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Up-Regulation of Secretory Leukocyte Protease Inhibitor (SLPI) in the Brain after Ischemic Stroke: Adenoviral Expression of SLPI Protects Brain from Ischemic Injury

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ABSTRACT

Secretory leukocyte protease inhibitor (SLPI) is a 12-kDa secreted protein initially identified from epithelial cells as an inhibitor of leukocyte serine proteases. In the present study, we described the identification of SLPI expression in ischemic cortex by suppression subtractive hybridization strategy. Our full-length rat SLPI cDNA shares 81% and 63% amino acid sequence identity with its mouse and human homologs, respectively, and with several polymorphisms to previous reported rat sequences. Northern blot analysis confirmed that SLPI mRNA was significantly induced in the ischemic brain tissue at 12 h (5.1-fold increase over sham controls, $n = 4$, $p < 0.05$), peaked at 2 days (26.1-fold increase, $p < 0.001$), and sustained up to 5 days (5.1-fold increase, $p < 0.05$). 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 immunohistochemical and confocal microscopy analysis. Administration of a recombinant adenovirus overexpressing 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 ($p < 0.01$, $n = 8$) and significantly improved functional outcome ($p < 0.01$). 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.

Differential gene expression plays a critical role in initiation, propagation, and maturation of ischemic brain injury. Several waves of de novo gene expression have been characterized after focal brain ischemia, including early response transcription factors (first wave), heat shock proteins (second wave), inflammatory mediators (third wave), and tissue remodeling/repairing proteins (fourth wave) (Wang and Feuerstein, 2000). In an effort to understand the molecular mechanism associated with focal stroke, we have applied suppression subtractive hybridization (SSH) method to identify genes that are specifically regulated after occlusion of the middle cerebral artery (MCAO) in rats. As illustrated in this work, a gene that encodes a rat homolog to human and murine secretory leukocyte protease inhibitor (SLPI), also termed antileukoproteinase, was identified in ischemic cortex using the SSH approach.

SLPI is an 11.7-kDa protein initially found in fluids secreted from the parotid gland (Stetler et al., 1986; Thompson and Ohlsson, 1986) and subsequently in seminal, cervical, nasal, and bronchial mucous (Fritz, 1988; Molhuizen and Schalkwijk, 1995). It was initially considered an epithelial cell product (Abe et al., 1991) but was later found in human neutrophils (Bohm et al., 1992) and peritoneal macrophages (Jin et al., 1997). 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 (Fink et al., 1986; Thompson and Ohlsson, 1986; Ohlsson et al., 1988; Molhuizen and Schalkwijk, 1995). Recent studies have revealed that SLPI functions as more than just a protease inhibitor. For examples, SLPI suppressed bacterial growth (Hiemstra et al., 1996) and inhibited HIV-1 infection of macrophages at physiological concentrations (Shine et al., 1997). SLPI exerts anti-inflammatory functions on macrophages (Jin et al., 1997), neutrophils (Grobmyer et al., 2000), and B cells (Nakamura et al., 2003). SLPI has been shown to play an important role in regulating inflammatory responses by reducing inflamma-

ABBREVIATIONS: SSH, suppression subtractive hybridization; MCAO, occlusion of the middle cerebral artery; SLPI, secretory leukocyte protease inhibitor; TGF, tumor growth factor; SHR, spontaneously hypertensive rat; MCA, middle cerebral artery; ECA, external common carotid; rpL32, ribosomal protein L32; bp, base pair(s); RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; NeuN, neuron specific nuclear protein; TTC, 2,3,5-triphenyltetrazolium chloride; NFκB, nuclear factor-κB.

tory gene expression and diminishing inflammatory cell accumulation after both hepatic and lung injuries (Lentsch et al., 1999; Ward and Lentsch, 2002). Mice deficient in SLPI showed impaired cutaneous wound healing with increased inflammation and TGF-β activity, as well as increased elastase activity and reduced matrix (Ashcroft et al., 2000). Recent studies suggest that SLPI attenuates inflammatory responses and contributes to balanced function of innate immunity (Zhu et al., 2002; Nakamura et al., 2003).

