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# Inflammation and Stroke: Therapeutic Effects of Adenoviral Expression of Secretory Leukocyte Protease Inhibitor

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## 1. ABSTRACT

Inflammation is a fundamental reaction of the organism aimed to protect, defend and amend diverse forms of injuries. Stroke, an ischemic condition of the brain, provokes acute and chronic inflammatory reactions composed of brain cells as well as circulating immune inflammatory cells. In response to ischemia, brain cells transcribe and express inflammatory proteins that orchestrate numerous biochemical pathways, some of which of potential detrimental effects. This review paper is aimed to delineate strategies that may enable the discovery of novel therapeutic targets for the discovery of drugs for stroke. Soluble Leukocyte Protease Inhibitor (SLPI) serves as a prime example for this strategy.

## 2. INTRODUCTION

Stroke is a major medical emergency due to the high rate of mortality and morbidity. In the USA alone, about 750,000 strokes, resulting in about 150,000 deaths are estimated annually, which makes stroke the third leading cause of death in the US. In addition, stroke management incurs high burden of health costs due to prolonged rehabilitation, permanent (~30%) residual deficits and loss of productive professional capabilities (1, 2). The incidence of strokes has been on the decline in the 1980-1990 primarily due to advances in aggressive pharmacological management of key risk factors such as hypertension, (all anti-hypertensive drugs reduce stroke rates), atherosclerosis (statins), life style modifications

(smoking) and in recent years, improved time to admittance to health facilities that are better equipped and organized to diagnose and treat strokes in specialized stroke care units. However, the incidence of stroke has not decreased in recent years in most of the industrialized countries while the prevalence has actually increased due to the aging population and increased rates of co-morbidities such as diabetes, heart failure and the metabolic syndrome (1, 2).

In spite of the medical severity of strokes, its prevalence and health burden, little progress has been realized in the treatment of this condition. In fact, none of the pharmaceutical houses has been able to realize a single drug for stroke so far. The only specific, pharmacological treatment registered for stroke is the pro-thrombolysis agents tPA (3-5). This agent however finds limited utility due to substantial limitation of time to administration (3 hrs), the need to exclude hemorrhagic condition (commonly requiring neurologist and radiology facilities) and toxicity, resulting in an overall 2-5% of stroke patients. In addition, no mortality benefits have been proven in prospective clinical trials with thrombolysis agents (3, 4).

The reasons for the difficulties to discover and develop drugs for treatment of stroke are complex. Detailed reviews on this matter have been published and are beyond the scope of the specific chapter on inflammation and stroke (6-7). It is sufficed to point out that revisions of the conceptual frames, new molecular targets, better animal models and clinical trials strategies are being conducted with the hope to accelerate breakthrough therapeutics within this decade.

This brief review focuses on the role of inflammation in stroke with special emphasis on inflammatory mediators derived from inflammatory cells. Such mediators include proteases, which are believed to play an important role in ischemic brain injury. Strategy to identify inhibitors for these proteases using directional gene transfer of endogenous protease inhibitors will be illustrated.

## 3. INFLAMMATORY CELL AND MEDIATORS IN BRAIN ISCHEMIA

### 3.1. General consideration

Ischemic stroke triggers an inflammatory reaction in the affected area. This inflammatory reaction is marked by complex and diverse genomic, biochemical and cellular components, which include both endogenous as well as circulatory elements. In addition, the inflammatory reaction is fashioned by temporal changes, which could progress over days, weeks and months (8). The presence of inflammation in perspective of other events that are initiated by ischemic brain injury is depicted in Figure 1. This scheme points out the very early engagement of inflammation in the ischemic area commencing by activation (within minutes) of transcription factors that regulate pro-inflammatory gene products (e.g., cytokines, adhesion molecules, inflammatory eicosanoids products).

