Exploratory Aim 3: To determine whether patients with TIA have the same peripheral APR as the patients with stroke over time.

Research Question. What is the group (TIA versus Stroke) by time (admission and 24 hours) interaction effect on the 7 dependent variables related to the acute phase response, the WBC count, neutrophil %, lymphocyte %, monocyte %, absolute neutrophil count, absolute lymphocyte count, and absolute monocyte count, after controlling for main effects to group, and time?

Significance

Stroke is the third leading cause of death in the United States, with a death from stroke occurring every 3 minutes (AHA, 2004). Heart disease and stroke continue to be among the leading causes of death, accounting for 41.1% of all the deaths in the US (AHA, 2004). Oregon's death rate from stroke is ranked as the third highest in the nation (AHA, 2004). Each year 500,000 new strokes and 200,000 recurrent strokes occur, averaging a stroke event every 45 seconds nationwide (AHA, 2004). In 2004, the estimated direct and indirect cost of stroke care was 53.6 billion dollars per year, and inpatient hospital costs account for 70 percent of the first year post-stroke costs (AHA,

2004). In 2001, an estimated $103 million was spent on all stroke related hospitalization in Oregon (Oregon Department of Human Services, 2001). Yet to date, the emerging stroke registries undertaken nationwide include few TIA events, making the financial or clinical impact of TIA on health and healthcare in the United States difficult to estimate.

Clinicians have to rely on the evolution of neurological deficits, and changes on serial CT or MRI imagining as clinical markers of stroke evolution. If new therapies are developed related to cellular level processes that are neuroprotective, then timing these new therapies may be problematic when the initial CT scan or MRI may initially be negative for ischemia or stroke, and neurological deficits continue to deteriorate over the first 3 days due to increasing cerebral edema. The peripheral acute phase response may provide a robust marker for the cellular process of ischemia, as this clinical measure is the only measure that changes over a long period of time in stroke evolution. The innate immune response, specifically the APR, is considered the major signaling pathway of injury and repair in the context of stroke (Sairanen, Carpen, Karjalainen-Lindsberg, Paetau, Turpeinen, Kaste, & Lindsberg, 2001). Determining the presence of the APR in TIA and its similarity to the APR in stroke extends the knowledge base related to the immune systems contribution to ischemic injury, repair and neuroprotection.

CHAPTER 2

Literature Review

TIA

TIA is "a brief episode of neurologic dysfunction caused by focal brain or retinal ischemia, with clinical symptoms typically lasting less than one hour, and without evidence of acute infarction."(Albers, 2002) TIA signs and symptoms are similar to stroke but the duration of the deficits is limited. Additionally there is no objective evidence of ischemia on CT scan, or on the retina. This means that the ischemic event did not leave a damaged area in the brain tissue. If the neurological deficits persist, lasting longer than one hour, brain imaging usually shows an area of infarction (Albers, 2004). An older definition based on time (i.e., symptoms lasting less than 24 hours) was problematic as there was a blurring of the patient populations that had evidence of infarction on head CT scan, but were classified as a TIA patient according to the duration of symptoms (Albers, 2002; Easton, et al., 2004). This new definition makes a shift in the focus from time, to tissue, and aligns the TIA and stroke definitions with the definitions of angina and myocardial infarction, respectively (Albers, 2002) (See Appendix I for Overview of Cerebral Ischemia and Infarction; see Appendix II for Definition of Terms in this literature review).

TIA as a Stroke Risk Factor and Prediction Stratification

Predictors of stroke include being older that 60, having diabetes, focal weakness or speech deficit, and TIA lasting longer than10 minutes in duration (Johnston, et al., 2000). TIA has emerged as a strong independent predictor in stokes models (Johnston, 2000; Kernan et al., 2000). TIA's are thought to increase the short-term risk of stroke. In

approximately 10.5% of people who had TIA's developed stroke within 90 days, 50% of those (5.25%) developed stroke in the first 2 days after TIA (Johnston, 2000). During this identified 90 day risk window 25% of all patients had another cardiovascular adverse event (i.e., myocardial infarction, 2.6%, death, 2.6%, and recurrent TIA, 12.7%). More recently, the risk period after TIA was reported as 7 days with 30-43% of the patients with stroke having had a previous TIA with this time frame. Seventeen percent of these stroke patients had a TIA the day of their stroke (Rothwell & Warlow, 2005). The evidence from this report clearly outlines a 7-day window of risk for stroke following TIA.

TIA: Risk Factor for Stroke or Neuroprotective

TIA can be thought of in two ways, as a risk factor for stroke that increases the risk of stroke occurrence, or as an ischemic preconditioning event that confers neuroprotection. Starting in 1994 there was a split in the research trend from thinking TIA was only a risk factor to investigating the events in the ischemic cascade as possible candidate mechanisms in developing tolerance to ischemia (Hakim, 1994). The notion that TIA events may be neuroprotective is a divergence from the concept that TIA events are only small ischemic events that can be tied to risk stratification (Hakim, 1994; Rejdak, Rejdak, Sidklucka-Dziuba, Stelmasiak and Grieb, 2001; Shaller, 2003; Dirnagl, 2003). Ischemic preconditioning and neuroprotection are important concepts to the investigation of the APR in TIA in this proposed study.

TIA as Neuroprotection

Many researchers have demonstrated that smaller infarction volumes occur when lethal ischemia is preceded by a sub-lethal ischemia, like prodromal TIA in stroke. This evidence was the turning point in the search for mechanisms of neuroprotection in stroke.

Using comparison groups based on the duration of TIA, Moncayo, de Freitas, Bogousslavsky, Altieri, and van Melle, (2000) reported favorable outcome in those with prior TIA, and with TIA within one week of stroke. TIA's lasting longer than 20 minutes reduced favorable outcome, and TIA's lasting longer than 60 minutes was the same as not having had a previous TIA. The authors concluded that the time of ischemic preconditioning in humans was between 10 and 20 minutes, and that ischemia of less than 10 minutes did not create tolerance and more than 20 minutes reduced favorable outcome but still provided some protection. This observational cohort study could not test mechanisms associated with TIA that induce tolerance against ischemic damage. However, the authors reported that the same time period (i.e., one week) was associated with both increased risk of subsequent stroke and the "ideal" time for conferring a protective effect via ischemic preconditioning between first time TIA and stroke.

Castillo et al., (2003) reported patients who had a stroke 72 hours after having a TIA had improved functional outcome, and smaller stroke volumes. Utilizing diffusion- and perfusion-weighted MRI, Wegener et al. (2004) studied patients with first time ischemic stroke. The findings of Wegener's study showed that prodromal TIA was the only parameter in a multiple regression analysis that predicted small final infarction size in this stroke sample. These findings were associated with less severe neurological deficits. Wegener suggested that protection by prodromal TIA was not explained by

changes in CBF, collateral recruitment, or enhances vascularization. Wegener proposed that an endogenous neuroprotective mechanism might be occurring that confers ischemic tolerance.

In an epidemiological study, Sitzer (2004) reported that 15.6% of patients admitted with stroke had a history of previous TIA. TIA patients had a 2.76 times higher likelihood of having a subsequent TIA, than having a subsequent stroke. Sitzer concluded that TIA was protective against stroke.

Several studies reported that TIA was associated with smaller infarction sizes or volumes (see Table 1) (Moncayo, 2000; Castillo, 2003; Sitzer, 2004; Wegener, 2004). The interval between stroke and the prodromal TIA was known in only one study to be 72 hours (Castillo, 2003). Prodromal TIA was associated with improved functional outcome after stroke (Moncayo, 2000; Castillo, 2003). One study reported that people with a history of prodromal TIA were almost three times more likely to have another TIA as have a subsequent stroke (Sitzer, 2004). In all, these studies support the proposal that TIAs are neuroprotective, and perhaps confer tolerance.

Ischemic Tolerance

Endogenous neuroprotection is the conceptual focus of many basic science investigations related to stroke. Ischemic tolerance is the primary model for this research, with many researchers interested in the goal of developing modes of ischemic preconditioning based on endogenous mechanisms. Dawson (2002) best defined ischemic preconditioning as a "powerful endogenous phenomenon in which brief episodes of a sub-toxic ischemic insult induces robust protection against future, lengthy, lethal ischemia." Research has shown that many stimuli that are potentially injurious, if

applied at a level lower than the injury threshold, confer tolerance to subsequent similar lethal stimuli (Dirnagl, Simon, & Hallenbeck, 2003). The sub-lethal stimulus is the preconditioning agent, or the agent capable of inducing tolerance. In the context of ischemia, the two terms, ischemic preconditioning (IP) and ischemic tolerance (IT) are used.

Preconditioning utilizes a stimulus that induces protective and not destructive signaling cascades. There are three steps in protective signaling: 1) induction of receptors, channels, and regulators, 2) transduction via specific protein kinases, transcriptions factors and paracrine and autocrine growth factors which amplify the signal, and 3) expression of proteins that provide a protective effect (i.e., anti-inflammatory, anti-apoptotic and anti-oxidative proteins) (Mergenthaler, 2004). Candidate mechanisms for ischemic preconditioning are summarized in Table 2. The mechanism of interest in this study is the APR.

Models of IP have been developed using various IP stimuli (i.e., global ischemia, hypothermia, hypoxia, intracisternal TNF-alpha injection, intraventricular IL-10 injection, pharmacological blockade of brain norepinephrine, thrombin injection, and humanized E- and P-selectin tolerization) (see Table 3). These IP stimuli reduce infarction sizes or volumes (all studies in table); reduce brain edema without changing CBF (Masada, 2000), reduce infarction size in a time- and dose-dependent manner (Nawashiro, 1997; Nishio, 1999); increase IL-1 receptor antagonist (IL-1ra) expression (Barone, 1998); decrease PMN infiltration in brain tissue, suppress systemic complement activation and induce immune suppression (Mocco, 2002). Some of these experimental models are based on acute phase response cytokine signaling, and acute phase protein

effecting downstream mechanisms. Collectively, these experimental studies provide strong evidence of mechanisms that can confer IT.

Ischemic Preconditioning Phenomenon – Window of Tolerance. Inquiries into effective timing for intra-ischemic and post-ischemic therapies have focused on windows of tolerance to ischemia (Nishio, et al. 1999). Early tolerance windows have been identified, 5-120 minutes after stimulation, known as classical preconditioning (Moncayo, et al., 2000). There also exists a delayed window of tolerance that starts at about 24 hours after injury and lasts about 72 hours, with a slower onset and a persistent effect lasting up to 7 days (Moncayo, et al., 2000; Dirnagl et al., 2003; Stenzel-Poore, et al., 2003). Barone, et al., (1998), and Nishio et al., (1999) reported that ischemic preconditioning required de novo protein synthesis in order to confer protection from subsequent ischemic insults. These researchers infer that this finding is a hallmark of the translation of ischemic preconditioning to ischemic tolerance. Barone et al. reported that the proteins pertinent to multiple mechanisms of protection appear in brain tissue between 1 and 7 days, and that severe permanent middle cerebral artery occlusion (MCAO) did not produce these same proteins. This is the same 7-day window that is associated with neuroprotection in the models of ischemic preconditioning.

The Acute Phase Response

The acute phase response (APR) is a specific early and immediate set of physiological reactions, which is part of the inflammatory process. The APR is activated to return the organism to normal function after an infective threat or a tissue injury (Baumann and Gauldie, 1994).

The cells that most often elicit the APR are the tissue macrophages or the blood monocytes. Both of these cells have the capability to release IL-1 and TNF-alpha in response to a threat to the host (Baumann and Gauldie, 1994; Hallenbeck, 1997). Additionally, injured brain cells release both IL-1beta and TNF-alpha. These two different sites of tissue release (leukocyte subsets, and brain tissue) are thought to produce functionally redundant cytokines (Dirnagl, Iadecola, & Meisel, 1999). Both of these cytokines act both locally and distal to the site of production. Locally, they activate the vascular endothelium (i.e., induce ICAM and selectins), and promote chemotaxis by promoting the release of IL-8 and monocyte chemoattractant protein, MCP. Other reactions at the site of injury include vasodilation via TNF-alpha induction of nitrous oxide and arachidonic acid metabolites, like the prostaglandins; leakage of blood and fluid into the tissue, and egress of leukocytes into the tissue. Later in tissue damage, IL-6 is released (Baumann and Gauldie, 1994; Dirnagl, 1999; Hallenbeck, 1997).

Circulating IL-1, Il-6, and TNF-alpha induce gene regulation in the liver to produce the acute phase proteins including C-reactive protein, fibrinogen, and mannan-binding lectin. C-reactive protein initiates the complement cascade via C1q of the classical pathway. Fibrinogen is converted to fibrin to make more clots. Mannan-binding lectin is a pattern recognition molecule that interacts with myelin-basic protein, a cell membrane molecule that is released from dying neurons. Mannan-binding lectin initiates the complement cascade via the Mannan-binding lectin pathway. All of which are needed to control the immunogenic threat (Baumann and Gauldie, 1994; Janeway, 2001).

Resolution of the APR is mediated by IL-10 and IL-1ra, which subdues the response over 24 to 48 hours. IL-10 inhibits this response by retarding the synthesis of IL-1, IL-6 and TNF-alpha by macrophages and monocytes; by inhibiting colony stimulating factors; and inducing the synthesis of IL-1ra (Baumann and Gauldie, 1994).

Cytokines and the APR in Stroke

Cytokines and the APR in Stroke – Experimental. Animal models of stroke have shown that plasma IL-6 peaks at 3 hours after permanent MCAO and remain elevated at least 12 hours (Clark, 1997). Leukocytes expressing IL-1beta, IL-6, and TNF-alpha, the major pro-inflammatory cytokines, infiltrate around cerebral blood vessels, ventricles, and meninges on histological examination of neurologically impaired rats (Siren et al., 2001). Additionally, TNF-alpha is decreased at 12 hours and remains low to at least 48 hours after stroke (Prass, 2003).

Cytokines and the APR in Stroke - Human. Studies of the acute phase cytokines in human subjects with stroke have focused on IL-6 and TNF-alpha predominantly (see Table 4). IL-6 has been shown to be present in the plasma and CSF on admission after stroke (Ferrarese et al., 1999; Vila, et al., 2000; Johansson et al., 2000, Perini, et al., 2001; Emsley, et al., 2003; Castillo, et al., 2003). A few studies present conflicting evidence that IL-6 was not significantly elevated on admission (Perini, 2001; Emsely, 2003), but others found IL-6 significantly elevated from admission, peaking at day 3, and remaining elevated through 3 months after stroke (Ferrarese, 1999; Johansson, 2000; Perini, 2001; Emsley, 2003; Castillo, 2003). IL-6 was correlated to cortisol levels at 48 hours (Johansson, 2000).

TNF-alpha is elevated on admission in clinical stroke and remains elevated in the plasma due to cytokine release from peripheral blood cells for up to 3 months after stroke onset (Ferrarese et al., 1999; Vila, et al., 2000). In a study of post mortem brain tissue, neuronal TNF-alpha expression peaks 2 to 3 days after stroke, and astrocyte TNF-alpha expression persists up to 17-18 days after stroke onset (Sairanen, et al., 2001). Because TNF-alpha is temporally expressed during the ischemic cascade, evidence links TNF-alpha with both promotion of inflammation and necrosis (via glial TNF-alpha) and also with later restorative processes in the penumbra (via astrocyte TNF-alpha) (Sairanen, et al., 2001; Hallenbeck, 2002).