Cerebral ischemia is a pathophysiological condition with a robust leukocyte and macrophage infiltration and accumulation in the lesions (Clark et al., 1993; Garcia et al., 1994). In addition to inflammation, brain ischemia results in considerable tissue remodeling including gliosis, necrosis/apoptosis, and neovascularization. All these pathophysiological changes may require de novo gene expression, protein synthesis, and their coordinated functions. In this report, we describe the cloning of the rat SLPI paralog from ischemic rat brain. We further explored its temporal and spatial distribution by means of Northern analysis and immunohistochemistry. Using the recombinant SLPI adenovirus gene transfer technology, the potential capacity of SLPI to convey neuroprotection in ischemic brain injury was investigated.

Materials and Methods

Animals. Rats were housed and cared for in accordance with the *Guide for the Care and Use of Laboratory Animals* (1996). Procedures using lab animals were approved by the Institutional Animal Care and Use Committees of SmithKline Beecham Pharmaceuticals and Bristol-Myers Squibb Company.

Models of Focal Brain Ischemia. Two widely used models of focal cerebral ischemia were used for this study. Male spontaneously hypertensive rats (SHR) weighing 250 to 330 g were subjected to permanent MCAO as described in detail previously (Barone et al., 1992). Briefly, the middle cerebral artery (MCA) was permanently occluded and cut dorsal to the lateral olfactory tract at the level of the inferior cerebral vein using electrocoagulation (Force 2 electrosurgical generator; Valleylab, Boulder, CO). In sham-operated rats, the dura was opened over the MCA but the artery was not occluded. The ischemic cortex (i.e., the cortex ipsilateral to surgery) and nonischemic (contralateral) cortex was dissected from rats at 1, 3, 6, and 12 h, and 1, 2, 5, 10, and 15 days after permanent MCAO or 12 h and 2 days after sham surgery. The cortical samples from this model were immediately frozen in liquid nitrogen, stored at −80°C, and used for SSH and Northern analysis.

In addition, a "thread" model of focal cerebral ischemia was carried out using male Sprague-Dawley rats weighing 250 to 330 g as described in detail previously (Wang et al., 2001). After ensuring the same SLPI mRNA expression profile as described above using SHR, this model was applied for immunohistochemical analysis and to assess the effect of the recombinant SLPI adenovirus in ischemic brain injury. Briefly, after anesthesia, an incision of the skin was made on top of the right common carotid artery region. The fascia was then blunt-dissected until the bifurcation of the external common carotid (ECA) and internal common carotid was isolated. A small incision was made on the ECA, and a 3-0 monofilament suture with a round tip was threaded into the internal common carotid via the ECA. The suture was advanced toward the MCA region to create focal ischemia. For permanent MCAO, the suture was maintained in the vessel and the wound was closed. Sham operation was performed using the same procedure except that no suture was inserted.

Suppression Subtractive Hybridization. Total RNA of the ipsilateral (ischemic) or normal (nonischemic) forebrain was prepared as described previously (Wang et al., 1999). Poly(A)$^+$ mRNA was extracted with an oligo-dT cellulose column from total cellular RNA pooled from 25 animals at 12 h after permanent MCAO or from normal cortex. SSH was carried out using a PCR-select cDNA subtraction kit (BD Biosciences Clontech, Palo Alto, CA) according to the manufacturer's instruction. Three micrograms of poly(A)$^+$ mRNA was extracted from ischemic cortex 12 h after permanent MCAO (as a tester) or from normal brain cortex (as a driver) for the subtraction. Procedures for SSH and differential hybridization have been described in detail previously (Wang et al., 1999).

Northern Blot Analysis. For Northern analysis, RNA samples (40 μg/lane) were resolved by electrophoresis, transferred onto a GeneScreen Plus membrane (PerkinElmer Life Sciences, Boston, MA), and hybridized to rat SLPI or ribosomal protein L32 (rpL32) as previously described in detail (Wang et al., 1999).