### 3.2. Inflammatory cells in stroke: microglia, astrocytes, neutrophils and monocytes

Cerebral ischemia is marked by infiltration of inflammatory cells into the ischemic territory (see Figure 1). Early accumulation of blood-borne inflammatory cells in the ischemic brain persists for days to weeks after the initial insult (9). The early infiltration of polymorphonuclear cells into the ischemic zone capillaries and parenchyma parallels the early activation of microglia and astroglia. Monocytes are arriving in the injury zone much later at around 12-24 hrs after onset of ischemic injury and are rapidly transformed into tissue macrophages displaying aggressive phagocytosis. Other immune/inflammatory cells such as lymphocytes are also observed at later time points. The significance of the exogenous leukocyte infiltration into the ischemic brain is controversial. It is likely that the significance is highly cell and time specific as well as the context and status of the tissue. The presence of leukocytes in the capillaries distal to the occlusion zone, especially in the penumbra was argued to contribute to low, or no-flow phenomenon (8, 10). Furthermore, the gamut of toxic mediators released by the activated leukocytes, e.g., reactive oxygen radicals, proteases and cytokines, have also argued to contribute to endothelial capillaries damage as well as to neurons and other brain cells (11). However, a cause and effect relationships between the extent of neutrophil trafficking and the severity of ischemic damage has not been established (12). The role of the microglia and the astroglia in the acute response to brain ischemia, while clearly evident from morphological and biochemical markers is even more difficult to assess and are likely to contribute to both repair and injury processes.

### 3.3. Cytokine

Extensive research in the past decade has shed much light on inflammatory mediators that are generated following brain ischemia by either endogenous or exogenous inflammatory cells. Such mediators include: eicosanoids (prostaglandins and leukotrienes), phospholipids (PAF), cytokines, chemokines, adhesion molecules (E-P-L-selectins, integrins and CD11/18 and others). Most studied in recent years focused on pro-inflammatory cytokines, interleukins and endothelium-leukocyte adhesion molecules (see Figure 1), which culminated in clinical trials.

The normal brain has virtually no or only extremely low levels of cytokines and interleukins mRNA transcripts and proteins as well as expressed leukocyte adhesion molecules. However, in response to ischemic injury, short or prolonged expression of a large number of cytokines, interleukins and leukocyte adhesion molecules are expressed in a typical time course (see Figure 1 and 13-25). The significance of the cytokines/interleukins is still debated, as many are plieotropic polypeptides that have been shown to mediate numerous cellular effects such as growth, proliferation, activation, apoptosis and specific functionalities. Some of these cytokines, e.g., TNF alpha or IL-1 have been implicated in ischemic neuronal death in some models but the opposite in others. Likewise, blockade of many leukocyte adhesion molecules by genetic