Measurement of IL-10 in stroke shows that IL-10 is decreased on admission and remains decreased through day 7 (Perini, 2001; Vila, 2003). This decrease in IL-10 is associated with neurological worsening (Vila, 2003). There is a negative correlation to IL-6 levels, which is the opposite of normal IL-6/IL-10 relationship in healthy people in association with inflammatory challenges (Perini, 2001). IL-10 is a prominent anti-inflammatory cytokine that suppresses the immune response after antigenic or immunogenic challenge. Finding IL-10 suppressed after stroke through day 7 means that the innate and adaptive immune response run unopposed initially in the context of stroke. This is an important finding that helps to explain the effectiveness of the immune response in stroke initiating early neuron cell damage via apoptosis.

Ischemic Preconditioning and the Acute Phase Cytokines – Human. Prodromal TIA reduces IL-6 during the subsequent stroke event, if the stroke is within 72 hours of the TIA (Castillo, Moro, Blanco, Leria, Serena, Lizasoain, and Davalos, 2003). In acute stroke patients with prodromal TIA the plasma concentrations of TNF-alpha were

increased and plasma concentrations of IL-6 were decreased (Castillo, et al., 2003). In stroke with prodromal TIA, increased TNF-alpha is associated with reduced infarction size (Castillo, et al., 2003). TNF-alpha, then, in stroke after a TIA, appears to be down regulated. It is not known whether TNF-alpha and IL-6 have to appear together to effect the tissue damage seen in the infarction core in stroke, but this evidence suggests that IL-6 is needed for this effect.

In summary, the APR during a stroke is anti-inflammatory, if preceded by TIA within a 7-day window. This is an important finding for future research of neuroprotective mechanisms in stroke and TIA. Signaling by the acute phase cytokines is pivotal to conferring tolerance, protecting intracellular metabolism, and producing genetic expression of protective proteins. The immune system as a whole can confer tolerance by many mechanisms, and taken collectively these studies demonstrate that the APR is associated with neuroprotection in non-lethal ischemia (i.e., temporary MCAO) in both animal models of stroke and in human stroke.

Cytokines and the APR in TIA

APR Cytokines in TIA – Animal. Wang, et al. (2000) reported that significantly lower levels of IL-1beta mRNA were present after temporary MCAO. These lower levels in the temporary MCAO model only, when compared to permanent MCAO, were consistent with the duration of IT (i.e., measured through 5 days).

APR Cytokines in TIA – Human. There are no reports of the acute phase cytokine measurements in human TIA. This is not the focus of this study, but the acute phase response phenomenon remains to be described in relation to cytokine signaling in TIA.

Mechanism of Interest

Mechanisms of Immune Activation in Stroke and Ischemic Preconditioning Affecting Peripheral Immune Cells

Ischemic preconditioning may confer ischemic tolerance through multiple mechanisms. The compelling evidence that the immune system is the major signaling mechanism needs further elaboration. There are alternate mechanisms that activate or suppress the immune system in the context of stroke evolution. The translation of stress by the autonomic sympathetic nervous system and the hypothalamic-pituitary-adrenal axis is one of them.

Immune system activation

The central nervous system translates a stressful stimulus into a variety of responses. Specific to this research is the transduction of stress to the immune system. A stressor activates the sympathetic autonomic nervous system to produce immediate reactions mediated by catecholamines (i.e., epinephrine and nor-epinephrine). If the stress is prolonged, the hypothalamic-pituitary-adrenal (HPA) axis is activated to induce cortisol release from the adrenal cortex.

Sympathetic Autonomic Nervous System. The anatomical innervation and functional results have been elucidated for sympathetic autonomic nervous system (SANS) neural-immune interaction. In order to determine the possibility that the SANS and their neurotransmitters (NT) could be the major neural-immune connection, criteria need to be satisfied. First, NT has to be in close proximity and be released in adequate amounts to allow for signaling. Lymphoid organs are innervated by nor-adrenergic nerve fibers of the SANS. This innervation is primarily to the T cell areas (i.e., paracortical

zone of the lymph nodes, and thymic cortex, peri-arteriolar lymphatic sheath in the spleen), and areas with many macrophages (i.e., marginal zone of the spleen and the medullary cords of the lymph nodes). It is important to note that this innervation has tended to avoid B cell zones in the lymphoid follicles. Second, immune effector cells must have receptors for the NTs of the SANS. Both lymphocytes and macrophages have receptors for alpha- and beta-adrenergic NTs, and granulocytes have receptors for beta-adrenergic NTs. T lymphocyte subsets have differing levels of expression of adrenergic receptors. Third, the immune system must have the capacity to reproduce the SANS signal. Nor-epinephrine addition to T cells produces different responses to the signal depending on the activation stage of the T cell. Beta-adrenergic stimulation inhibits T cell proliferation, antibody secretion, NK cell activity, and production of pro-inflammatory cytokines by macrophages, whereas, alpha-adrenergic stimulation does the opposite. Fourth, the intracellular second messenger must be responsive to the catecholamine stimulation, and catecholamines have been reported to increase second messenger cAMP (Felten, Felten, Bellinger, & Madden, 1993; Felten and Felten, 1994; Madden, Sanders, Felten, 1995; and Madden, 2003).

For the neural-immune system interaction to be important to the evolution of transient ischemia (i.e., TIA) and cerebral infarction, the total capacity of the immune system must be compromised is such a way that the host's health status is challenged. The resultant effector cell changes must contribute to the evolution of vulnerability to disease or damage by the initiating stimulus. Catecholamine stimulation decreases neutrophil phagocytosis, release of lysosomal enzymes, chemotaxis and diapedesis (Felten, Felten, Bellinger, & Madden, 1993; Felten and Felten, 1994; Madden, Sanders,

Felten, 1995; and Madden, 2003). It is important to know that B cell areas are not innervated by the SANS. The summative result of the neural-immune interaction in stroke is prolonged immune suppression over several days, including suppression of the adaptive immune response and the ability of the host to respond to an antigenic or immunogenic threat. Prass, et al., (2003) has described stroke-induced immunodeficiency syndrome (SIDS) as mediated by the SANS and the HPA; and results in an increased window of immune suppression that is correlated with pneumonia, bacterial infection and spontaneous septicemia 3 days after ischemia.

Hypothalamic-Pituitary-Adrenal Axis. Research has linked the HPA axis activation and the resultant cortisol production to cytokines release and delayed neutrophilia. Hypercortisolism, physiologic evidence of stress has been identified in stroke (Olson, Marklund, Gustafson, & Nasman, 1992). Elevation of glucose and cortisol levels occurs early after ischemic stroke (Murros, Fogelholm, Kettunen, & Vuorela, 1993). Hypercortisolism has been linked with the inflammatory process and with cytokine production in stroke (Johansson, Ahren, Masman, Carlstrom, & Olsson, 2000; Johansson, Olsson, Carlberg, Karlsonn, & Fagerlund, 1997; Slowik et al. 2002). IL-1, and possibly IL-6 and TNF alpha, known acute phase cytokines, can induce HPA axis glucocorticoid (i.e., cortisol) secretion (Felten and Felten, 1994).

Peripheral Acute Phase Response in Stroke

The peripheral APR is a composite of effects that reflect an activation of the innate immune response. The laboratory data that reflect the peripheral APR are: elevations of neutrophils, decreased lymphocytes, and activated monocytes (Barone, & Feuerstein, 1999; Emsley, et al., 2003; Siren, et al., 2001; and Vila, et al., 2003). The

peripheral acute phase response has been reported in stroke patients (Emsley, Smith, Garvin, Georgiou, Vail, Bareran, Hallenbeck, del Zoppo, Rothwell, Tyrrell, & Hopkins, 2003) (see Tables 5 & 6).

Peripheral Leukocytes and the APR – Animal. Decreased lymphocyte subsets are known to occur by the first 12 hours after MCAO in an animal model of stroke (Prass, et al., 2003). Specifically, B-, T-, and NK-cells reach the nadir by 12 hours and this lymphocytic depression lasts up to 5-7 days post MCAO (Prass, et al., 2003). Decreased lymphocytes are associated with stroke-induced immune deficiency, and provide a context of risk for bacterial infections; inferring that the adaptive immune response is suppressed (Prass, et al., 2003) (see Table 5).

Peripheral Leukocytes and the APR – Human. The peripheral APR has been reported as occurring at the onset of stroke symptoms (Barone and Feuerstein, 1999). The APR is an integral mechanism in ischemia and infarction maturation. Leukocytosis has long been a marker for an inflammatory response. Peripheral immune cells are a major source of increased IL-6 and TNF-alpha production after ischemia (Ferrarese, Mascarucci, Zoia, Cavarretta, Frigo, Begni, Sarinella, Frattola, De Simoni, 1999). Total WBC count is increased on admission, at 24 hours, and continuing to day 7, with increased neutrophils accounting for the majority of this rise (i.e., also elevated on admission and at 24 hours) after stroke onset in humans (Emsley, et al., 2003). Monocyte activation has been reported in ischemia, which is a cell surface activity that is evidenced by increased monocyte binding with endothelial cells and increased expression of intercellular adhesion molecules (McCarron, et al., 1994; Hallenbeck, 1997). Monocyte

elevation occurs later with the first increase in numbers reported at 24 hours and persisting to day 7 after stoke onset (Emsley, 2003) (see Table 6).

Peripheral Acute Phase Response in TIA

It is not known if the peripheral APR is present in TIA patients. TIA occurs in the context of the innate immune response readiness. Blood vessel endothelium is activated by risk factors for stroke (i.e., hypertension). Effector cells are available in adequate numbers to effect cytokine production, diapedesis, migration to areas of potential issue injury, initiation of complement, and phagocytosis of neural membrane bound antigens, like myelin basic protein, for antigen presentation. Presence of cytokines and cytokine signaling occurs in human cerebral infarction. Cytokines and cytokine signaling may play a role in transient ischemia (TIA); this may be a neuroprotective mechanism. If the peripheral APR occurs in TIA then cytokine signaling may be present also.

Neural-immune activation via the SANS, either prior to stroke, or as a result of ischemia (stroke) suppresses both the innate and adaptive immune system responses, but to a greater degree, T cells (prominent effectors of the adaptive immune response). It is interesting to note that 12 hours after MCAO in animals, lymphocytes (B-, T-, & NK-cells) are suppressed and that this lasts for 7 days. If this occurs in TIA, then the suppression of the adaptive immune response via fewer numbers of lymphocytes may account for the window of ischemic tolerance. If this peripheral APR is present in TIA as is described in animal models of stroke, then it is possible that the suppression of the adaptive immune response to antigenic challenge (MBP usually seen by vigilant B- and T-cells in a competent immune system) may account for this window of neuroprotection. Since, blood cell phenotyping and the adaptive immune system is not the focus of this

study, but is a long-range goal of this line of research; a first step is needed to determine if the peripheral APR is present in TIA.

To summarize, in cerebral infarction, tissue damage occurs in the context of a suppressed adaptive immune response, and the APR is the dominant signaling mechanism. In transient ischemia, specifically clinical TIA, no cerebral tissue damage is seen. It is not known if the APR exists as a signaling mechanism in TIA. If there is evidence of the peripheral APR in TIA, then the APR may be a signaling mechanism that potentially has dual capabilities, of translating the signal for protection or for damage (see Figure 1).

Variables of Interest

Leukocyte count, differential counts, and absolute counts of neutrophils, lymphocytes and monocytes are the most often reported measures and these are routinely available in the clinical setting of stroke (Emsley, et al., 2003). WBCs of stroke patients are elevated on admission, at 24 hours, and through day 7 (Emsley, et al., 2003). Neutrophils account for the majority of the rise in WBC count (i.e., neutrophils are also elevated on admission and at 24 hours) after stroke onset (Emsley, et al., 2003). Monocytes are significantly elevated starting at 24 hours, with elevations lasting to day 7 (Emsley et al., 2003). Decreased lymphocyte subsets, Specifically, B-, T-, and NK-cells, reach the nadir by 12 hours and this lymphocytic depression lasts up to 5-7 days after MCAO in an animal model of stroke (Prass, et al., 2003). The peripheral acute phase response in stroke patients and animal stroke models mirrors the reports of the cellular level of signaling by acute phase cytokines (i.e., IL-1, IL-6, and TNF-alpha). The signaling done by IL-1, IL-6 and TNF-alpha occurs over a long time period and is

responsible for inflammation and repair processes in ischemic brain tissue. Investigation of these variables in TIA needs to be in relation to the reported time course of these variables in the context of cerebral infarction.

These measures are sensitive to a multitude of confounding variables, like concomitant infection, steroid and anti-inflammatory drug use, having had recent surgery, a cancer diagnosis, and chemotherapy. These confounding variables need to be controlled in a study related to the APR (Ferrarese, 1999). It is expected that the WBC count, neutrophil percent, monocyte percent, absolute neutrophil count, and absolute monocyte counts will be elevated on admission in stroke patients in this study. It is expected that the lymphocyte percent and the absolute lymphocyte count will be decreased on admission in stroke patients in this study.

Significance

The investigation of the acute phase immune response is pivotal in the study of the physiological events of TIA and Stroke. Laboratory investigation of neuroprotective agents and endogenously occurring mechanisms of ischemic tolerance have shown therapeutic potential, but translation to the clinical setting has been problematic (Wegener, et al., 2004). Researchers have called for investigation of the several candidate mechanisms of ischemic preconditioning as possible avenues of therapy that broaden the window of intervention beyond the initial 3 hours, which is the currently accepted time frame for administration of tPA. It is well known that this narrow window of therapy with tPA is problematic with approximately 5% of the people presenting to the emergency department eligible to receive this drug (Barone and Feuerstein, 1999).

The inflammatory condition may contribute to the development of stroke or exacerbate ischemia in brain tissue. The summative report of the peripheral acute phase immune response in stroke is an important finding for clinicians. Currently, there are no direct measures of the cellular level biochemical events, or the cytokine signaling of the acute phase response in the clinical setting. Therefore, in the clinical setting the evolution of the stroke deficits, serial CT scan, and MRI imaging are the primary assessments of brain injury due to ischemia. There is little variation in any of the laboratory findings in stroke, and the only variations of laboratory findings in stroke relate to the stress response (i.e., the above described peripheral acute phase response and elevations in glucose). The development of therapies based on ischemic preconditioning needs to be tied to the clinical course of stroke, specifically, screening for risk factors, recognition of risk factors, identification of high-risk co-morbidities, and evidence of evolving disease. Timing of new therapies needs elucidation as well, in the care delivery model (Barone and Feuerstein, 1999).

To date, in TIA the peripheral APR has not been reported in a clinical setting. There is evidence that signaling by the acute phase cytokines and a peripheral immune response in animal models of ischemic preconditioning is important to the model of ischemic tolerance. Yet, no investigation has occurred of the peripheral APR, or the acute phase cytokine contribution to ischemic tolerance in TIA patients. The proposed study is a novel first step in describing the evidence of the peripheral APR in human transient ischemia. It is important that this description be defined by its comparison to the peripheral APR response in human stroke (see Figure 1). The pattern of the peripheral APR in TIA and stroke over time is foundational to the investigation of the

APR as a candidate mechanism of neuroprotection. This study is the first report of the peripheral APR in human transient ischemia.

Purpose

The purpose of this study was to explore whether there is a peripheral APR in TIA patients.

Specific Aims and Research Question

Aim 1: To determine if there is sufficient evidence to conclude that the peripheral APR exists in transient ischemic attack patients on admission.

1. On admission, is the WBC count of TIA patients significantly different from the mean of the laboratory normals of 7.8 K/UL?
2. On admission, is the neutrophil % of TIA patients significantly different from the mean of the laboratory normals of 52.5%?
3. On admission, is the lymphocyte % of TIA patients significantly different from the mean of the laboratory normals of 35%?
4. On admission, is the monocyte % of TIA patients significantly different from the mean of the laboratory normals of 6%?
5. On admission, is the absolute neutrophil count of TIA patients significantly different from the mean of the laboratory normals of 4.45 K/UL?
6. On admission, is the absolute lymphocyte count of TIA patients significantly different from the mean of the laboratory normals of 2.7 K/UL?