Isolation of the Rat SLPI Full-Length cDNA. DNA sequencing analysis revealed that our SSH cDNA clone contains only 503 bp of the 3′ cDNA. To isolate the 5′-end cDNA, 5′-RACE was applied using a RACE kit from Invitrogen (Carlsbad, CA) according to the manufacturer's specification. A gene-specific primer (5′-TTCTTCACTGGTCCACGAATG-3′) was used for the initial PCR and a second primer (5′-GTATCTTGGCAGCATCTCTGC-3′) was used for the nested PCR. The 5′-RACE PCR product was subcloned into a pCR2.1 vector. DNA sequencing revealed that our 5′-RACE clone overlapped the initial SSH clone, with an additional 218 bp extended at the 5′-end. The full-length SLPI cDNA was generated using a pair of forward (5′-TCAGAGCTACCCTGCCTTCA-3′) and reverse (5′-TTTTTTTTTTTTTTTAGAGAAATG-3′) primers located at each end of the SLPI cDNA. A 665-bp cDNA fragment containing the full-length SLPI mRNA was isolated. DNA sequencing was performed in both strands using both universal primers and gene specific primers. Nucleotide sequences were analyzed using the DNASTAR software system (DNASTAR Inc., Madison, WI).

Immunohistochemical Analysis of SLPI. Brain tissues were collected from rats ($n = 4$) at 6, 12, 24, and 72 h after MCAO or sham operation. Immunohistochemical analysis was performed as previously described in detail (Wang et al., 2001). Rabbit anti-human SLPI (1:200; HyCult Biotechnology, The Netherlands) was used to detect SLPI expression in rat brain tissues. Double-labeling immunofluorescence was carried out using rabbit anti-human SLPI in combination with either mouse anti-rat CD11b/c (OX42, 1:1000; BD PharMingen, San Diego, CA), mouse anti-rat monocytes/macrophages (ED1, 1:250; Chemicon, Temecula, CA), mouse anti-glial fibrillary acidic protein (GFAP, 1:1000; Chemicon) or mouse anti-neuron specific nuclear protein (NeuN, 1:1000; Chemicon) as described in detail previously (Wang et al., 2001). Confocal microscopy (Wang et al., 2001) was used to determine the cellular localization of SLPI in the brain tissue.

Recombinant Adenovirus Construction and Cortical Injection. The full-length coding sequence of the rat SLPI cDNA was subcloned into the *Bam*HI and *Xba*I sites of the shuttle plasmid pAdv/CMV (a kind gift from Dr. Kenneth Chien, University of California at San Diego, La Jolla, CA). The resulting plasmid, pAdv/CMV-SLPI was cotransfected with a helper plasmid, pJM17, into human embryonic kidney 293 cells to generate the recombinant adenovirus Adv/SLPI, as described previously (Xia et al., 1999; Xu et al., 2002). The recombinant adenoviruses were plaque-purified and amplified in human embryonic kidney 293 cells. Concentrated adenoviruses were prepared by CsCl gradient centrifugation, followed by desalting with chromatography in 1 mM $MgCl_2$ in phosphate-buffered saline. The titer of the adenovirus was measured from DNA content of the viral solution with 1.0 OD_{260} as approximately 1.0×10^{12} particles/ml. The adenovirus construct Adv/GFP, containing the cDNA of green fluorescent protein (GFP), was generated by a similar strategy.

Cortical injection of Adv/SLPI and Adv/GFP was carried out using a stereotaxic instrument (Xu et al., 2002). Each rat was subjected to four cortical injections in the following locations: point 1, 1 mm caudal to the bregma, 4.6 mm lateral to the midline

of the skull, and 4 mm ventral to the exterior surface of the skull; point 2, 2 mm caudal to the bregma, 4.3 mm lateral to the midline of the skull, and 4 mm ventral to the exterior surface of the skull; point 3, 3 mm caudal to the bregma, 4.6 mm lateral to the midline of the skull, and 4 mm ventral to the exterior surface of the skull; and point 4, 4 mm caudal to the bregma, 5.2 mm lateral to the midline of the skull, and 4 mm ventral to the exterior surface of the skull. All the target points were in the right hemisphere (i.e., ipsilateral to the MCAO). Two microliters of adenoviral suspension containing 1×10^{11} particles/ml was injected in each point at a rate of 0.2 μl/min. The needle was withdrawn over a course of 10 min. Forty-eight hours after injection of adenoviruses, rats were subjected to MCAO using the "thread" model as described above. Immunohistochemical study confirmed the active expression of SLPI by Adv/SLPI at 24 and 48 h after the adenovirus injection in naive animals.