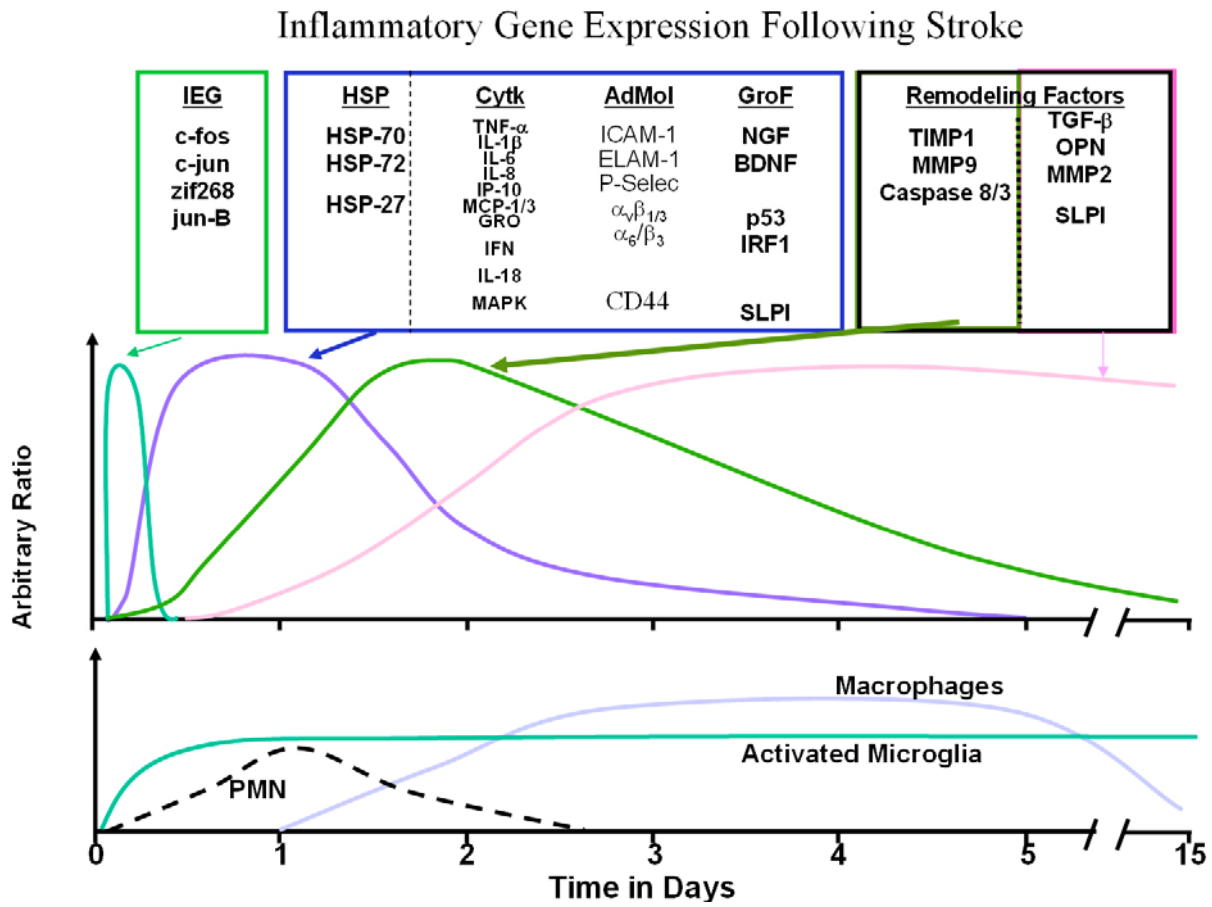


**Figure 1.** Gene expression in response to brain ischemia. The figure depicts 4 "waves" which represent diverse functions. Wave 1 includes transcription factors which up regulate many of the inflammatory genes depicted in later waves. Wave 2 includes a large number of genes that could serve pro or anti-inflammatory functions. Wave 3 and 4 include genes associated with matrix regulation and apoptosis while wave 4 includes genes that might be associated with tissue repair and rehabilitation.

or pharmacological manipulations in *in vivo* experimental stroke models suggested a detrimental role of these receptors in acute ischemic brain injury. However, clinical trials with several different inhibitors of neutrophil adhesion molecules that enable polymorphonuclear traffic across the capillary endothelium into the ischemic brain (anti-ICAM-1 antibody, enlimomab; LeukArrest, Hu23F2G, a monoclonal antibody that targets the neutrophil CD11/CD18 cell adhesion molecule; rNIF, recombinant neutrophil inhibitory factor, a hookworm-derived agent that reduces the inflammatory cascade by inhibiting the polymorphonuclear leukocyte adhesion to the endothelium) have failed to show the hoped for efficacy (26). No clinical trials have yet been conducted with specific anti-cytokine/interleukins in stroke.

### 3.4. Chemokines

The Chemokines are a family of more than 50 structurally and functionally related proteins involved in inflammatory cell recruitment. The relatively small (8-10kDa) proteins are usually subdivided into four subfamilies on the basis of their structural conservation of cysteine residues (for review of the structural, functional, genomics and proteomics see 27-30). Chemokines and their receptors are constitutively expressed in microglia, astrocytes and neurons throughout the CNS albeit at very low levels (29). As shown in Figure 1, rapid expression of many Chemokines takes place in response to acute ischemic brain injury (29-30), which corresponds with cytokines and leukocyte adhesion molecule expression. Most prominent activation has been documented for MCP-1 (Monocyte Attractant Protein –1) (31), IL-8 (32), IP-10 (Interferon-inducible protein-1, 33), MIP (Macrophage Inflammatory Protein-1) (34) and fractalkine (35). While these Chemokines and others seem to be expressed in the location and at the time that could lend them a role in propagation, aggravation or repair of the ischemic injury, no agent to day has been deployed in clinical trials in stroke or other brain injury conditions. Experiments with certain tool-compounds that block certain chemokines have shown the potential for better recovery following stroke (29-30) yet proof of concept has not been obtained in clinical studies.