7. On admission, is the absolute monocyte count of TIA patients significantly different from the mean of the laboratory normals of 0.6 K/UL?
8. On admission, is the WBC count of Stroke patients significantly different from the mean of the laboratory normals of 7.8 K/UL?
9. On admission, is the neutrophil % of Stroke patients significantly different from the mean of the laboratory normals of 52.5%?
10. On admission, is the lymphocyte % of Stroke patients significantly different from the mean of the laboratory normals of 35%?
11. On admission, is the monocyte % of Stroke patients significantly different from the mean of the laboratory normals of 6%?
12. On admission, is the absolute neutrophil count of Stroke patients significantly different from the mean of the laboratory normals of 4.45 K/UL?
13. On admission, is the absolute lymphocyte count of Stroke patients significantly different from the mean of the laboratory normals of 2.7 K/UL?
14. On admission, is the absolute monocyte count of Stroke patients significantly different from the mean of the laboratory normals of 0.6 K/UL?

The evidence is considered sufficient if there are significant differences from normal laboratory values in the TIA patients on admission. Additionally, the following exploratory aims were investigated.

Exploratory Aim 2: To determine whether patients with TIA have the same peripheral APR as the patients with Stroke.

Research Question. On admission, what is the main effect of group (TIA versus Stroke) on the 7 dependent variables related to the acute phase response, the WBC count, neutrophil %, lymphocyte %, monocyte %, absolute neutrophil count, absolute lymphocyte count, and absolute monocyte count?

Exploratory Aim 3: To determine whether patients with TIA have the same peripheral APR as the patients with stroke over time.

Research Question. What is the group (TIA versus Stroke) by time (admission and 24 hours) interaction effect on the 7 dependent variables related to the acute phase response, the WBC count, neutrophil %, lymphocyte %, monocyte %, absolute neutrophil count, absolute lymphocyte count, and absolute monocyte count?

CHAPTER 3

Method

Design

In order to identify the presence of the peripheral APR in the TIA and stroke population, this descriptive cross-sectional two-group study was conducted. A two-tier retrospective chart review of incident hospitalized patients admitted with first time TIA, and first time stroke from January 1, 2000 to August 31, 2005 was done. This time frame was used because older records were from patients being cared for outside of the Neurologist's oversight in a burgeoning stroke program. These charts were reviewed for the clinical markers of the peripheral APR and a description and analysis was done to compare this phenomenon in these two study groups (i.e., TIA versus Stroke).

The design employed is classified as cross-sectional because the identification and classification of cases for the two groups (i.e., TIA patients and stroke patients charts) was done simultaneously to the collection of data on the dependent variables of interest (i.e., WBC count, neutrophil %, lymphocyte %, monocyte %, absolute neutrophil count, absolute lymphocyte count, and absolute monocyte count) (Gordis, 2004).

Setting

Rogue Valley Medical Center is a 305-bed regional medical center in southern Oregon. All laboratory analyses and diagnostic testing was done on site. The radiologist and the neurologist read all head CT scans. Both the admitting physician and the neurologist, if consulted, had dictated history and physicals on the chart. The emergency medical services and the emergency department physician's records were retained in the medical record.

Sample

The sample for this study was drawn from the medical records of adults admitted to Rogue Valley Medical Center between January 1, 2000, and August 31, 2005, who have experienced stroke or TIA. Since this was the first study to describe the presence of the APR in TIA, and the two studies of the APR in stroke reported statistics related to risk stratification, insufficient data was available to conduct a power analysis and determine optimum sample size a priori. The sample was a referent group for incidence of TIA and stroke in the population. Since, the population from which hospitalized incident cases were a part cannot fully be described, it was appropriate to compare two cohort groups from the same hospital, because these likely come from the same population. Therefore it was assumed that factors affecting referral, transport, admission, emergency department care, and inpatient care were similar between and within the groups; bringing homogeneity to the demographic data. Thus, the differences found on the dependent variables were more readily attributed to the different study groups (i.e., TIA versus Stroke) (Gordis, 2004). The advantage of this design is that using one hospital's data controls for these factors, but the disadvantage is that it limits the generalizability of the findings.

Sampling Procedural Analysis

Sampling Procedure

Sampling had two tiers, an electronic tier and a manual tier. The electronic search schemes identified a large set of medical records for retrospective review by the researcher using the electronic search scheme (N=1041). A manual review followed using the inclusion and exclusion criteria. Determination of case inclusion and exclusion,

and assignment to study groups was done according to the definitions of qualifiers presented in Table 11. Prior to the review process an expert reviewer (i.e., Neurologist) and the primary researcher met and discussed the definitions of the study group categories and the variables of interest.

Electronic Search Scheme. The electronic search method was explored by Coull, Silver, Bull, Giles, and Rothwell (2004) and found to lead to near-complete ascertainment of stroke cases. Two electronic search schemes were done to identify two separate lists (i.e., one for TIA and one for stroke) of possible medical records for review. Separating the electronic searches early in the search process helped to separate the cohort groups for inclusion and exclusion by manual review. Many TIA cases were admitted as stroke cases, but as neurological deficits resolved quickly, the patients were then discharged as TIA cases. Electronic search criteria for identifying charts of patients with TIA included: 1) primary admission diagnosis of TIA, or stroke, 2) primary discharge diagnosis of TIA or stroke, and 3) discharge diagnosis of stroke with an admission diagnosis of TIA. Electronic search criteria for identifying charts of patients with ischemic stroke included: 1) primary admission diagnosis of stroke, 2) primary discharge diagnosis of stroke, and 3) primary discharge diagnosis of stroke with an admission diagnosis of TIA. The electronic query for stroke charts was limited with the following two qualifiers: 1) no hemorrhagic stroke, and 2) no history of stroke. Both electronic searches included the strategy of 'discharge diagnosis of stroke with an admission diagnosis of TIA', as many patients were admitted with improving neurological deficits and the diagnosis of stroke is made 24 to 48 hours after admission when either the neurological deficits did not resolve, or serial brain imaging showed

evidence of stroke. There exists a known difficulty in coding the admission and discharge diagnosis of TIA and stroke; therefore no patients were excluded from the electronic query for the possible charts of patients with stroke or TIA. This identified 1041 cases in the medical record database.

Case Selection and Classification into Study Groups. Case lists were generated for TIA and Stroke admissions as described. There were a total of 1041 admissions with 208 admissions coded as TIA and 833 admissions coded as stroke in the hospital database. One hundred forty-eight replicate listings of coded admissions were excluded from the initial 1041 coded admissions to produce 893 total discrete first-time admissions for TIA (n = 125) and Stroke (n = 768) patients during the study period.

Cases were classified as TIA if in the discharge diagnosis on the admission sheet or in the discharge summary was listed as TIA, there was no evidence on head CT or MRI scan report of stroke, area of ischemia, ischemic changes, infarction, old stroke, stroke of in determinant age, and the neurological deficits lasted less than 1 hour. Cases were classified as stroke if in the discharge diagnosis on the admission sheet, or in the discharge summary their discharge diagnosis was listed as stroke; there was evidence on head CT or MRI scan of acute stroke, area of acute ischemia, ischemic changes, infarction, and that neurological deficits lasted longer than 1 hour. Cases included in the stroke group, did not have words referring to an old stroke, or stroke of in determinant age on their head CT or MRI scan report. All TIA and stroke event cases were then pulled for review, and explored for inclusion and exclusion, which is summarized in Table 12.

Manual Review and Inclusion Criteria. Adult patients over 21 years of age with TIA (n = 125) or stroke (n = 768) were included as cases for manual review for inclusion. Cases included patients that were: 1) over 21 years of age, 2) first time TIA or ischemic stroke, 3) admitted and have their admission labs drawn within 12 hours of onset of symptoms, 4) have no evidence of previous stroke on CT or MRI scan, 5) afebrile and infection free, 6) not diagnosed or have a history of cancer, 7) not presently taking steroids, non-steroidal anti-inflammatory drugs, or immune suppressive drugs, and 8) not recovering from a recent surgery or procedure.

Manual Review and Exclusion Criteria. For the patients included in the TIA and stroke groups, any head CT scan evidence of an old stroke were excluded, n = 44 and n = 253 excluded, respectively. Patients with history of TIA or stroke in the TIA and stroke group were excluded, n = 26 and n = 84 excluded, respectively. A proportionally larger group of patients in the stroke group presented after having symptoms lasting longer than 24 hours prior to admission (n = 147) than TIA patients whose symptoms occurred greater than 24 hours prior to admission (n = 12).

Patients who were admitted with, or developed in the first seven days of hospital admission: fever, concomitant atelectasis or pneumonia on chest X-ray, rales, and evidence of urinary tract infection (i.e., urinalysis positive for bacteria, protein, RBC's, WBC's, or cloudiness) were excluded (for TIA n = 6; and for stroke n = 81). This was evidence that there may be an impending bacterial or viral infection that would affect the WBC count, the differential count and the absolute counts of the WBC subsets. Further if the patient was on non-steroidal anti-inflammatory drugs, steroids, immune suppressive

drugs, was a cancer patient, or had a recent surgery or procedure in the last 4 weeks were excluded (for TIA n = 17; and for stroke n = 110) (Ferrarese, 1999).

Patients on non-steroidal anti-inflammatory drugs may have had a pre-existing inflammatory condition that may have affected the primary variables of interest (i.e., the WBC count, the differential count and the absolute counts of the WBC subsets). Steroids mimic a cortisol type stress response in the body and suppress the immune system affecting the WBC count and the neutrophil count predominantly. Cancer patients and patients on immune suppressive drugs also have suppression of selected WBC cell subsets. Patients who have had recent surgery have undergone a strong cortisol driven stress response and are in the process of healing; both processes dependent on ongoing inflammatory responses affecting the primary variables of interest (Ferrarese, 1999).

Final diagnosis of medical other included patients who were admitted for neurological deficits that were attributed to seizures, electrolyte imbalances, and drug overdose (TIA n = 4; and stroke n = 17). Hemorrhagic stroke was noted in one TIA case and in 16 stroke cases and these were excluded. There was missing laboratory data for three TIA cases and 14 stroke cases; and additionally there were three missing medical records in the stroke group for the listed admission on the electronic search lists. The application of the inclusion and exclusion criteria resulted in 12 adult first-time TIA cases and 43 adult first-time stroke cases.

Inter-rater Reliability and Verification of Data Plan.

Case Inclusion and Exclusion Reliability. The Neurologist reviewed 5% of the total case charts selected by electronic search, after duplicate medical record numbers were deleted, on a random basis for reliability of inclusion and exclusion of cases

utilizing the described criteria. There was 100% agreement on the inclusion and exclusion of these randomly selected cases.

Case Assignment to Study Groups Reliability. The researcher and Neurologist had less than satisfactory agreement of included TIA and stroke cases in their assignment to study groups, 78.57% and 81.25%, respectively during a preliminary review of the case assignment process. The discrepancy was related to duration of symptoms and CT scan findings. At this point, all included cases were scrutinized for duration of symptoms and CT scan findings again by the researcher. This resulted in a re-classification of study cases by the researcher, based on criteria outlined by the reviewing Neurologist (for TIA n = 125; and for stroke n = 768). The researcher pulled all final included charts for review by the Neurologist for a second time at the end of the manual review process. This review resulted in a 100% consensus of assignment of cases to each study group. The researcher sent the data for the sample of 12 TIA cases and 43 stroke cases to the data analyst for data verification.

Data and Analysis Verification. The data analyst reviewed and compared the data in the charts, data collection sheets and database as described. There was 100% agreement between the chart and the data collection form and between the data collection form and the database. This process resulted in 3 checks of the data: 1) entry by the researcher, 2) a crosscheck by the analyst, and 3) a final check by the researcher after the analyst had verified the data. All analyses were run twice and the results compared.

Variables for Study

The charts were manually reviewed for the following data on the variables for description of the study sample, determination of study inclusion and exclusion, and the

primary dependent variables. The variables for the description of the study sample included: 1) demographics (i.e., age, gender, and ethnicity), 2) medical history (i.e., diabetes, smoking, hypertension, myocardial infarction, and atrial fibrillation), 3) length of stay, 4) neurological deficits (i.e., motor, speech, cognitive, and visual deficits), 5) duration of symptoms and 6) diagnostic results (i.e., carotid ultrasound studies, and echocardiography). The variables for inclusion and exclusion of cases included: 1) diagnostic results (i.e., CT scans, MRI scans, chest X-rays, and urine analysis, both microscopic and microbiological), 2) body temperature, 3) history of TIA or Stroke, and 4) concomitant medications confounding primary outcome variables, specifically on admission and during hospitalization. The variables for assignment of cases to the independent variable of study group included: 1) neurological deficits (i.e., motor, speech, cognitive and visual deficits), 2) duration of symptoms, and 3) diagnostic results (i.e., CT scans, and MRI scans). The date and times of lab work were collected to determine timing of the results in relation to the onset of signs and symptoms of TIA and stroke.

Measurement

Measurement of Primary Dependent Variables of Interest

Clinical evidence of the peripheral APR were measured by the following dependent variables: WBC count; differential counts (i.e., percentages of 100 cell counts for neutrophils, lymphocytes, and monocytes); and absolute counts of neutrophils, lymphocytes, and monocytes. The Coulter® GEN•S™ System measured these variables. Admission data on the primary outcome variables of interest were designated as time 1

data, and the data on these same variables at 24 hours after admission were designated as time 2 data.

Rationale for the Selection of Measures. Primary outcome variables were chosen to reflect the peripheral measures of the APR. Based on the data from laboratory and clinical studies of the peripheral APR in stroke, the variables were chosen that were measured clinically on a routine basis.

The peripheral APR needs to be tied to the cellular physiology that is being elucidated by laboratory research. Several disciplines involved in the care of the TIA and stroke patients monitor these variables, and it would be pertinent to describe the peripheral APR as foundational to any planning of an intervention that required initiation of therapy based on these measures or required monitoring of these measures in a clinical setting. Multiple factors influence the variability of these measures (i.e., infection, fever, diagnosis of cancer, steroid use, chemotherapy, and use of non-steroidal anti-inflammatory drugs) and these were controlled for in the exclusion criteria of the sampling scheme.

Utilizing the differential proportional count is problematic to report because changes in one cell percentage will change all the other cell proportions in the sample report (Freedman, Flanders, Barboriak, Malarcher, and Gates, 1996). The differential count then is correlated within the sample due to the proportionality of the numbers (Freedman, Flanders, Barboriak, Malarcher, and Gates, 1996). Therefore the differential count will not reflect true proportion of the total count of subtypes of white blood cells, whereas the absolute count is in relation to the total population of white blood cells (NCCLS, 1997). Absolute counts of neutrophils, lymphocytes and monocytes are

reported as a product of the total WBC count and the proportional differential count. Coulter® GEN•S™ System did this calculation (Coulter® GEN•S™ Manual, 1988).

Clinical Lab Standards. The Clinical and Laboratory Standards Institute formerly the National Committee for Clinical Laboratory Standards have produced the guidelines "Reference Leukocyte differential Count (Proportional) and Evaluation of Instrumental Methods; Approved Standard" (1997) that governed the laboratory at the study site. Although, clinicians use the differential count, which is a proportional report of the number of neutrophils, lymphocytes, monocytes, eosinophils, and basophils in a sample of 100 cells, there is some difficulty with this measure when used for rigorous reporting. Therefore, the laboratory at the study site reported both the proportional count and the absolute count of these cells, as the absolute count would be the preferred method of reporting in the future (NCCLS, 1997).