Ischemic Lesion and Neurological Deficit Measurement. Ischemic lesion was evaluated 24 h after MCAO using 2,3,5-triphenyltetrazolium chloride (TTC) staining of 2-mm thick brain slices. The stained brain tissue was fixed in 10% formalin in phosphate-buffered saline. The image was captured using a Microtek ScanMaker 4 DUO Scanner (Microtek International, Inc., Hsinchu, Taiwan) within 24 h and quantitated using Image Pro Plus 4.1 software (Media Cybernetics, Silver Spring, MD). Total ischemic lesion, the cortical region per se (the adenovirus infected area), and the subcortical region (from caudate putamen to lat preoptic area) were measured.

Neurological deficits were examined for the same groups of animals before TTC staining using the following criteria (Wang et al., 2003): no neurological deficit = 0; right Horner's syndrome counts 1 point; failure to extend left forelimb and hindlimb, 1 point each; turning to left, 1 point; and circling to left, 1 point.

Statistical Analysis. Data were illustrated as mean ± S.E. Statistical comparisons were made by analysis of variance (Fisher's protected least-squares difference), and values were considered to be significant at $p < 0.05$.

Results

Identification of SLPI Gene Expression in the Brain after MCAO. SSH was used to subtract cortical samples (12 h after permanent MCAO) from normal cortex as described in detail previously (Wang et al., 1999). The subtracted cDNA clones were analyzed by comparative Southern dot-blot hybridization. Figure 1 illustrated a panel of SSH clones hybridized to an ischemic probe (Fig. 1A) and a nonischemic probe (Fig. 1B). One of the clones, indicated by an arrow in Fig. 1, was confirmed for its ischemia-induced expression by Northern analysis (see Fig. 3). DNA sequencing analysis and database search found that the sequence represented an unreported cDNA (at the time of cloning) but shared significant sequence identity to mouse SLPI cDNA (GenBank accession no. U73004; 80% nucleotide sequence identity in 515-bp overlap).

Isolation and Characterization of the Rat SLPI cDNA. Because the cDNA clone contained only partial sequence of the coding region along with a complete 3′-untranslated region, including the poly(A) tail, the full-length cloning was pursued using a 5′-RACE approach. The RACE experiments were carried out using poly(A) RNA isolated from both ischemic brain tissue and spleen, where it was confirmed to be abundantly expressed by Northern analysis (data not shown). 5′-RACE resulted in a 218-base extension at the 5′-region of the mRNA. To confirm the full-length cDNA, a pair of primers was synthesized according to the most 5′ and 3′ ends of the sequence to amplify the rat SLPI mRNA. The compiled nucleotide sequence of the SLPI cDNA from the SSH cDNA clone, 5′-RACE clone, and the full-length PCR clone was deposited to GenBank accesssion no. AF421377 and used to predict the coding sequence, which contains 22 bp of the 5′-untranslated sequence, 393 bp of coding sequence, and 236 bp of 3′-untranslated region. A polyadenylation signal (AATAAA) was identified 19 bp upstream of the 3′ end. The full-length rat SLPI cDNA shares 83% and 72% nucleotide sequence identity with mouse and human SLPI, respectively. The entire coding sequence shares 81% amino acid sequence identity (or 89% similarity) with mouse SLPI and 63% identity (or 71% similarity) with human SLPI (Fig. 3). The predicted amino acid sequence contains 25 residues of signal peptide and 105 residues of the secreted peptide. All the cysteine residues (total of 16) and 12 prolines, which are known to be critical to the SLPI protein structure (Molhuizen and Schalkwijk, 1995), are highly conserved among human, rat, and mouse species (Fig. 2). Based on their striking sequence similarity and conservation, we suggest that our clone represents the rat homolog of human SLPI.