### 3.5. Proteases and matrix proteins

The matrix metalloproteinases (MMPs) and serine proteases including plasmin activators (tPA and uPA) are two important neutral protease gene families that

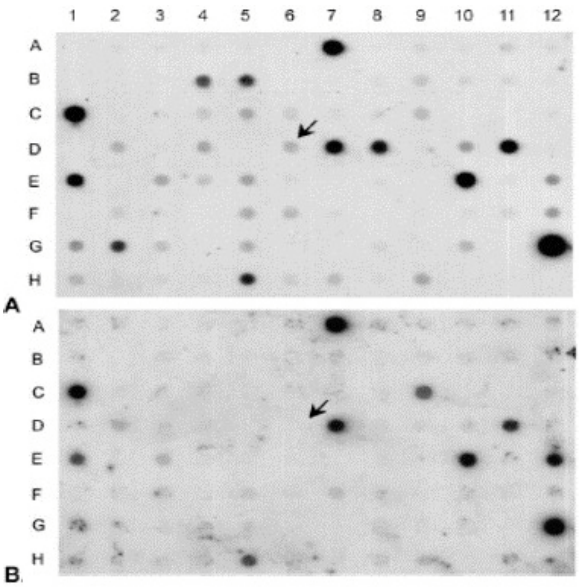


**Figure 2.** Identification of the induced SLPI mRNA expression in rat ischemic. cortex using suppression subtractive hybridization strategy. CDNA clones were generated by suppression subtractive hybridization, transformed into bacteria, and cultured in a 96-well dish. The bacterial cultures were transferred onto a nylon membrane using a dot-blot apparatus and then analyzed by Southern hybridization. A, Southern hybridization of the membrane with a probe generated from ischemic cortical samples. B, Southern blot analysis of the same membrane with a probe generated from normal cortex. Note that one clone (indicated with an arrow) was identified as rat SLPI by DNA sequencing analysis.

are involved in neuro-inflammation after cerebral ischemia. Activation of many members of this large family of proteases in response to brain ischemia and other brain injuries has been repeatedly demonstrated (36-37). These proteases metabolize a broad spectrum of matrix proteins (collagen, elastin, fibronectin, proteoglycans, and others zymogens), which impact on essential cell adhesion, interaction and blood brain barrier function (36-37). MMPs are produced and released from endogenous and exogenous inflammatory cells in response to diverse stimuli such as cytokines, interleukins, growth factors, which are rapidly up regulated by ischemia. However, activation of MMPs is also carefully regulated by multiple protease inhibitors that are abundant in the matrix or released by local or infiltrating inflammatory cells. Several endogenous tissue metalloprotease inhibitors have been shown to be up regulated by ischemia (39) possibly as an attempt to regulate the MMPs actions. The overall balance between proteolytic and anti-proteolytic activities is clearly tilted towards proteolytic action during the acute ischemic condition.

The overall significance of the proteolytic activities unleashed by ischemic injury in respect to short and long-term tissue repair and rehabilitation is unclear. In short-term experiments, inhibition of MMPs by relatively selective MMP inhibitors (40) have shown tissue salvage. However, long term benefits from MMPs inhibition has not been explored as yet in proper stroke studies due to lack of suitable pharmacological agents. In the following section (3.6) a potential therapeutic modality – soluble leukocyte protease inhibitor will be presented.