Coulter® GEN•S™ System. The use of the automated Coulter counter system improved precision over the results that have been previously performed by hand counting and classification (NCCLS, 1997). The automated system had the ability to flag for review any samples that have abnormal or difficult to classify cells. To reduce subjectivity in this measurement, the automated counts were used. Whole blood samples collected according to the guidelines were prepared by the automated system with numerous sub-samples within the sample, as repeated sampling is needed within one sample to generate the most precise automated counts (Coulter® GEN•S™, 1988). The published ranges for the Coulter® GEN•S™ by the company are listed is Table 7.

The intra-assay statistics were determined on 31 replicate determinations of stabilized blood control material and normal whole-blood specimens, using repeated

counting of 80,000 cells to determine the leukocyte counts and 8,000 cells to determine the proportional counts. The automated range of intra-assay precision for the proportional counts was reported in standard deviation units, range 0.12-1.22, the intra-assay precision within run is listed in Table 8. The inter-assay statistics were determined on 189-paired samples, using repeated counting of the same number of cells as described above. The automated mean difference range for the inter-assay accuracy was –1.06-1.93, the inter-assay accuracy is listed in Table 9. Additionally, the automated coulter counter flags a sample when the accuracy tolerance limits exceed ±3.0% mean difference for WBC count, lymphocyte and monocyte proportion; ±2.0% mean difference for neutrophils, and ±1.0% mean difference for eosinophil and basophil proportions. The laboratory and the Coulter® GEN•S™ System did not have published inter- and intra-assay statistics for the absolute counts that were generated by this Coulter counter.

Both the intra-assay and the inter-assay standard deviations were small which denote stable robust measures that were appropriate for statistical analysis. It was assumed that variability of these measures would reflect random variability in the human presentation of the acute phase response to an ischemic event, and not systematic error of measurement.

Clinical laboratory policy for defining and verification of the population reference ranges that are used at the study site were determined initially on more than 120 samples from healthy outpatient volunteers (Montgomery & Groshong, 1993). The data was plotted to determine normality of distribution. If normal distribution was established, the mean and standard deviation (SD) were calculated. Any values outside of 3 SDs from the mean were eliminated and the mean and SD was again calculated. The new reference

range was based on this second calculation and reflects the mean +/- 2 SDs. Review of reference ranges is done annually on a minimum of 120 healthy outpatient volunteers (Montgomery, 1998). Normal reference ranges used by the study site for the dependent variables are listed in Table 10.

Sample Collection and Processing. Whole venous blood was collected in purple top tubes containing tripotassium ethylenediamine tetra-acetate (K_3EDTA) 1.5 ± 0.15 mg (in liquid or powder form) per mL. Blood was collected by skin venapuncture or by removal of blood specimens from angiocatheter-based intravenous lines. Specimens were rejected for specimen analysis if macroscopically visible clots were seen, or the automated machine detected microscopically visible platelet clumps.

Institutional Review Board Approval

No personal identification was used on the data collection tools. The data was coded for data verification at a later date. The OHSU IRB approved this study with oversight waived to the Southern Oregon IRB, which oversees research at Rogue Valley Medical Center. All data and coding was kept confidential and no publication of results of this study identified the medical record or the patient.

Analysis Plan

Description

The collected data was managed in the database component of and analyzed by SPSS version 11.5. Descriptive statistics were used to describe the sample on the variables of age, gender, ethnicity, medical history, length of stay, duration of symptoms (on TIA sample only), CT scan findings, other diagnostic tests (i.e., carotid ultrasound), and neurological exam. The data on the 7 dependent variables (i.e., the WBC count,

neutrophil %, lymphocyte %, monocyte %, absolute neutrophil count, absolute lymphocyte count, and absolute monocyte count) was checked for normal distribution using histogram graphing.

Preliminary Analysis. The TIA and stroke groups were compared on the descriptive variables for differences that might influence the dependent variables. Independent sample two-tailed t-tests were conducted on age, duration of symptoms, and length of stay. Chi-square tests were conducted on gender, ethnicity, medical history, CT scan findings, other diagnostic tests and neurological exam. Level of significance was set at $\alpha = 0.05$.

After this preliminary analysis covariates emerged. Age, history of hypertension, high cholesterol, diabetes and smoking may affect the independent variable of group membership, with the stroke having older patients with more risk factors, or more severe co-morbidities. This may be a function of the evolution of cerebrovascular disease in this group. Age, gender, history of diabetes, and smoking may affect the dependent variables of the peripheral acute phase response. Some of these variables had differing impact on either, or both the independent variable and the dependent variables (Tabachnick and Fidell, 2001). Only smoking status emerged as a covariate.

Analysis for Aim 1

One sample two-tailed t-tests with a significance level of $\alpha = 0.05$ were done to test the research questions for Aim 1. The TIA patient's data on the 7 dependent variables as reported on the automated CBC were compared to the means of the normal range for the same 7 dependent variables published by the clinical lab of the study site, see Table 10. A significant t-value indicated that the TIA group was significantly

different from the mean of the laboratory normals on the dependent variable of interest. A significant t-value was sufficient evidence to conclude that the peripheral acute phase response exists in TIA patients on admission, and further analyses of the exploratory Aims were undertaken.

Analysis for Exploratory Aim 2

To determine whether patients with TIA have the same peripheral acute phase response as the patients with stroke, Multivariate Analysis of Covariance (MANCOVA) was used. MANCOVA was an appropriate method of analysis for the research question posed for investigation of aim 2 because the statistical test determined if the categorical independent variable, group membership (TIA versus stroke), accounted for differences in the 7 continuous data dependent variables (listed in the analysis for Aim 1 section) controlling for key covariates (Tabachnick and Fidell, 2001). After the preliminary analysis was completed, one covariate emerged, smoking, and a Multivariate Analysis of Covariance (MANCOVA) was used (Tabachnick and Fidell, 2001). A significance level of $\alpha = 0.05$ was set for both the MANOVA and the MANCOVA.

Examining the omnibus tests associated with the MANCOVA assessed if the groups differed on a linear combination of the dependent variables. If significant, further assessment of the individual dependent variables was done with univariate ANCOVA. This second step determined which dependent variables (listed in the analysis for Aim 1 section) differed between the study groups (TIA versus stroke) (Tabachnick and Fidell, 2001).

Analysis for Exploratory Aim 3

To determine whether patients with TIA have the same peripheral acute phase response as the patients with Stroke over time, MANCOVA was proposed. This statistical techniques allows for interaction terms to be used to test group (TIA versus stroke) by time (admission, Time 1; and at 24 hours, Time 2) interaction, and also allows for inclusion of control of the main effects of group, and/or time (Tabachnick and Fidell, 2001). However, exploratory Aim 3 was not undertaken due to too few cases having time 2 data on the 7 dependent variables of interest (i.e., n = 0 for TIA and n = 18 for Stroke). This would have resulted in insufficient data in the MANCOVA or ANCOVA cells for the comparison of admission and 24-hour data on the 7 variables, both between and within groups.

Assumptions for MANCOVA

The assumptions of MANCOVA are that the observations be independent; that the observations are normally distributed in the population; that there is linearity of the dependent variables, the covariates and the dependent variable/covariate pairs; and that there is homogeneity of variance (French, Poulsen, & Yu, 2002). The assumptions of MANCOVA were met with a priori planning. First, the observations were independent due to the persons being separate and that there were no people included in both groups. When testing for the assumption of normal distribution, it was expected that the differential percentages of the WBC subsets were not normally distributed. However, it was expected that the absolute counts of the WBC subsets were normally distributed. Testing for normal distribution (i.e., using histograms) and presence of skewing was done prior to data analysis and no skewing was found. (Tabachnick and Fidell, 2001).

Homoscedasticity and linearity of the relationships among all pairs of the dependent variables, covariates, and the covariate and dependent pairs were tested with scatterplots of these data (Tabachnick and Fidell, 2001). Testing for the assumption of homogeneity of variance (i.e., that the variance in the groups are equal to the variance in the population) (and covariance if applicable) was done by checking for the equality or inequality of variance in the MANCOVA tables. (Salkind, 2000; Tabacknick and Fidell, 2001)

Significance Level

An alpha (α) of .05 means that there was a 5% chance of committing a Type I error, rejecting the null hypothesis when it was true (i.e., conclude that the groups were different, when in fact they were not different) was appropriate for the majority of the analysis. The univariate ANCOVAs may have needed an adjustment of the α level dependent on the number of dependent variables (Tabachnick and Fidell, 2001, p. 348). This is done by dividing the multivariate α by the number of dependent variables, which results in all α's being equal, or adjusting for the importance of the dependent variable by giving more important variables more liberal α's (Tabachnick and Fidell, 2001, p. 349). Since this was a small study of this phenomenon, and it was estimated that there would be little power, this adjustment would be too severe, and would lead to dismissal of possible main effects. Thus, adjustment of the alpha level was not done.

Computation of Effect Size

Computation of effect sizes and a power analysis was conducted for use in the future planning of prospectively designed studies of this phenomenon. Effect size is a measure of the magnitude of effect. The effect size converts data to standardized units

for comparison between studies. An effect size of .2 means that the mean of the intervention group's distribution is .2 standard deviation units above the mean of the control group's distribution. Meaning, 58% of the comparison group (i.e., stroke group) would be below the mean of the group in which we are testing for an effect (i.e., TIA group) (Cohen, 1988). The determination of clinical significance was explored in the discussion of the results of this study with clinical experts. For Aim 1, effect sizes were calculated by hand using the formula below and categorization of effects sizes were based on Cohen's (*d*) effect sizes (1988). Cohen defined the effect sizes as follows: small ES = 0.2, medium or moderate ES = 0.5, and large ES = 0.8 (1988). For this study, effect sizes greater than 1.0 are described as substantial, and effect sizes less than 0.2 are described as miniscule.

$$\text{Cohen's } d = M_{\text{Study Group}} - M_{\text{Laboratory Normal}} / \sigma_{\text{Laboratory Normal}}$$

For the alternate analysis of Aim 2, effect sizes were calculated using the web-based effect size calculator at the University of Colorado at Colorado Springs (2000). This calculation is based on Cohen's d equation below. Categorization of effects sizes were based on Cohen's (*d*) effect sizes (1988).

$$\text{Cohen's } d = M_1 - M_2 / \sigma_{\text{pooled}}, \text{ where } \sigma_{\text{pooled}} = \sqrt{[(\sigma_1^2 + \sigma_2^2) / 2]}$$

The power analysis focused on determination of power on univariate t-tests, and ANCOVA, and for the multivariate MANCOVA.

Power Analysis and Estimated Sample Sizes

Power was calculated using a web-based calculator from the statistics department at University of California, Los Angeles (2005). For Aim 1, power calculations were based on one-sample normal distribution equations with the laboratory mean used for the

null hypothesis and the sample means for the alternate hypotheses, and the laboratory standard deviation for the standard deviation of the population. For the alternate analysis of Aim 2, power calculations were based on two-sample unequal variance equations utilizing the TIA and stroke group's means and standard deviations appropriately.

Sample sizes were estimated using a Web-based calculator, the Power Calculator from the statistics department at University of California, Los Angeles (2005). These estimates were based on a significance level of 0.05; and a power of .8 using a two-tailed approach. An estimation of sample size based on the observed effect size for a power of 0.8 was calculated for all non-significant findings.

CHAPTER 4

Results

Sample

Demographics

The retrospective non-probability sample was predominately Non-Hispanic White (98.2%), male (61.8%), and had an average age of 66.44 years. This weighting of the ethnicity of the sample reflects the population at large (i.e., non-Hispanic white 88.7%, Hispanic 6.3%, Asian 2.3%, and African American 0.6%) in the Southern Oregon catchment area (www.Oregon.gov, 2004).

History of hypertension was the predominant cerebrovascular risk factor (70.9%), followed by currently smoking status (27.3%), history of myocardial infarction (16.4%), and diabetes (14.5%). There were no significant differences between groups on all demographic data, although these tests had low power associated with them, with the exception of smoking status. The TIA group had the most non-smokers, 66.7%, versus 44.2% in the stroke group, $p = .168$. In the TIA group there were no current smokers, but current smokers comprised 34.9% of the stroke group, $p = .016$. ' Currently smoking' status was investigated as a covariate in Aim 2 of this study. Demographic characteristics are summarized in Table 13.

Hospital Course

The neurological exam on admission revealed a significant difference between TIA and stroke groups with the stroke group having motor deficits more often reported, $p = .003$. All other exam findings were not significantly different between study groups. The descriptive data related to the hospital course is summarized in Table 14.

CT scan findings are reported for the difference between the reports of stroke versus no stroke on admission. None of the TIA cases and 65.1% of the stroke cases had evidence of stroke on the admission CT scan. This finding is consistent with the inclusion criteria. TIA cases had almost double the reports of small vessel disease on admission CT scan than stroke cases, but this was not statistically significant.

Duration of symptoms and length of stay were significantly different between groups, $p < .001$. This is a reflection of the TIA neurological deficits by definition lasting less than one hour and resulting in the patient going home sooner as the patient's function has returned to baseline.

Aim 1

T-tests

To test that there were no differences between the means of each study group (i.e., TIA and Stroke), and the laboratory means on the 7 dependent variables of interest (i.e., WBC count, proportional counts of neutrophils, lymphocytes, and monocytes; and the absolute counts of neutrophils, lymphocytes, and monocytes), a one sample, two-tailed t-test was used. The analysis for Aim 1 is summarized in Table 15.

The null hypotheses were rejected for the differences between the sample and laboratory means on proportional counts of neutrophils, lymphocytes, and monocytes; and absolutes count of lymphocytes in both the TIA and Stroke groups. Additionally, the null hypothesis was rejected for the differences between the sample and laboratory means in the absolute count of neutrophils in the Stroke group. No significant differences were found for WBC counts, absolute count of monocytes in both study groups, and in the absolute count of neutrophils in the TIA group.

Effect Sizes and Power

Substantial effect sizes were noted in the difference between the means for proportional counts of neutrophils (i.e., for TIA = 1.76 and for stroke = 1.42), and lymphocytes (i.e., for TIA = -2.76 and for stroke = -2.05); and the absolute count of lymphocytes (i.e., for TIA = -1.61 and for stroke = -1.01) and the laboratory means in both study groups, see Table 15. There was adequate power (i.e., all observed power statistics were = 1.0) to detect the substantial effect sizes greater than 1.01. A large effect size was noted for the proportional count of monocytes in the TIA group (i.e., ES = .90), moderate effect sizes were noted in the difference between the means for proportional counts of monocytes, and the laboratory means in the stroke group (i.e., ES = .57), and between the means for the WBC count and the laboratory mean in the TIA group (i.e., ES = -59). The power was adequate in the stroke group (power = .95), but was less than adequate in the TIA group (power ≤ .63) to detect these effects. Small effect sizes were noted in the difference between the means for the absolute count of neutrophils and absolute count of monocytes of the TIA group (i.e., ES = .26 and -.27, respectively), and the absolute count of neutrophils in the stroke group (i.e., ES = .48), and the means of the laboratory normals. These analyses were underpowered to detect a small effect size. The miniscule effect size for the stroke group for the difference between the means for WBC count (i.e., ES = .007), and absolute count of monocytes (i.e., ES = 0) when compared to the laboratory mean implies that these differences are probably not clinically significant.

Estimated Sample Sizes

Sample sizes were adequate for the rejection of the null hypotheses for the proportional counts of neutrophils, lymphocytes, and monocytes; and absolute count of

lymphocytes in the Stroke group; and for proportional counts of neutrophils, lymphocytes and absolute counts of lymphocytes in the TIA group. Estimated sample sizes for the stroke group suggest that the non-significant differences in these variables from the laboratory mean are probably not worth further investigation in future studies. These estimates are summarized in Table 16. In order to detect all small to large effect sizes, the estimated sample size for the TIA group would be 119. The small to moderate effect sizes are probably worth further investigation in future studies with adequate sample sizes, as these differences may be clinically significant.