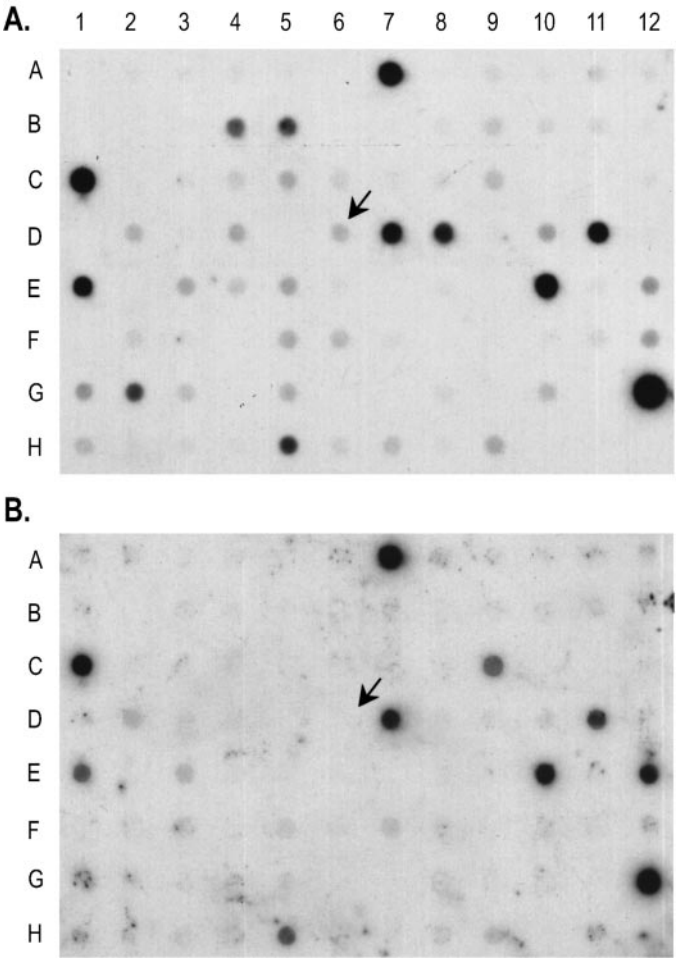

Fig. 1. Identification of the induced SLPI mRNA expression in rat ischemic cortex using suppression subtractive hybridization strategy. cDNA clones were generated by suppression subtractive hybridization, transformed into bacteria, and cultured in a 96-well dish. The bacterial cultures were transferred 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.

To date, three additional rat SLPI sequence homologs have been reported and/or deposited (AF151982; Gipson et al., 1999; Song et al., 1999). However, each of these sequences differs from the others (Fig. 2). The identical amino acid sequences were identified between one deposited sequence (GenBank accession no. AF151982; D. H. Chen, X. P. Xu, M. K. Bagchi, and I. C. Bagchi, unpublished data) and that in our present report, except for differences in one base located in the coding region and seven bases in the 3′-untranslated region. Six (Song et al., 1999) and 20 amino acids (Gipson et al., 1999) were identified to be different from our present sequence (Fig. 2). Despite the high variability among these rat SLPI sequences, no change occurs in the conserved cysteine and proline residues.

Temporal Expression of SLPI mRNA in Rat Cortex after MCAO. Northern analysis was used to measure the temporal expression of SLPI mRNA after MCAO. Figure 3A illustrates a representative Northern blot for SLPI mRNA expression in the ipsilateral (ischemic) and contralateral (nonischemic) cortical samples after MCAO. Quantitative Northern blot data ($n = 4$), after being normalized to an rpL32 probe, are illustrated graphically in Fig. 3B. Very low levels of SLPI mRNA expression were detected in normal, sham-operated, and contralateral of ischemic cortical samples. Significant SLPI mRNA induction was observed in the ischemic cortex at 12 h after MCAO (5.1-fold increase compared with sham, $n = 4$, $p < 0.05$), peaked at 2 days (26.1-fold increase, $p < 0.001$), and was sustained for up to 5 days (5.1-fold increase, $p < 0.05$).