### 3.6. Protease inhibitors as potential therapeutics in acute ischemic stroke

#### 3.6.1. Therapeutic gene transfer- SLPI

Over the background provided in section 3.5, it appears that inhibition of certain MMPs at an appropriate time frame might provide significant benefits at least in respect to BBB protection, edema formation and neuronal distress. In the following study, a promising example is presented that supports this notion.

Differential gene expression plays a critical role in initiation, propagation, and maturation of ischemic brain injury. As indicated in section 2 (vide supra) several waves of de novo gene expression have been characterized after focal brain ischemia (see Figure 1) where several MMPs and MMP inhibitors (TMPI) are typically expressed in later stages (wave 4, Wang & Feuerstein 41). In an effort to identify novel endogenous MMP inhibitors that could serve as therapeutics, we have applied suppression subtractive hybridization (SSH) method to identify genes that are specifically up-regulated at the time when MMPs are activated after occlusion of the middle cerebral artery (MCAO) in rats. As illustrated in Figure 2, a gene that encodes a rat homolog to human and murine secretory leukocyte protease inhibitor (SLPI), also termed anti-leukoproteinase, was identified (42).

SLPI is an 11.7-kDa protein initially found in fluids secreted from the parotid gland (43,44) and subsequently in seminal, cervical, nasal, and bronchial mucous (45-46). It was initially considered an epithelial cell product (47), but was later found in human neutrophils (48) and peritoneal macrophages (49). SLPI has been recognized as a potent inhibitor of leukocyte serine proteases, including elastase and cathepsin G from neutrophils, chymase and tryptase from mast cells, as well as trypsin and chymotrypsin from pancreatic acinar cells (46, 50, 51). Recent studies have revealed that SLPI functions as more than just a protease inhibitor. For example, SLPI suppressed bacterial growth (52) and inhibited HIV-1 infection of macrophages at physiological concentrations (53). SLPI exerts anti-inflammatory functions on macrophages (54), neutrophils (54), and B cells (55). SLPI has been shown to play an important role in regulating inflammatory responses by reducing inflammatory gene expression and diminishing inflammatory cell accumulation after both hepatic and lung injuries (56-57). Mice deficient in SLPI showed impaired cutaneous wound healing with increased inflammation and TGF-beta activity, as well as increased elastase activity and reduced matrix (58). Recent studies suggest that SLPI attenuates inflammatory responses and contributes to balanced function of innate immunity (55, 59).

The full-length rat SLPI cDNA identified by SSH shares 81% and 63% amino acid sequence identity with its

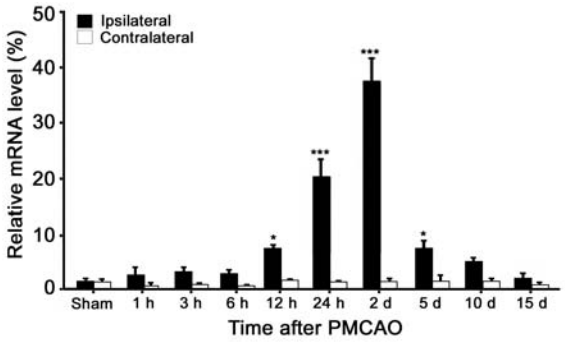


**Figure 3.** Time-course study of SLPI mRNA induction in rat ischemic cortex after MCAO. A, a represent Northern blot for SLPI and rpL32 cDNA probes to the samples from spontaneously hypertensive rats (SHR) after MCAO. Total cellular RNA (40microg/lane) was resolved by electrophoresis, transferred to a nylon membrane, and hybridized to the indicated cDNA probe. Ipsilateral and contralateral cortical samples from individual rats with sham surgery (S; 24 h) or after 1, 3, 6, 12, and 24 h and 2, 5, 10, and 15 days of permanent MCAO are depicted. B, quantitative Northern blot data (N=4) for SLPI mRNA expression after focal stroke. The data were generated using phosphoimaging analysis and displayed graphically after being normalized with rpL32 mRNA signals. *, p =0.05; ***, p = 0.001, compared with sham-operated animals.