Exploratory Aim 2

Multivariate and Univariate Analysis of Variance and Co-variance

The results of the MANOVA and MANCOVA using the 7 dependent variables of interest are summarized in Table 17. The initial MANOVA, which included all 7 dependent variables of interest, was not statistically significant. A MANCOVA including all 7 dependent variables of interest with current smoking status added as a covariate was also not statistically significant. These results provide evidence that the peripheral APR as an immune profile in TIA and stroke are clinically similar.

Alternate Analysis of Aim 2

T-tests, Effect Sizes and Power. Alternate analysis of the differences between the study group means of the 7 dependent variables of interest was done utilizing a two-tailed independent sample t-test with a significance level of 0.05. This alternate analysis of Aim 2 is summarized in Table 18. Differences between the study group means on 6 dependent variables of interest were not statistically significant. There was a statistically significant difference between study group means on absolute counts of lymphocytes, p =

.039. However, this analysis approach does not control for the experiment-wise Type I error rate.

A moderate effect size was noted between the study groups on the absolute count of lymphocytes and monocytes, ES = -.72 and ES = -.50 respectively. Small effect sizes were noted between the study groups on WBC count, ES = -.45; and the proportional counts of neutrophils, ES = .27; lymphocytes, ES = -.32; and monocytes, ES = .21. All analyses were underpowered to detect these effect sizes, range of power .07 to .63. The miniscule effect size, ES = -.17, noted between the study groups on the absolute count of neutrophils reflects the fact that these differences are probably not clinically significant.

Estimated Sample Sizes

Estimated sample sizes were computed for TIA and Stroke samples based on a significance level of 0.05, and a power of 0.8. These estimates based on the study group's means and standard deviations of the 7 dependent variables of interest are summarized in Table 19.

Estimated sample sizes for WBC count, proportional counts of neutrophils and lymphocytes, monocytes and absolute neutrophils and monocytes would optimally be 560 per group for a power of 0.8. Sample sizes in the range of 80 to 560 are feasible in a clinical setting, if the setting is a large Primary Stroke Center, or a multi-center consortium with yearly admission rates are 200 cases and above per center.

Secondary Analysis of the Effect of Smoking Status on Monocyte Percent

The effect of smoking status on the proportional count of monocytes on admission was analyzed using currently smoking versus not currently smoking as the independent variables. Those currently smoking (n = 15) had a proportional monocyte count (mean =

6.80, SD = 1.66), which was less than those currently not smoking (n = 40; mean = 8.18 and SD = 1.85). This yielded a statistically significant mean difference of 1.38%, p = .015. Smoking accounted for 9.6% of the variance of the proportional count of monocytes by simple regression analysis, adjusted $r^2 = .096$, p = .015.

CHAPTER 5

Discussion

Conclusions

The major findings of this study are: 1) the peripheral APR exists in TIA patients, 2) the peripheral APR in TIA and stroke patients is similar with the exception of the absolute count of lymphocytes, 3) there is evidence of clinically significant decreased proportional counts of lymphocytes in TIA and in stroke, 4) there are moderate effect sizes for the differences between TIA and stroke patients on WBC counts and absolute counts of monocytes, and 5) in this study current smokers had lower absolute counts of monocytes than current non-smokers. The scope of this study provided the findings for hypothesis generation, which can later be further refined and tested in appropriately powered prospective studies.

Peripheral APR exists in TIA patients. This is the first report of the peripheral APR in TIA patients. The previously reported peripheral APR in stroke was hypothesized to be present due to the ischemic damage of stroke and subsequent inflammatory reaction to this damaged tissue. This study provides evidence that the peripheral APR exists in the absence of residual tissue damage associated with ischemia. Not only did the peripheral APR exist in the context of transient ischemia, but it was also similar to the peripheral APR in permanent ischemia (i.e., stroke). The finding that the peripheral APR exists in both of these contexts generates the hypothesis that the APR, cytokine or peripheral, may be a signaling mechanism that potentially has dual capabilities of translating the signal for protection or damage.

The mean time for the duration of symptoms for the TIA group was 21.6 minutes (i.e., 0.36 hours), and all cases with duration of symptoms greater than one hour were classified as a stroke, as per the proposed definition of TIA by Albers, et al., (2002). This study may provide more questions regarding the definition by Albers et al. (2002) that still has a time-based context, that the neurological deficits last less than one hour. There is evidence in the literature suggesting that necrosis and apoptosis occur simultaneously in cells within the ischemic area (Unal-Cevik, Kilinc, Can, Gursoy-Ozdemir, and Dalkara, 2004). This evidence was found in the context of transient ischemia (i.e., animal model with 30 minutes of transient ischemia, followed by 72 hours of reperfusion) and permanent ischemia (i.e., animal model with 72 hours of permanent MCAO). This avenue of research suggests that physiological mechanisms leading to cellular death can occur in the time frame proposed by Albers, et al. It is possible that transient ischemia in the absence of tissue damage may be less than the 30 minutes used in the study by Unal-Cevik, Kilinc, Can, Gursoy-Ozdemir, and Dalkara. More research regarding cellular damage and death sub-routines needs to be done to outline the time frame for non-damage producing transient ischemia.

It is possible that the mean time of 21.6 minutes of transient ischemia really reflects damage-producing ischemia and that the peripheral APR is due to tissue damage. Moncayo, de Freitas, Bogousslavsky, Altieri, and van Melle, (2000) showed that the time frame to confer ischemic tolerance is between 10 and 20 minutes after onset of symptoms in humans. The mean time for this study lies just outside of this reported range. A study that had a large enough sample could be stratified for different durations of symptoms to

determine if the peripheral APR exists in all contexts of ischemia, and if this phenomenon is associated with the time frame that confers ischemic tolerance.

Confounders were controlled for in this study by the strict exclusion criteria. The criteria were not violated so that there was a minimization of the impact of confounders on the findings related to the outcome variables of interest. This is a major strength of the study design, which increases the believability that the findings were not spurious. The exclusion of patients with concurrent signs and symptoms of infection, immune suppression and steroid use strengthened the emergence of the plausible pathophysiology, which was the mechanism of interest in this study. This explicit exposure of the peripheral APR in both the TIA and stroke sample was achieved by strict control of confounders to the dependent variables of interest, and supports the validity of the results (Gordis, 2004). However, at the same time the application of strict exclusion criteria limits the generalizability of the findings to a larger population of TIA and stroke patients.

Clinically, few patients are hospitalized for TIA. It is common for patients to ignore transient symptoms and merely report these to their healthcare provider at the next convenient office visit. Many studies in the literature that explore phenomenon in TIA state that there is a known difficulty in obtaining a sufficient sample size in this population. Two examples in the literature, the European Atrial Fibrillation Trial Study Group (EAFT, 1993); and Johnston and the Kaiser Northern California TIA Group (2000), used multi-institutional designs to get an adequate sample size. These two studies explored treatment for stroke risk reduction and short-term risk of stroke after TIA in TIA populations. The EAFT study enrolled 1007 patients over 43 months (3.6

years) from 108 centers in 13 countries, with an average of 9.3 patients per center (range 1-49) equaling 2.6 patients per year per center. The Kaiser Northern California TIA study identified 1707 patients in 16 hospitals over 1 year, with an average of 106.7 patients per center. Neither of these used electronic databases other than to generate the case lists. Neither study used the stringent exclusion criteria that were needed in this study to maintain a rigorous sample of patients without confounding variables to the 7 dependent variables of interest in this study. This study included 12 TIA cases over 5.67 years averaging 2.12 cases per year. This number is only slightly less than the EAFT study that mobilized 108 centers to yield their sample. Further, physiological studies done in a clinical setting do have sample sizes less than 35 (Zapoliska-Downar, Naruszewicz, Zapolski-Downar, Markiewski, Bukowska and Millo, 2000; Bergman, Siekmeier, Mix and Jaross, 1998).

The number of stroke cases was considerably higher over the study time period, but many were excluded due to previous evidence or history of stroke, and concomitant infection. A recent research report by Marquardt, Ruf, Mansmann, Winter, Buggle, Kallenberg and Grau (2005) investigated the inflammatory response after stroke using 50 patients, 30 age and gender matched controls, and 20 risk factor matched controls. These researchers excluded patients with recent trauma, surgery, and organ ischemia within 3 months prior to admission; severe liver disease, renal failure, cancer, chronic inflammatory disease and fever, or infection at the time of study entry (i.e., admission to the hospital). These researchers chose to leave in their analysis 8 participants with signs and symptoms of infection during the week prior to admission and 22 participants who developed fever or signs and symptoms of infection on at least one measurement during

the study. Had the 30 people with signs and symptoms of infection been excluded the stroke study group sample size would have been smaller than in this study's stroke group, N=20 and N=43, respectively. This study emphasizes the issues related to obtaining an adequate sample size when studying the immune response in the context of humans with stroke and TIA.

The final sample sizes did not provide adequate power in all comparisons to detect significant differences in the variables of interest between the TIA group, and the stroke group and the laboratory mean, and between the two study groups. The small sample size that resulted from the various exclusions that were applied weakened the generalizability of the findings. The small sample sizes can be considered a major limitation to this study.

Incident hospitalized cases exclude those who died en route to hospital, or as an outpatient, or who were not brought to the hospital because they were treated in the primary care setting and returned home. These incident cases are moderately severe in the context of this study, as the mildly impaired may not have come to the hospital setting for treatment and those who were critical died outside of the hospital. Additionally, inaccurate coding of charts might yield a less representative sample of incident cases. This is the second major limitation of this study. A priori planning for a broad electronic search conducted early in the selection of cases for each cohort in order to be as inclusive of all medical records yields the best ascertainment of cases (Coull, et al., 2004). Additionally, many specialists admit patients to the hospital with TIA or Stroke (i.e., Primary Care Physicians, Internists, Family Practitioners; and Emergency Department physicians in conjunction with Hospitalists). Because of the differing levels of expertise

in the diagnosis and care of the patients with TIA and stroke, a neurologist reviewed the selection of cases, the inclusion and exclusion of cases; and the assignment of cases to study groups. Inter-rater reliability for inclusion and exclusion, and classification of cases were good, which strengthen the reliability of the findings of this study.

The quality and the extent of information may be limited by missing data, the timing of lab work, the completeness of the history and physical, and the discharge summary being dictated by a variety of physician sub-specialties, and CT scan reporting done by multiple readers. This is a known limitation of clinical studies of this type and impacts the interpretation and generalizability of this study's findings (Gordis, 2004).

In this study, causality was not determined due to the measures of the variables of interest being distal to the intracellular events, and the cross-sectional design negating any determination of a temporal relationship. This study is unable to elucidate alternate explanations for this phenomenon (i.e., that the observed peripheral APR exhibits a dose-dependent relationship to the amount of ischemic stress, or antecedent stress). This study was not designed to determine causality, as this was a first step in the process to describe the peripheral APR in transient ischemia. This study represents the first evidence on the description of the peripheral APR based on study group membership.

With some variables, the sample size was adequate to produce the power needed to detect the corresponding effect size. Determination of the estimated sample sizes was part of the scope of this study and provides data on which to base future research on this phenomenon. It is likely that given adequate sample size (N=560 per group) the TIA and stroke group may have emerged with statistically significant differences on the 7 dependent variable so interest, but these smaller sample sizes may have avoided making a

finding statistically significant when it is not significant clinically (Salkind, 2000; Tabacknick and Fidell, 2001). The findings that the variables of interest were different from the laboratory mean denotes the direction of the shift in differential counts of the WBCs, but the finding that the proportional count of lymphocytes was out-of-range of normal, lower than the lowest cut off for the normal range may be clinically significant. This is a major finding in this study.

Multivariate analysis of variance and covariance showed no statistically significant effect of group (i.e., TIA versus Stroke) on the 7 dependent variables of interest. However, the alternate analyses of group differences, which did not control for experiment-wise Type I error, showed the means of the absolute count of lymphocytes were significantly different between groups at a significance level of .05. Had we adjusted for the number of tests performed this would not have been a significant finding. This has not been previously reported in the literature. It is interesting to note that the absolute lymphocyte count in the TIA group was significantly lower than in the stroke group. This may be evidence that the sympathetic autonomic nervous system suppression of lymphocyte centers in lymphoid tissue was unopposed by a competing mechanism for lymphocytes to be elevated, in the TIA group. Whereas in the stroke group, the higher absolute lymphocyte counts may be a reflection of a competing mechanism to elevate lymphocyte number in response to cytokine production secondary to an incompetent blood brain barrier and exposure of brain tissue antigen.

The Peripheral APR in TIA and Stroke is Similar. All comparisons of the 7 dependent variables of interest with the laboratory means were in identical directions for both the TIA and stroke groups (i.e., elevated neutrophils, decreased lymphocytes, and

elevated monocytes) that were the hallmarks of the previously described peripheral APR. Additionally, there were significant differences from the laboratory means for the proportional counts of neutrophils, lymphocytes and monocytes in both study groups. There was sufficient power in both groups to detect these large effect sizes. To date, the literature has only stated that monocytes were activated in the context of stroke, or stroke animal models, which infers that their surface receptors are specifically different than in the inactive state, or that fewer monocytes are measured in the blood due to their extravasation to the tissue and conversion to macrophages. This report includes data on both proportional and absolute counts because clinicians commonly use the proportional counts in decision-making.

Finding the mean of the WBC count not significantly different from the mean of the laboratory normals is inconsistent with the report by Emsley, et al. (2003), who found the WBC counts to be elevated on admission. This difference is interesting to note due to the exclusion criteria being different in this study from the criteria used in Emsley's study. Emsley excluded patients with evidence of malignancy, but 8 patients developed infections in the first week after admission and an additional 3 patients had inflammatory responses prior to admission (i.e., 1 with myocardial infarction 5 days prior to admission and 2 with infection with in 6 weeks prior to admission). These 11 patients constituted 31% of Emsley study sample on admission. Whether Emsley recruited first ever stroke patients in unclear and this may have influenced the difference between Emsley findings and this report.

The findings from this study are consistent with Vila (2000) who used similar inclusion and exclusion criteria (i.e., first ever stroke, with exclusion of patients with

concomitant infection, malignancy, using anti-inflammatory drugs, etc.) to this report and found that in the stroke group the mean of the WBC count was within the range of normal, and was not significantly different from the mean of the laboratory normals.

Other findings consistent with Emsely, et al. were the significantly higher means of the absolute count of neutrophils and monocytes than the means of the laboratory normals in the stroke group. This was not found in the TIA group. The effect sizes in both groups were small to non-existent in the stroke group and the TIA group, respectively.

Clinically Significant Decreases in Proportional Counts of Lymphocytes. The means of the proportional count of lymphocytes and the means of the absolute count of the lymphocytes were significantly lower than the means of the laboratory normals in both the TIA and stroke groups; and there was sufficient power to detect these substantial effect sizes. This was the only variable to be outside of the range of normal in this study. Proportional counts of lymphocytes have not recently been reported in the literature, this may reflect the move to report the absolute counts in the research literature. These findings are consistent with the report by Prass, et al. (2003) that lymphocytes subsets (i.e., B-cells, T-cells and NK-cells) are decreased maximally at 12 hours after onset of stroke in an animal model of permanent ischemia. Inference must be made from the report by Prass, that the total counts, and proportional counts of lymphocytes would be decreased as well.

Moderate Effects Sizes Found for Differences between TIA and Stroke Patients. There were moderate effect sizes noted for the differences between the WBCs and the absolute monocytes between the TIA and the stroke groups. Had the study had an

adequate sample size then these findings may have been significant. However, the moderate effect sizes, or the differences in the WBC count between groups may be a reflection of the differences between the study groups on the absolute counts of neutrophils, lymphocytes, and monocytes. And the moderate effect sizes between the absolute counts of monocytes may reflect the influence of current smoking status on the monocyte counts of the stroke group. Although the smokers had lower absolute monocyte counts, the literature holds that the monocytes are activated, and there was an overall finding that the monocytes were elevated in stroke to a greater degree than in TIA. Elucidation of these correlations needs to be undertaken in an adequately powered study of these variables in TIA and stroke populations.