Immunohistochemical Analysis of SLPI Expression in the Ischemic Lesion. Time-course study confirmed a parallel induction of SLPI peptide with mRNA in the brain after MCAO. The increase in SLPI immunoreactivity was not observed until 24 h and extended up to 72 h after MCAO. No immunoreactive signal was observed for SLPI in the ischemic core. The induction of SLPI was restricted only to the peri-infarct area. Double-labeling immunohistochemistry followed by confocal microscopy analysis demonstrated that the cellular sources of SLPI expression were mainly in astrocytes, as well as a number of neurons (about 10% neurons showed SLPI-immunoreactivity, ~ 80% of which were double-stained with NeuN) in the peri-infarct area (Fig. 4). SLPI staining was identified in both neuronal cells and astrocytes 24 h after MCAO but later (72 h) limited to astrocytes. No SLPI immuno-signal was detected in either microglia or macrophages at various time points observed after MCAO (Fig. 4). Immunohistochemical study was also carried out to assess the expression of SLPI, along with the specific cellular markers, in the brain after sham operation. There is no basal expression of SLPI in the uninjured brain (Fig. 4).

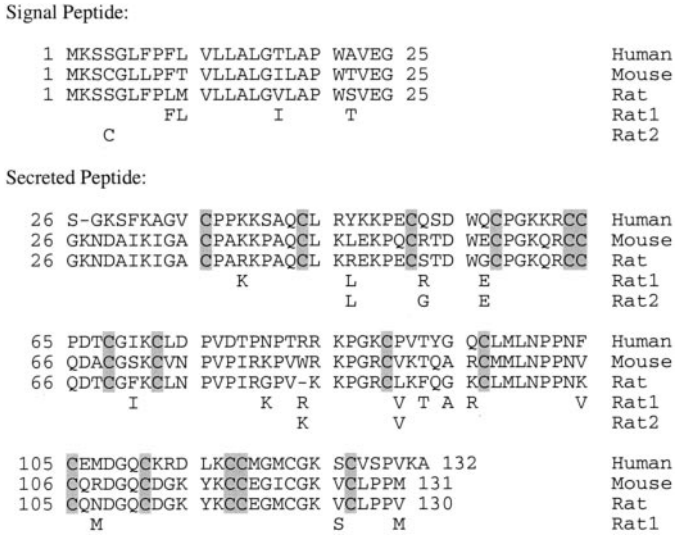

Fig. 2. Sequence alignment of rat, mouse, and human SLPI homologs. Mouse SLPI is from GenBank accession number U73004 (Jin et al., 1997) and human SLPI is from GenBank accession number X04502 (Stetler et al., 1986). Rat SLPI sequence alignment is based on our present report (GenBank accession number AF421377); the differences of our sequence from two reported rat homologs are illustrated; Rat1 is from Gipson et al. (1999); Rat2 is from Song et al. (1999). The conserved cysteine residues are shaded.

Expression of Recombinant Adenoviral SLPI Protects Brain from Ischemic Injury. To explore a potential role of SLPI in ischemic brain injury, the adenovirus was constructed to express the recombinant SLPI. Adv/SLPI was delivered into discrete brain regions, and its expression was confirmed by immunohistochemical analysis (Fig. 5). Expression of Adv/SLPI in the cortex significantly protected the brain from ischemic injury (Fig. 6), with a 22% reduction in total ischemic lesion ($n = 8$, $p < 0.05$ over Adv/GFP-infected controls) or a 58% reduction in the up-cortical region (where the adenovirus was injected; $p < 0.001$). A marked reduction in neurological deficits (32% reduction over controls, $n = 8$, $p < 0.01$) was also observed in the Adv/SLPI-injected rats (Fig. 6). In contrast, no difference was observed in infarct size in the non-virally infected subcortical regions between the Adv/SLPI- and Adv/GFP-injected rats after MCAO (Fig. 6).