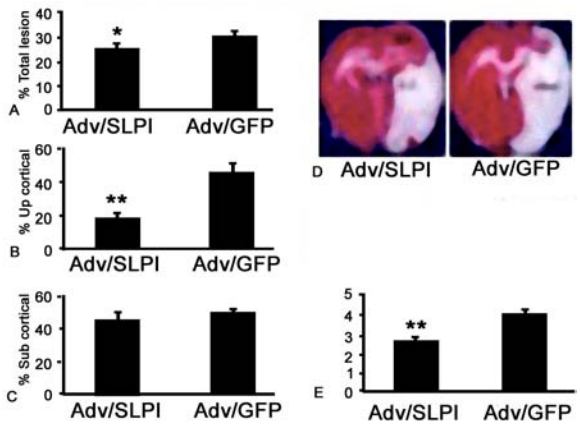


**Figure 4.** Effect of cortical Adv/SLPI injection on ischemic brain injury. Recombinant adenoviruses Adv/SLPI and Adv/GFP were injected into four up-cortical positions in the ipsilateral hemisphere as described in detail under Materials and Methods reference 42. Two days after adenovirus injection, rats were subjected to MCAO. Neurological deficits (E) and ischemic lesion (A–D) were measured 24 h after MCAO. Brain slices (2 mm) were TTC-stained to define the infarct area (the white area illustrated with dotted lines in D). Total ischemic lesion (A), as well as the lesion at the up-cortical [about a third of the ipsilateral cortex (U.C.)] and subcortical (S.C.) regions was also illustrated (B and C). *, p = 0.05; **, p =0.01, compared with Adv/GFP-treated animals.

mouse and human homolog, respectively, and with several polymorphisms to previous reported rat sequences. Northern blot analysis (Figure 3) confirmed that SLPI mRNA was significantly induced in the ischemic brain tissue at 12 h (5.1-fold increase over sham controls peaked at 2 days (26.1-fold increase) and sustained up to 5 days (5.1-fold increase). SLPI was localized in neurons and astrocytes in the peri-infarct zone from 24 to 72 h after middle cerebral artery occlusion by means of immunohistochemistry and confocal microscopy analysis (see Figure 4, 42). Administration of a recombinant adenovirus over-expressing SLPI (Adv/SLPI) into the cortical tissue resulted in up to 58.4% reduction in ischemic lesion over controls at the site of Adv/ SLPI expression and significantly improved functional outcome. These data suggest that the ischemia-induced expression of SLPI might play a neuroprotective role in focal stroke, possibly because of rapid inhibition of activated proteases and its suppression in inflammatory response.

## 4. PERSPECTIVE

The past 20 years have witnessed marked advances in stroke research sponsored by governments, industry, academic and many private for profit and non-profit organizations. Billions of dollars have been spent in discovery and development of drugs for stroke yet only very modest progress has been realized in actual pharmacological treatment of acute stroke. However, enormous knowledge has been gained in many critical fronts that laid solid foundation for important breakthrough within this decade. In this respect, major strides in understanding the regulation of tissue matrix proteins, their synthetic and metabolic degradation have enriched the number of molecular targets that might be subject for pharmaceutical exploitations with major interest in certain MMPs that govern BBB integrity, cell adhesion and cell, axons and stem cell migration biology. Gene transfer technology has made major contributions to research aimed to provide proof of concept on the efficacy of these targets in modulation of ischemic brain injury. The studies commenced with SLPI are the first to demonstrate the potential of SLPI to convey neuroprotection and improve outcome in stroke model. This breakthrough study enabled by gene transfer methodology has a long way to go towards maturation into drug discovery. Delivery of SLPI into the injured brain region via systemic administration of the protein per se, has not been tried as yet not has it been shown that SLPI could provide benefits if administered after the onset of the ischemic injury. However, based on the principle demonstrated in the studies with SLPI, design of small molecules that could enhance rapid up-regulation of SLPI might provide sufficient SLPI in the site of injury to curb excessive activation of MMPs that are injurious to the integrity of the microcirculation and brain matrix health.

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