The findings do provide evidence that the peripheral APR in TIA does have the same direction of change in the peripheral immune profile as previously reported in studies of the peripheral APR in stroke. These previous reports in stroke were summarized by Barone and Feuerstein (1999), and were reported by the work of Hallenbeck (1997) and his associates, del Zoppo, et al., (2001), Siren et al., (2001), and Emsley, et al., (2003). Further, in this study the lymphocyte depression early after the ischemia event is greater in first-time TIA than in first-time stroke.

Current Smokers had Lower Proportional Counts of Monocytes. An additional confounder, emerged as a covariate, and was identified during the analysis of the demographic data. This included current smoking status, which can possibly affect the inflammatory response, or the magnitude of the APR. It is interesting to note that the stroke group included all the current smokers in this study. When currently smoking status's effect was investigated as a covariate that influenced monocyte percent, it

contributed to only a small portion of the variance in monocytes. The means of the monocytes in the smokers was less than the mean of the monocytes in the non-smokers. This is consistent with Vayssier-Taussat, Camilli, Aron, Meplan, Hainaut, Polla and Weksler (2000) who reported that smoking decreases or detrimentally interferes with oxidative metabolism in monocytes resulting in fewer monocytes in circulation. Or, that in the context of stroke smoking has pre-activated monocyte integrins and the monocytes then migrate and attach to the cytokine-activated endovascular epithelium ICAMs, thus reducing the number of circulating monocytes (Berman, Siekmeier, Mox and Jaross, 1998; Berliner, et al., 1999). In either case, circulating monocytes are reduced, which is consistent with the findings of this study.

The overall influence of smoking on the variance in monocyte percent was small. This finding supports the proposal that monocytes are, for the major part, influence by the signal of the APR in ischemia, and only to a minor degree by the covariance of smoking in these populations.

The scope of this study did not explore the clinical significance of the peripheral APR to outcome, or presence of subsequent infection beyond 7 days. Additionally, this study did not explore the risk stratification of subsequent stroke following TIA based on inflammatory markers. Nor did this study explore the presence of the peripheral APR antecedent to the TIA event, as a risk factor for ischemia as outlined by Grau, et al. (2004). Further, it was clearly not in the scope of this study to investigate the links between the cytokine APR and the peripheral APR. Lastly, this study did not explore the whether the peripheral APR is evidence of the conference of tolerance and provision of

neuroprotection. This study provided a critical first step in these preponderances, and serves only to aid in hypothesis generation.

Implications for Practice

As in the study by Prass, et al. (2003) lymphocyte depression early after the onset of stroke like symptoms may increase the risk of infection after TIA as in stroke. The outcome of infection after TIA is difficult to determine in a retrospective design, where the length of stay is so short that infections due to the phenomenon are not evident prior to discharge from the hospital. Since the neurological deficits resolve quickly risk of aspiration pneumonia exist only during the symptomatic episode complicated by dysphagia. Care should be taken to protect the airway during the period that the patient has a swallowing sensorimotor deficit in the context of TIA as in stroke. Teaching staff about this risk, and teaching patients who have frequent TIA episodes involving swallowing difficulty, not to feed anything by mouth until this deficit can be evaluated could potentially reduce aspiration pneumonia as a recurrent complication in both the TIA and Stroke populations.

Implications for Future Research

Two major hypotheses were generated from the results of this study. First, there is no difference in the signal pathophysiology of the peripheral APR in TIA and stroke. Both activate the innate immune system (i.e., elevate neutrophils and monocytes) and both suppress the adaptive immune system (i.e., decrease lymphocyte numbers). Implied in this hypothesis is that the clinically decreased lymphocytes may confer ischemic tolerance and account for the 7-10 day window of neuroprotection. Second, if the peripheral APR reflects tissue damage, then there is no difference between the context of

tissue damage in stroke and TIA. Implied in this hypothesis is that transient ischemia with symptoms lasting less than 30 minutes (i.e., duration of symptoms in this study was 21.6 minutes) may be associated with brain tissue damage.

To test the first hypothesis that there is no difference in signal pathophysiology and both TIA and Stroke result in clinically significant adaptive immune suppression a prospective multi-institutional trial needs to be undertaken. The target variables for the acute phase signal pathophysiology would be: 1) the acute phase cytokines, IL-1, IL-6, TNF-alpha, 2) the peripheral APR response as outlined in this study, and 3) the acute phase reactants, CRP, MBL, and fibrinogen. To illustrate that the sympathetic ANS is stimulated would require serial determinations of epinephrine and nor-epinephrine. To test that stress is translated by the sympathetic ANS stimulation to lymphoid tissue would require lymphocyte phenotyping of T and B cells. Lastly, to detect immune system suppression would require elicited white blood cell responses, and a reduced response to application of antigenic vaccine.

To test the second hypothesis, that the peripheral APR reflects tissue damage, and that there is tissue damage in both TIA and stroke would require a prospective multi-institutional trial over approximately 2 weeks using biomarkers of tissue damage. This would require measurement of the peripheral APR, serum proteomic determination of a biomarker of brain tissue damage (e.g., myelin basic protein, or p- or e-selectin), and brain imaging that can be quantified to detect blood brain barrier competence and ischemic damage.

In closing, this study provided new information on the existence of the peripheral APR in the context of transient ischemia, infers that there may be a common

pathophysiological mechanism (i.e., possibly a signal) in stroke and TIA in humans, and generates new avenues for future research.

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Appendix I. Cerebral Ischemia and Infarction

Stroke Definition. Stroke is defined as a clinical syndrome of rapidly or gradually developing signs and symptoms that coincide with neurological deficits focal to a known vascular territory (Warlow, 1998). When these symptoms are persistent, lastly longer than one hour, the neurological deficits persist and brain imaging usually shows an area of brain infarction (Albers, 2004).

Types of Stroke. There are two broad categories of stroke, ischemic and hemorrhagic. Hemorrhagic stroke has two subtypes, intracerebral hemorrhage, and subarachnoid hemorrhage accounting for 9% and 3% of all strokes, respectively. Ischemic stroke accounts for 88% of all stroke types. There are five subtypes of ischemic stroke, atherothrombotic, cardioembolic, lacunar, crytogenic, and other (includes vasospasm, arteritis, and altered coagulation) accounting for 20%, 20%, 25%, 30% and 5% of all strokes, respectively (AHA, 2005).

Signs and Symptoms. Stroke signs and symptoms include: sudden onset headache, blurred vision, difficulty speaking or understanding speech, numbness or weakness in the face, arm or leg on one side of the body, and dizziness (AHA, 2005). The stroke deficits sometimes evolve over 3 days (i.e., worsen) and then become less pronounced indicating that tissue repair may be taking place in the area surrounding the infarction (i.e., penumbra) or that brain edema in the area of ischemia is resolving (Dirnagl, Iadecola, and Moskowitz, 1999).

Risk Factors for Stroke. Risk factors for Stroke have been identified and include hypertension, diabetes, high cholesterol, smoking, overweight and obesity, physical inactivity, atrial fibrillation, and transient ischemic attack (TIA) (AHA, 2005). Primary

prevention of stroke is commonly enacted through stringent risk factor management (AHA, 2005).

Tissue Context of Ischemia

Stroke risk factors (i.e., hypertension, diabetes, advanced age and genetic predisposition to stroke) provide a context of tissue vulnerability linked to a modified Shwartzman reaction, which includes blood vessel activation, thrombogenesis, ischemia in a vascular territory, but not microvascular hemorrhages characteristic of the Shwartzman reaction (Hallenbeck, et al., 1988; Hallenbeck, 1997).

Early Blood Vessel Activation. Blood vessel activation involves the inflammatory response at the level of the blood vessel endothelium. Hypertension promotes activation of vascular endothelium, activation of monocytes and monocyte adhesion to cerebral vascular endothelium (McCarron, Wang, Siren, Spatz, & Hallenbeck, 1994; Liu, Liu, McCarron, Spatz, Feuerstein, Hallenbeck and Siren, 1996). Effective levels of tumor necrosis factor-alpha (TNF-alpha), and Interleukin-1 (IL-1) induces an inflammatory process where thrombogenesis can take place (Goetz, 2003; Mergenthaler, Dirnagl and Meisel, 2004).

Thrombogenesis. The area of inflammation in the blood vessels in the brain provides the foundation for the accumulation of platelets, fibrin and the formation of a thrombus. Branching points in the cerebrovasculature are common sites for the development of atherosclerosis. During the process of atherosclerosis, sub-intimal layers of the blood vessel wall (i.e., fibrous and smooth muscle layers) overgrow and with foam cells induced by TNF-alpha, provide the environment for fatty plaques to form (Hallenbeck, 2002). The plaques themselves serve as a framework for platelets

adherence. Fibrin, and thrombin deposition ensues and clot formation occurs, ending in thrombus evolution. The thrombus itself can occlude the blood vessel, reduce local cerebral blood flow (CBF), and produce ischemia. Or the thrombus may break off, creating a mobile embolus that travels through the blood vessel in the microvasculature to a point where occlusion takes place (Goetz, 2003).

Ischemia Overview. Ischemia of the brain tissue distal to this point follows with irreversible ischemia at the infarction core. Ischemia results from the CBF decreasing to less than 30 ml/100gm/min (normal CBF = 30-50 ml/100gm/min) (Minhas, Menon, Smielewski, Czosnyka, Kirkpatrick, Clark, Pickard, 2003). Neurological deficits are apparent when CBF is less than 20 ml/100gm/min (Powers, Grubb, Baker, Mintun, Raichle, 1985). Due to the reduction in blood flow to the core, electrical activity changes (CBF < 20ml/100gm/min) (Powers, et al., 1985), cellular homeostatic metabolic processes deteriorate, cellular energy supply is depleted, and ion balance is disrupted due to ion pump failure (CBF = 10-12 ml/100gm/min), the endpoint of which is the resulting cellular death within minutes (i.e., necrosis) (CBF < 10 ml/100gm/min) (Minhas, et al., 2003; Powers et al., 1985; Sughrue, Mehra, Connolly Jr, D'Ambrosio, 2004).

Ischemia and Infarction Maturation. Just like in myocardial infarction, the area surrounding the infarction core is an environment of reversible ischemia, and in the context of brain tissue this is known as the penumbra. In this region some collateral blood flow is reserved, and the disruption of cellular homeostasis produces a stepwise cellular death, with approximately 50% of the penumbra going onto infarction (Mergenthaler, Dirnagl and Meisel, 2004).

Late Blood Vessel Activation. Later in the inflammatory response, selectins support adhesion of leukocytes to the endovascular epithelium and enhance diapedesis (Bevilacqua, 1993; Bevilacqua, Nelson, Mannori, & Cecconi, 1994; Okada et al., 1994). Endothelial cells have been established as expressing cell surface molecules P-selectin and E-selectin in response to cytokines, specifically IL-1 and TNF-alpha (Bevilacqua & Nelson, 1993). This results in the upregulation of both P-selectin and E-selectin (Barkalow, Goodman, Gerritsen, & Mayadas, 1996; Takeda et al., 2002). P-selectin and E-selectin may be functionally redundant in the interaction of leukocytes with the vascular endothelium during leukocyte trafficking (Labow et al., 1994). Within hours of ischemia intracellular adhesion molecules, ICAM-1 and the vessel wall, VCAM-1, moderate leukocyte interaction, leading to neutrophils accumulating in the microvascular circulation and further disrupting blood flow (del Zoppo, 1991).

Monocyte activation predispose brain blood vessels to further thrombogenesis and hemorrhage (Siren et al., 2001). Leukocytes expressing IL-1beta, IL-6, and TNF-alpha, the major pro-inflammatory cytokines, infiltrate around cerebral blood vessels, ventricles, and meninges on histological examination of neurologically impaired rats (Siren et al., 2001). Il-6 acts with TNF-alpha, and IL-1 to increase acute phase protein production by the liver (Janeway, 2001). Acute phase proteins act to promote inflammation systemically (Janeway, 2001). The expression of IL-1, IL-6 and TNF-alpha and the resulting effects on the liver and peripheral immune response is known as the acute phase response (APR) (Baumann & Gualdie, 1994, Janeway, 2001). This peripheral acute phase response is thought to upregulate the body's capability to mount an inflammatory

response of longer duration with all pertinent effector cells mobilized (i.e., neutrophils and monocytes) (Baumann & Gualdie, 1994).

Blood Brain Barrier (BBB). Soon after onset of ischemia the BBB opens due to induction of matrix metalloproteases (MMPs), which breakdown the cellular matrix of the basal lamina between the endothelial cells of the blood vessel, and the podocytes of the astrocytes. TNF-alpha has been implicated in activating MMP and increasing vascular permeability (Hallenbeck, 2002). The immune privileged area of the brain tissue is now open to the immune system, allowing immigration of leukocytes, and promotion of vasogenic edema. The expression of MMP has been correlated to the risk of hemorrhagic transformation of the ischemic infarction. Inhibition of MMP reduces damage to the BBB and infarction size (Mergenthaler, 2004).

Cellular Excitotoxicity. Local cellular energy deficits result in depolarization of neurons and glia. This depolarization activates voltage dependent calcium channels; excitotoxic glutamate is released from the cell. Glutamate, directly or indirectly increases the influx of calcium into the cell through activation of its receptors (i.e., NMDA- and AMPA-). Stimulation of these receptors also allows influx of sodium and chloride into the cell, which results in further disturbance of ionic homeostasis, intracellular and tissue edema. These massive ionic shifts lead to osmotic lysis (i.e., necrosis). This process of excitotoxicity may be the initial step in induction of inflammation and apoptosis (i.e., programmed cell death, or delayed cell death). Peri-infarct depolarizations are seen in the penumbra, with propagation of waves away from the core several times an hour (Dirnagl, Iadecola, and Moskowitz, 1999). These depolarizations further stress the

deteriorating metabolic processes of the penumbra and lead to infarct maturation of this region (Mergenthaler, 2004).

Failing Cellular Metabolism and Necrosis. In the area of ischemia there is elevated glucose in the tissue in a context of hypoxia, this results in lactate production leading to intracellular acidosis. Therefore, cellular injury due to acidosis indirectly results from oxygen deprivation. With out oxygen, ATP dependent cell functions cease, like oxidative metabolism, resulting in increasing intracellular acidosis, which further inhibits mitochondrial function. Hypoxia may impair reuptake of glutamate, a pre-synaptic neurotransmitter resulting in excitotoxicity, perpetuating the process described earlier. This excitotoxicity opens Na+, Cl- and Ca++ channels increasing the influx of calcium into the cell through stunned opened calcium channels. With intracellular swelling ensuing, concurrent increased intracellular calcium dependent degradation enzymes accumulate to further inhibit mitochondrial activity. The summation of these events reduces ATP manufacture. A reduction of ATP affects protein synthesis, which is needed to maintain cellular viability (Dirnagl, 1999; Mergenthaler, 2004).

Reperfusion Injury. Ischemia, followed by reperfusion, produces increased reactive free oxygen radicals (i.e., superoxide, peroxide, and hydroxyl ions) intracellularly. Nitric oxide synthase (NOS) activation produces a highly reactive peroxy-nitric radical from the product of superoxide and nitric oxide. These radicals can destroy many intracellular components (i.e., carbohydrates, proteins, DNA and phospholipids). Free oxygen radicals also promote the destruction of mitochondrial ATP processes by assisting in the formation of the mitochondria permeability transition pore

(MPTP). The mitochondria swell, lyse, and release molecules leading to apoptosis (Mergenthaler, 2004).