Discussion

In the present report, we describe the discovery of induced SLPI expression in the brain after ischemic injury by means of a suppression subtractive strategy and confirmed by Northern blotting and immunohistochemical analysis. This finding was also in agreement with a recent report that showed a significant increase in serum levels of SLPI in patients after ischemic stroke (Ilzecka and Stelmasiak, 2002). Using the recombinant adenovirus overexpressing SLPI, our present study provides direct evidence that SLPI could reduce brain infarct and improve functional outcome after ischemic insult. It should be pointed out that these data should be interpreted with caution because the viral expression of SLPI does not necessarily represent its ischemic-induced expression (in the temporal, spatial, and cellular characteristics); therefore, although the data indicate that SLPI can be neuroprotective, they do not prove the role of ischemia-induced SLPI in ischemic injury.

The mechanisms of the potential neuroprotective effect of SLPI in ischemia-induced brain injury remain to be explored. Based upon the known functions for SLPI and the pathophysiologic features associated with ischemic stroke, the following possible mechanisms might be hypothesized. First, ischemia-induced SLPI in the brain may contribute to the regulation of serine proteases by interfering with their proteolytic activities. Previous studies demonstrated that SLPI could function as an inhibitor of leukocyte serine proteases, including elastase, chymase, tryptase, and trypsin (Fritz, 1988; Molhuizen and Schalkwijk, 1995). The source of such proteases could be of infiltrating inflammatory cells or brain cells activated by

ischemic injury. In fact, inhibition of neutrophil elastase by a specific small-molecule inhibitor was shown to reduce ischemic brain damage (Shimakura et al., 2000) in a similar rat model of focal stroke. In airway inflammatory disorders, the activity and balance between proteases and anti-proteases, such as SLPI and elafin, are thought to provide the first line of defense against proteinase (such as neutrophil elastase) attack (Knight et al., 1997; Tomee et al., 1998).

Second, SLPI might regulate leukocyte infiltration and suppress inflammatory responses induced by brain ischemia and therefore reduce ischemic damage. One of the key functions of SLPI has been defined as anti-inflammatory actions, as demonstrated on macrophages (Jin et al., 1997) neutrophils (Grobmyer et al., 2000), and B cells (Nakamura et al., 2003) in vitro, as well as hepatic and lung injuries (Lentsch et al., 1999; Ward and Lentsch, 2002). A robust leukocyte and macrophage infiltration and accumulation has been documented in the ischemic brain tissue (Clark et al., 1993; Garcia et al., 1994). The ischemia-induced expression of SLPI may suppress leukocyte accumulation and regulate inflammatory response, as has been demonstrated after hepatic ischemia and reperfusion injury (Lentsch et al., 1999). It has recently been suggested that the role of SLPI, along with interleukin 10 and interleukin 13, on the suppression of inflammatory response may depend on the suppression of NFκB activation (Kubes and Ward, 2000). The activation and role of NFκB in focal cerebral ischemia have also been demonstrated (Schneider et al., 1999), and suppression of NFκB by overexpression of dominant-negative IκBa (as a selective inhibitor) protected brain from ischemic damage (Xu et al., 2002). Likewise, the important role of SLPI in suppression of both inflammatory response and serine proteases (such as elastase) activity has been demonstrated in cutaneous wound healing using SLPI-deficient mice (Ashcroft et al., 2000).

It is of interest that the temporal expression of SLPI revealed that its expression was delayed in comparison with a number of inflammatory cytokines (e.g., interleukin-1β and tumor necrosis factor-α), chemokines (e.g., monocyte chemo-