When perfusion to the area is re-established the build up of ADP and pyruvate from the cessation of oxidative metabolism steals hydrogen ion from the cell membrane and deforms neuronal cells. This is known as reperfusion injury (Mergenthaler, 2004).

Cellular Death, via Apoptosis and Necrosis. Stepwise cellular death refers to the early necrosis, already described, and the later occurring apoptosis and necrosis sequences that are described here. Extrinsic activation of apoptosis via the FAS-receptor and Fas-ligand interaction induces intracellular caspases. Additionally, cytokines can activate caspases. Caspases are a family of resident intracellular proteins that are inactive until induced to initiate cascades of intracellular protein cleavage mediated by caspase-activated DNAse (CAD), which leads to cleavage of DNA into 200 base-pairs ladders characteristic of apoptosis. The cytosolic protein, cytochrome c, depends on the Bcl-2 protein family pro-apoptotic proteins (i.e., Bid, Bax, Bad, and Bag) for induction. Intracellularly following a signal from Fas-receptor stimulation, Bid, Bad, or Bax move to the mitochondrial membrane and initiate the MPTP, cytochrome c is released, which destroys the mitochondria leading indirectly to cellular death by necrosis (Dirnagl, 1999; Janeway, 2001; and Mergenthaler, 2004). Complement has also been identified marking neurons for apoptosis (D'Ambrosio, Pinksy, and Connolly, 2001).

Infarction Size and Associated Markers. Evidence suggests that CSF markers (e.g., myelin-basic protein, [MBP], and IL-6) vary with specific neurological pathology (Matias-Guiu et al., 1986; Strand, Alling, Karlsson, Karlsson, & Winblad, 1984). Increased IL-6 production occurs predominantly in areas of neuronal cell loss (Clark, et

al., 2000). More recently, CSF and serum IL-6 has been associated with infarct size in stroke in mice (Clark, 1997). Leukocytes, specifically monocytes, macrophages, TH2 cells and stromal cells of the bone marrow; microglial cells, astrocytes, endothelial cells and exercising muscle cells secrete IL-6 (Clark, 1997; Steensberg, et al., 2000; Perini, et al., 2001). It is unknown whether IL-6 produced from different tissues is functionally redundant in the process of inflammation and the resultant systemic effects.

Cytokines and Control of the Inflammatory Response. It is known that elevated IL-6 precedes the secretion of IL-10, a dominant anti-inflammatory cytokine that serves to regulate the inflammatory response by inhibiting TNF-alpha and the production of cytokines form TH1 cells, and the expression of cyclo-oxygenase-2 (Perini, et al., 2001). IL-10 levels are low in stroke patients and may indicate that the anti-inflammatory response is down regulated in acute stroke (Perini, et al., 2001). In animal models, IL-10 reduces infarction size in focal stroke (Spera, Ellision, Feuerstin & Barone, 1998). IL-10 confers neuroprotection through its anti-inflammatory effects (Spera, et al., 1998). Development of therapies that increase or maintain normal IL-10 production might benefit people at risk for stroke (Perini, et al., 2001).

Immunological tolerance. Cytokines, like IL-10, may be important in the "immunological tolerance of cerebral ischemia", which is induced by MBP, and other immunogenic proteins. This immunological tolerance is mediated by lymphocytes that express transforming growth factor-β1 (TGF-β1). Cerebral immune tolerance is thought to confer protection for up to 1 month. Microglial cells are the primary immune effector cell in the immune privileged brain tissue. Microglial cells are capable of producing pro-inflammatory cytokines and chemokines. Following transmigration of monocytes from

the blood to the brain tissue, a portion of these cells differentiates into microglial cells within 14 days. Additionally, astrocytes are capable of Janus-like roles by producing proinflammatory cytokines and inducing neuroprotective factors (i.e., erythropoietin, and TGF-β1) (Mergenthaler, 2004).

Appendix II.

Proteins of Interest

Proteins	Secreted from	Action
IL-1α; IL-1β	Macrophages; epithelial cells	Produces fever; induces T cell activation; macrophage activation
IL-1ra	Monocytes; macrophages; neutrophils; hepatocytes	Antagonizes IL-1; actively binds to IL-1 receptor and blocks action
IL-6	Macrophages; T cells; endothelial cells	Induces acute phase response; possibly anti-inflammatory
IL-8	Damaged tissue	Chemokine; Target Tissue: Neutrophil; basophil; T cell
IL-10	Macrophages; T cells	Potent suppressor of macrophage function; inhibits cytokine release
TNF-α	Macrophages; NK cells; T cells	Local inflammation; endothelial cell activation; induces NO production
TGF-β	Monocytes; T cells	Inhibits cell growth; anti-inflammatory; inhibits activation of macrophages; activates neutrophils
Bcl proteins	Bcl-2 genes in nucleus	Small family of genes that promote or prevent cell death; Bad, Bax, Bid promote cell death
C1q	Collectin family protein	Ca^{++} dependent sugar binding protein; binds sugar on pathogen surface; 1st complement component of Classical pathway; binds to Antigen:Antibody complexes & pathogen surfaces; key effector between innate & adaptive immune systems
MBL	Liver; found in serum	Mannan binding lectin; binds to mannose residue on pathogen; opsonization; activation of complement by MBL pathway
CRP	Liver	Pathogen recognition molecule; opsonization; activation of complement by binding to C1q
Fas-receptor; Fas-ligand	T cells; stroma	Tumor Necrosis Factor family of proteins; Binding with Fas-ligand initiates apoptosis; Ca^{++} independent cytotoxicity

Appendix II. Continued

Proteins of Interest

Proteins	Secreted from	Action
Caspases	Found in cytosol	Related to cysteine proteases; in cascade for apoptosis via CAD (Caspase-activated deoxyribonuclease)
MBP	Myelinated cells	Cell surface antigen
NF-kappaB	Found in cytosol	Migrates to nucleus; induces transcription
IL-1; IL-6; TNF-α		Collectively is responsible for initiating Acute Phase Response by: 1. Inducing acute phase protein release from liver CRP, MBL; which opsonize and activate complement 2. Mobilize neutrophils to increase phagocytosis 3. Increases body temperature and the mobilization of fat and energy stores to support the increase body temperature 4. Stimulates the mobilization of dendritic cells to lymph nodes to initiate adaptive immune response

*Apoptotic program present in all cells; absence of survival signaling or presence of death signal by Fas will initiate cascade.

Table 1.

Decreased Cerebral Infarction Size and Ischemic Preconditioning/TIA - Human

Citation	Design	IP Stimulus Duration	Interval to Stroke	Additional Findings
Moncayo, 2000	Retrospective review N=2490 prospective stroke bank patients; N=293 TIA in 3 groups IV: groups based on duration of TIA DV: infarct volume; outcome	TIA 10-20 minutes	Unknown	↑ favorable outcome
Castillo, 2003	Prospective N=283 Stroke N=38 w/ TIA IV: group DV: infarct volume; Barthel Index	TIA	w/in 72 hrs	↑ favorable outcome
Sitzer, 2004	Retrospective review N=4969 Prospective stroke bank; N=332 w/ prodromal TIA IV: group DV: infarct volume; Barthel Index, Modified Rankin Score	TIA	Unknown	TIA pts, had a 2.76 times higher chance of another TIA than a stroke
Wegener, 2004	Retrospective review N=65 Prospective stroke bank; N=16 w/ prodromal TIA IV: group DV: perfusion maps, CBF, CB volume, infarct size	TIA	Unknown	TIA pts that went on to stroke had small infarct volumes; diffusion- and perfusion weighted MRI study

Table 2.

Candidate Mechanisms for Ischemic Preconditioning

Mechanism	Authors
Neural Cell Membrane	
activation of NMDA receptors	Nishio, 1999
activation of adenosine triphosphate-regulated potassium channels	Xiong, 2003
mediation of NF-κB via erythropoietin receptor activation	Dawson, 2002; Prass, 2003
inhibition of brain nor-epinephrine	Nellgard, 1999
regulation or modulation of ion channel or intracellular ion function	Dhodda, 2004
Transcription and Translation	
induction of early immediate genes	Tang, 2003
induction of apoptosis suppressor genes	Gillford, 2004
induction of transcription factors, hypoxia-inducible-factor-1 (HIF-1)	Prass, 2003
induction of growth factors: TGF-β1; VEGF	Dhodda, 2004; Sun, 2005
de novo protein synthesis; including heat shock protein synthesis	Moncayo, 2000; Nishio, 1999; Barone, 1998 Masada, 2001; Xu, 2002; Gillford, 2004
Neural Cell Metabolism	
enhanced antioxidant activity	Dhodda, 2004
mitochondrial function preservation	Zhang, 2003
hypoxia, or hypothermia induced preconditioning	Dijkhuizen, 1998; Nishio, 1999; Miller, 2001

Table 2. Continued

Candidate Mechanisms for Ischemic Preconditioning

Mechanism	Authors
Inflammatory Response	
intracellular adhesion molecules; acute phase response	Hallenbeck, 2003
regulation of TNF-alpha to synthesize ceramide and induce hypoxic tolerance	Liu, 2000; Ginis, 2002

Table 3.

Decreased Cerebral Infarction Size and Ischemic Preconditioning (IP) – Animal

Citation	Design	IP Stimulus Duration	Interval to MCAO	MCAO Duration	Additional Findings
Matsushima, 1995	RCT N=36 in 4 groups Male Wistar rats	Global ischemia 30 minutes	4 days	180 minutes	No change in CBF
Nawashiro, 1997	RCT N= 61 in 10 groups BALB/C mice	TNF-alpha intracisternal Injection	48hrs	24 hrs	↓ infarct size in time- and dose-dependent manner
Spera, 1998	RCT N=27 in 3 groups SHR rats	IL-10 injection into ventricle 30 minutes and 3 hrs after MCAO		Permane nt MCAO	Both times protective & ↓ infarct volume
Barone, 1998	RCT N= 72; 7-9 per group SHR rats	Temporary MCAO 10 minutes	2,6,12 hrs &1,2,7,14, 21 days	PMCAO	↓reduced infarct size only from Day 1-7 after IP; IL-1ra & protein expression ↑ only during IT, day 1-7
Nishio, 1999	RCT N=45 in 7 groups Sprague-Dawly rats	Intra-ischemic hypothermia 20 minutes	6 hrs to 7 days	60 minutes	↓ infarct volume 24 hrs> 48 hrs> 6 hrs
Nellgard, 1999	RCT N=45 in 2 groups Male Wistar rats	Block NorEpi in brain w/ DSP-4 Infusion	3 days	75 minutes	↓ sub-cortical infarct

Table 3. Continued

Decreased Cerebral Infarction Size and Ischemic Preconditioning (IP) – Animal

Citation	Design	IP Stimulus Duration	Interval to MCAO	MCAO Duration	Additional Findings
Masada, 2000	RCT N=54 in 3 groups Sprague-Dawly rats	Thrombin Injection, 1u into caudate nucleus	7 days	Permanent MCAO	↓brain edema; improved neurological deficits
Miller, 2001	RCT N=25 in 2 groups New mouse model	Hypoxia, 11% oxygen 2 hrs	48hrs	90 minutes	
Mocco, 2002	RCT N=19 in 2 groups Non-human primate	Humanized E/P selectin immediately after onset of IP; Tolerized		Re-perfused ischemic stoke model	1 hr after ischemia, ↓ PMN infiltration in brain tissue; no systemic complement activation; + immune suppression

MCAO = Middle Cerebral Artery Occlusion
PMCAO = Permanent MCAO
TMCAO = Temporary MCAO

Table 4.

Cytokines and Acute Phase Response – Human

Citation	Inducer Design	Findings	Admission	24 hrs	48 hrs	Other time intervals
Ferrarese, 1999	Stroke, RCT N=55 stroke; N=20 controls IV: groups DV: IL-6, TNF-alpha cytokine release from peripheral blood cells	IL-6, TNF-alpha Induced by LPS		↑ ↑	↑ ↑	↑ at 1 mo ↑ at 1 & 3 months
Vila, 2000	Stroke First ever N=231 N=83 patients w/ neurological worsening IV: groups DV: neuro deficits; IL-6 CSF & plasma; TNF-alpha	IL-6 plasma IL-6 CSF TNF-alpha Associated with Neurological worsening & infarct size	↑ ↑ ↑			
Johansson, 2000	RCT N=8 men; N=4 women; N=10 controls IV: groups DV: levels & diurnal variations of plasma IL-6; TNF-alpha	IL-6 & cortisol		Correlation		
		TNF-alpha & Leptin		Correlation		
		IL-6 & paresis				Correlation Day 7
		IL-6 & MMSE		Correlation		Correlation Day 7

Table 4. Continued

Cytokines and Acute Phase Response – Human

Citation	Inducer Design	Findings	Admission	24 hrs	48 hrs	Other time intervals
Perini, 2001	Stroke RCT N=42 stroke N=39 controls IV: groups DV: IL-6; IL-10 on admission, day 1, 3, 7, and 14	IL-6	NS	NS	↑ peak 3 days	↑ day 7 > control & admission ↓ to day 14 peak day 7
		IL-10 + correlation between IL-6& IL-10 at day 3 IL-6 and IL-10 no difference between men & women	↓	↓ nadir	↓	↓ compared to controls
Sairanen, 2001	Stroke post mortem brain tissue RCT N=16 stroke; N=3 controls IV: groups DV: Neuronal TNF-alpha; Glial TNF-alpha; Astrocyte TNF-alpha; in situ cell death; peripheral blood TNF-alpha; CSF TNF-alpha	Neuronal TNF-alpha			↑ peak day 2-3	
		Astrocyte TNF-alpha				↑17-18 days
Emsley, 2003	Stroke RCT N=36 stroke N=36 controls IV: groups DV: CRP; ESR; WBC count; IL-6; cortisol; aural temperature; infection	IL-6	NS	↑		↑

Table 4. Continued

Cytokines and Acute Phase Response – Human

Citation	Inducer Design	Findings	Admission	24 hrs	48 hrs	Other time intervals
Vila, 2003	Stroke N=231 N=83 patients w/ neurological worsening IV: groups DV: IL-10; IL-4	IL-10 associated with neurological worsening IL-4 no association		↓		
Castillo, 2003	TIA & Stroke w/in 72 hrs Stroke w/o TIA Prospective N=283 Stroke N=38 w/ TIA IV: group DV: infarct volume; Barthel Index	TIA & Stroke w/in 72 hrs TNF-alpha IL-6 TNF-alpha/IL-6 index Stroke w/o TIA TNF-alpha IL-6 TNF-alpha/IL-6 index		 ↑ ↓ + - ↑ ∅		At 5-7 days ↓ infarct volume

+ TNF-alpha/IL-6 index = TNF-alpha >30pg/ml and Il-6<30pg/ml

Table 5.

Peripheral Measures of Acute Phase Response – Animal

Citation	IP Stimulus Design	Findings	Immediate	24 hours	48 hours	5-7 days
Prass, 2003	Stroke model 60 minutes MCAO RCT N=25; 4-14 in each group IV: group, RU486, inderal, IFN-gamma DV: cell phenotyping; TNF-alpha after LPS; IL-4; histopath & bacterial colonies of lung tissue; Lymphocyte subset count	B cells	↓ nadir at 12 hrs		↓	↓
		T cells	↓ at 12 hrs		↓	↓
		NK cells	↓ at 12 hrs		↓	↓

Table 6.

Peripheral Measures of Acute Phase Response – Human

Citation	Inducer Design	Findings	Admission	24 hrs	48 hrs	5-7 days
Vila, 2000	Stroke First ever N=231 N=83 patients w/ neurological worsening IV: groups DV: neuro deficits; IL-6 CSF & plasma; TNF-alpha	WBC count Associated w/ neurological worsening	↑; but NS			
Emsley, 2003	Stroke RCT N=36 stroke N=36 controls IV: groups DV: CRP; ESR; WBC count; IL-6; cortisol; aural temperature; infection	WBC count ABS Neutrophils Monocytes ESR Cortisol AT 3 months WBC count ABS Neutrophils Monocytes ESR Cortisol	↑ ↑ NS NS ↑	↑ ↑ ↑ NS NS		↑ ↑ ↑ ↑ ↑ ↑ ↑ NS ↑ NS

Table 7.

Whole Blood Reference ranges, Normal Population Study (N=240 samples, males and females)

Variable	Units	Mean	95% Confidence Low Limit	95% Confidence High Limit
WBC	x 10^3 cell/μL	6.11	3.58	11.07
Neutrophils	%	58.15	43.18	71.52
Lymphocytes	%	30.10	16.75	43.43
Monocytes	%	7.95	4.63	12.37
Eosinophils	%	2.74	0.70	7.8
Basophils	%	0.59	0.22	1.21
Neutrophils	x 10^3 cell/μL	3.58	1.86	7.17
Lymphocytes	x 10^3 cell/μL	1.81	1.10	2.74
Monocytes	x 10^3 cell/μL	0.48	0.29	0.84
Eosinophils	x 10^3 cell/μL	0.17	0.04	0.49
Basophils	x 10^3 cell/μL	0.04	0.01	0.08

Table 8.

Intra-assay Statistics on the variables of interest—Whole Blood

Variable	N= # of samples	Mean	CV%*	2 SD
WBC	31	6.87	1.13	
Neutrophils	31	51.09		1.22
Lymphocytes	31	37.07		1.09
Monocytes	31	8.23		0.78
Eosinophils	31	3.25		0.40
Basophils	31	0.35		0.12

*CV% = cell volume percent

Table 9.

Inter-assay Statistics on the variables of interest—Whole Blood

Variable	N= # of samples	Mean Difference	SD	Mean percent difference
WBC	256	0.12	0.26	1.93
Neutrophils	189	-0.72	3.2	NR
Lymphocytes	189	-1.06	3.22	NR
Monocytes	189	1.8	1.74	NR
Eosinophils	189	0.15	1.02	NR
Basophils	189	-0.16	0.53	NR

* the magnitude of the mean difference denotes accuracy

Table 10.

Automated CBC Normal Range, Male and Female Adults – RVMC

CBC Auto	Units	Mean	Range
WBC	K/UL	7.8	4.8 – 10.8
Neutrophils	%	52.5	35 – 70
Lymphocytes	%	35	25 – 45
Monocytes	%	6	0 – 12
ABS Neutrophils	K/UL	4.45	1.6 – 7.3
ABS Lymphocytes	K/UL	2.7	1.1 – 4.3
ABS Monocytes	K/UL	0.6	0 – 1.2

Table 11.

Criteria for Manual Inclusion and Exclusion; and Classification into Study Groups*

	TIA		Stroke	
	Included	Excluded	Excluded	Included
Medical History				
History of TIA		x	x	
History of Stroke		x	x	
Neurological Exam				
Duration of Symptoms				
Transient	x		x	
Less than 1 hour	x		x	
Motor deficits > 1 hr		x		x
Sensory deficits but improving < 12 hours as only symptom	x		x	
Aphasia Only Expressive or Receptive <24 hrs	x		x	
Impaired Swallowing > 1hr		x		x
CT/MRI scans				
Negative for Stroke or normal	x			x
Old Stroke		x	x	
For TIA only; later admission in chart shows second CT or MRI that shows Old Stroke from TIA admission		x		
Stroke age indeterminate		x	x	
Stroke acute		x		x
Ischemic changes		x		x
New Infarction		x		x
Chest X-ray				
Clear	x			x
Infiltrates		x	x	
Areas of Atelectasis		x	x	
Areas of Consolidation		x	x	
Pulmonary Edema		x	x	
Coarse or Fine Breath Sounds on exam		x	x	

*From discrete electronically derived chart lists; both lists may include patients charts that have both TIA and stroke admissions. Emphasis is on finding the first time TIA and Stroke event for each patient.

Table 11. Continued

Criteria for Manual Inclusion and Exclusion; and Classification into Study Groups*

	TIA		Stroke	
	Included	Excluded	Excluded	Included
Urine analysis w/ microscopic				
Negative	x			x
Positive for Bacteria		x	x	
Positive for WBC's		x	x	
Positive for RBC's		x	x	
Positive for Protein		x	x	
Positive for Cloudiness		x	x	
Body Temperature				
Less than 38.5°C	x			x
Greater than 38.5°C		x	x	
Concomitant medications/therapies				
Steroids		x	x	
Anti-inflammatory drugs		x	x	
Chemotherapy		x	x	
Cancer patient		x	x	
Recent Surgery < 1 month prior to admission		x	x	

*From discrete electronically derived chart lists; both lists may include patients charts that have both TIA and stroke admissions. Emphasis is on finding the first time TIA and Stroke event for each patient.

Table 12.

Reasons for Exclusion from Entire Cohort

	Total Admissions n=1041	
	TIA n=208	Stroke n=833
	Repeat Admissions Discarded n=148	
	Discrete Admissions n=893	
Exclusion Criteria:	TIA Cases n=125	Stroke Cases n=768
Old Stroke on CT/MRI	44	253
History of TIA	16	37
History of Stroke	10	47
Symptoms > 24 hrs. PTA*	12	147
Evidence of Infection		
Urinary Tract Infection	2	45
Pneumonia	2	25
Fever of unknown origin	2	11
History of Cancer	6	61
On Steroids or NSAIDS	1	14
Recent/Current Admission for Invasive Procedure	10	35
Final Differential Diagnosis Medical Other	4	17
Hemorrhagic Stroke	1	16
No Labs on Chart	3	14
No Charts Available	0	3
Research and Neurologist Re-classification of cases	TIA n=12	Stroke n=43

* PTA = prior to admission

Table 13.

Demographics of Sample

	TIA (n=12)	Stroke (n=43)	p value
Age (Mean & SD)*	68.67 (13.01)	65.81 (13.66)	.521
Female (%)**	33.3	39.5	.696
Ethnicity (%)**			
White	100	97.7	.594
Asian	0	2.3	
History of Diabetes (%)**	16.7	14.0	.814
History of Hypertension (%)**	66.7	72.1	.714
History of MI (%)**	25	14	.360
History of Smoking (%)**			
Non-smoker	66.7	44.2	.168
Ex-smoker	33.3	20.9	.371
Currently smoking	0	34.9	.016

* p for t-test

** p for Chi-square = asymptotic significance under null hypothesis

Table 14.

Characteristic of Case Presentation and Hospital Course

	TIA (n=12)	Stroke (n=43)	p value
Motor Deficit (%)**	50	88.4	.003
Speech Deficit (%)**			
Slurred Speech	16.7	41.9	.109
Expressive aphasia	25	11.6	.245
Receptive aphasia	0	2.3	.594
Global aphasia	0	4.7	.447
Confusion (%)**	16.7	9.3	.469
Dizziness (%)**	8.3	7.0	.873
Visual Deficit (%)**			
Blurred vision	8.3	7.0	.873
Field cut Right/Left	8.3/0	2.3/4.7	.326/.447
Atrial Fibrillation on ADM (%)**	8.3	16.3	.669
CT scan (%)**			
Negative for stroke	100	65.1	.016
Positive for new stroke	0	34.9	.016
Small vessel disease	33.3	16.3	.192
Carotid Ultrasound (%)**			
Positive for Stenosis	55.6 (n=5/9)	60 (n=24/40)	.806
Length of Stay (Hrs)*			
Mean (min-max)	30.22 (18-55)	71.42 (15.5-206.8)	< .001
Duration of Symptoms (Hrs)*			
Mean (min-max)	0.36 (.03-1.0)	60.23 (1.0-207.5)	< .001

* p for t-test

** p for Chi-square = asymptotic significance under null hypothesis

Table 15.

Aim 1: Variables on Admission Compared to the Laboratory Mean

	Mean (SD)	Laboratory Mean (SD)	Significance Two-tailed	Effect Size	Observed Power
		TIA (n=12)			
White Blood Cells (K/UL)	6.92 (1.67)	7.8 (1.50)	.094	-.59	.46
Neutrophils (%)	67.92 (12.67)	52.5 (8.75)	.001	1.76	1.0
Lymphocytes (%)	21.67 (10.51)	35 (5)	.001	-2.67	1.0
Monocytes (%)	8.17 (2.76)	6 (3)	.020	.90	.63
Absolute Neutrophils (K/UL)	4.82 (1.82)	4.45 (1.43)	.490	.26	.13
Absolute Lymphocytes (K/UL)	1.41 (0.59)	2.7 (0.8)	< .001	-1.61	1.0
Absolute Monocytes (K/UL)	0.52 (0.15)	0.6 (0.3)	.078	-.27	.13
		Stroke (n=43)			
White Blood Cells (K/UL)	7.81 (2.26)	7.8 (1.50)	.984	.007	.03
Neutrophils (%)	64.95 (9.09)	52.5 (8.75)	< .001	1.42	1.0
Lymphocytes (%)	24.74 (8.36)	35 (5)	< .001	-2.05	1.0
Monocytes (%)	7.70 (1.60)	6 (3)	< .001	.57	.95
Absolute Neutrophils (K/UL)	5.13 (1.88)	4.45 (1.43)	.022	.48	.86
Absolute Lymphocytes (K/UL)	1.89 (0.73)	2.7 (0.8)	< .001	-1.01	1.0
Absolute Monocytes (K/UL)	0.60 (0.17)	0.6 (0.3)	.848	0	.03

Power and Effect size calculated using the study group SD; Significance = .05

Table 16.

Table 16.

Estimated Sample Size for Aim 1

	Sample Size TIA
	Power of 0.8
White Blood Cells (K/UL)	25
Absolute Neutrophils (K/UL)	119
Absolute Monocytes (K/UL)	112
	Sample Size Stroke
	Power of 0.8
White Blood Cells (K/UL)	176,600
Absolute Monocytes (K/UL)	No estimate

Based on Significance of 0.05; two-tailed

Table 17.

Aim 2: Multivariate and Univariate Analysis with Covariate Influence

	Wilks'Lambda	F	df	Significance	Observed Power
MANOVA *7					
Group	.846	1.23	7	.308	.47
MANCOVA *7					
Group	.850	1.16	7	.345	.44
Covariate:					
Current Smoker	.810	1.55	7	.176	.58

* 7 Variables = White Blood Cells (K/UL); Neutrophils (%); Lymphocytes (%); Monocytes (%); Absolute Neutrophils (K/UL); Absolute Lymphocytes (K/UL); Absolute Monocytes (K/UL)

Table 18.

Aim 2: Alternate Analyses for Variables on Admission Compared by Independent Sample t-test

	TIA (n=12) Mean (SD)	Stroke (n=43) Mean (SD)	Significance Two-tailed	Effect Size	Obs.* Power
White Blood Cells (K/UL)	6.92 (1.67)	7.81 (2.26)	.210	-.45	.30
Neutrophils (%)	67.92 (12.67)	64.95 (9.09)	.365	.27	.11
Lymphocytes (%)	21.67 (10.51)	24.74 (8.36)	.292	-.32	.14
Monocytes (%)	8.17 (2.76)	7.70 (1.60)	.452	.21	.08
Absolute Neutrophils (K/UL)	4.82 (1.82)	5.13 (1.88)	.614	-.17	.07
Absolute Lymphocytes (K/UL)	1.41 (0.59)	1.89 (0.73)	.039	-.72	.63
Absolute Monocytes (K/UL)	0.52 (0.15)	0.60 (0.17)	.153	-.50	.32

* Observed Power

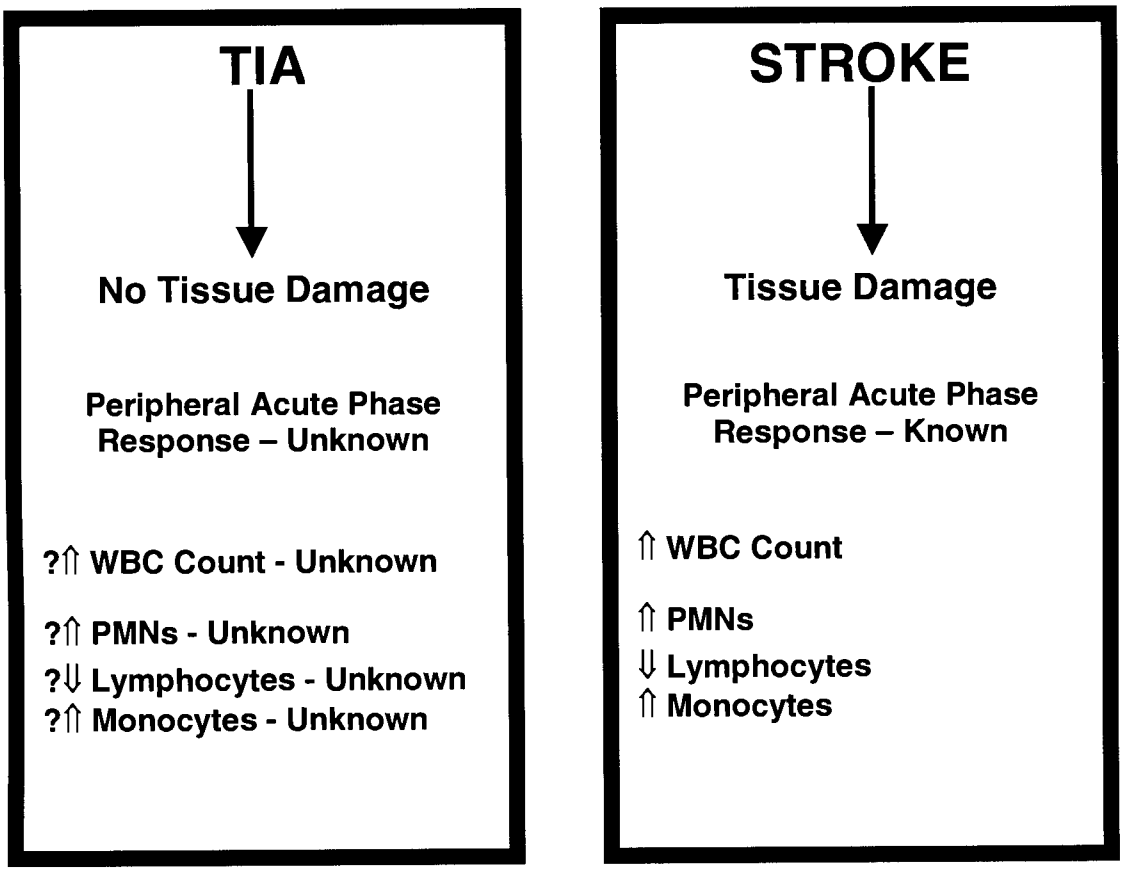
Table 19.

Estimated Sample Size for Aim 2

	Sample Size
	Power 0.8
White Blood Cells (K/UL)	80
Neutrophils (%)	217
Lymphocytes (%)	151
Monocytes (%)	362
Absolute Neutrophils (K/UL)	560
Absolute Monocytes (K/UL)	65

Based on Significance of 0.05; two-tailed

Figure 1. Model for Study



Captions for Figures

Figure 1. TIA does not produce tissue damage, as evidenced by infarction on brain imaging, or residual neurological deficits. Stroke produces tissues damage with infarction on brain imaging, and residual neurological deficits. The presence of the peripheral acute phase response is known to be elevation of WBC count, neutrophils, and monocytes; and depression of lymphocytes on admission and at 24 hours after onset of stroke symptoms. The presence of the peripheral acute phase response is not known in TIA. Evidence of the peripheral acute phase response in TIA is the focus of this study; a comparison to the peripheral acute phase response in stroke was done.