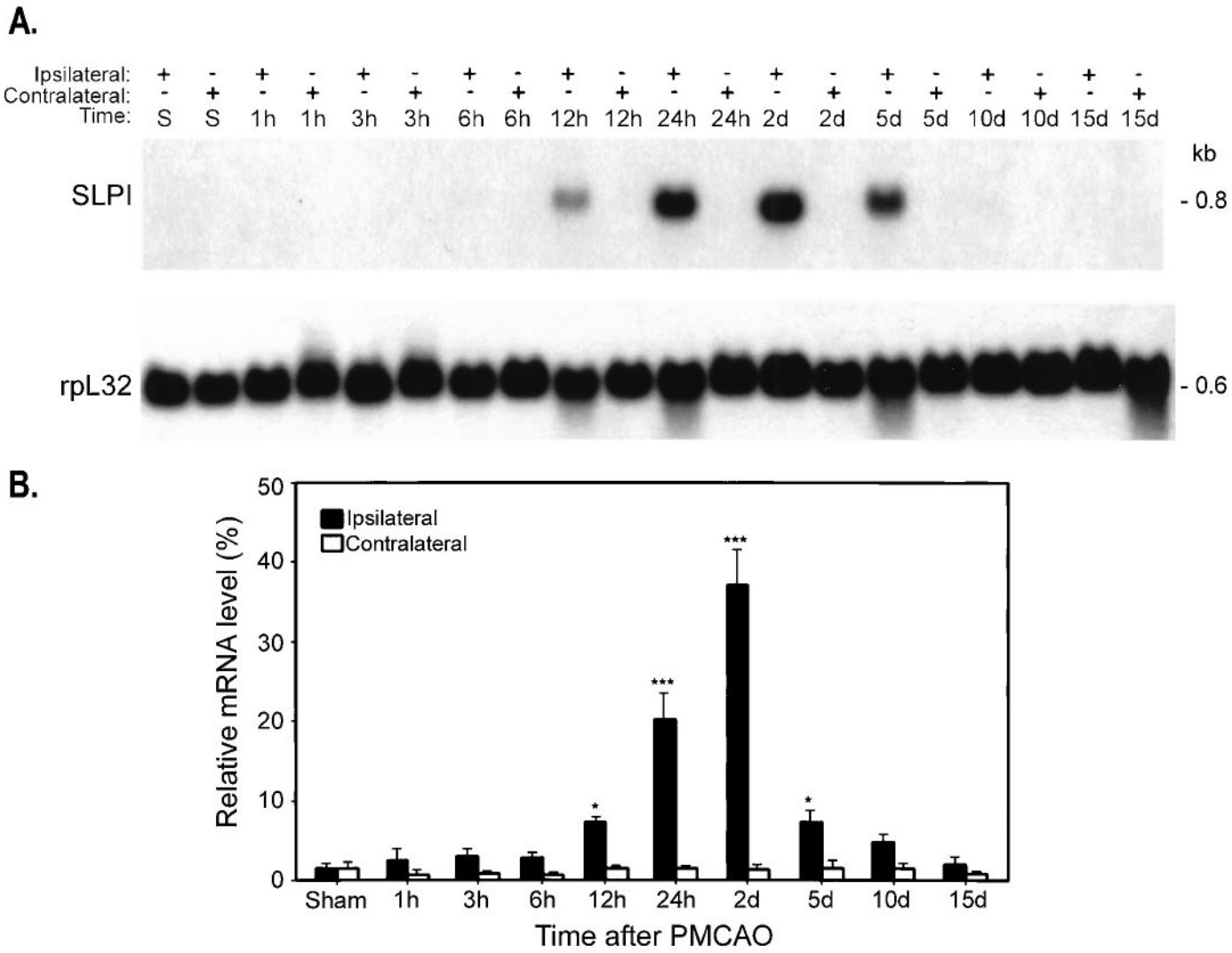

Fig. 3. Time-course study of SLPI mRNA induction in rat ischemic cortex after MCAO. A, a represent Northern blot for SLPI and rpL32 cDNA probes to the samples from spontaneously hypertensive rats (SHR) after MCAO. Total cellular RNA (40 μg/lane) was resolved by electrophoresis, transferred to a nylon 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.

tactic protein-1 and interferon inducible protein-10), and cell adhesion molecules (e.g., intracellular adhesion molecule-1 and endothelial-leukocyte adhesion molecule-1), which reach a peak level at 12 h for transcripts and 24 h for protein after focal stroke (del Zoppo et al., 2000; Wang and Feuerstein, 2000). Thus, SLPI induction in the brain paralleled with macrophage accumulation, which followed neutrophil infiltration (Clark et al., 1993; Garcia et al., 1994) in the ischemic brain tissue. It is possible that inflammatory cytokines might be responsible for the induction of SLPI, as has been demon-

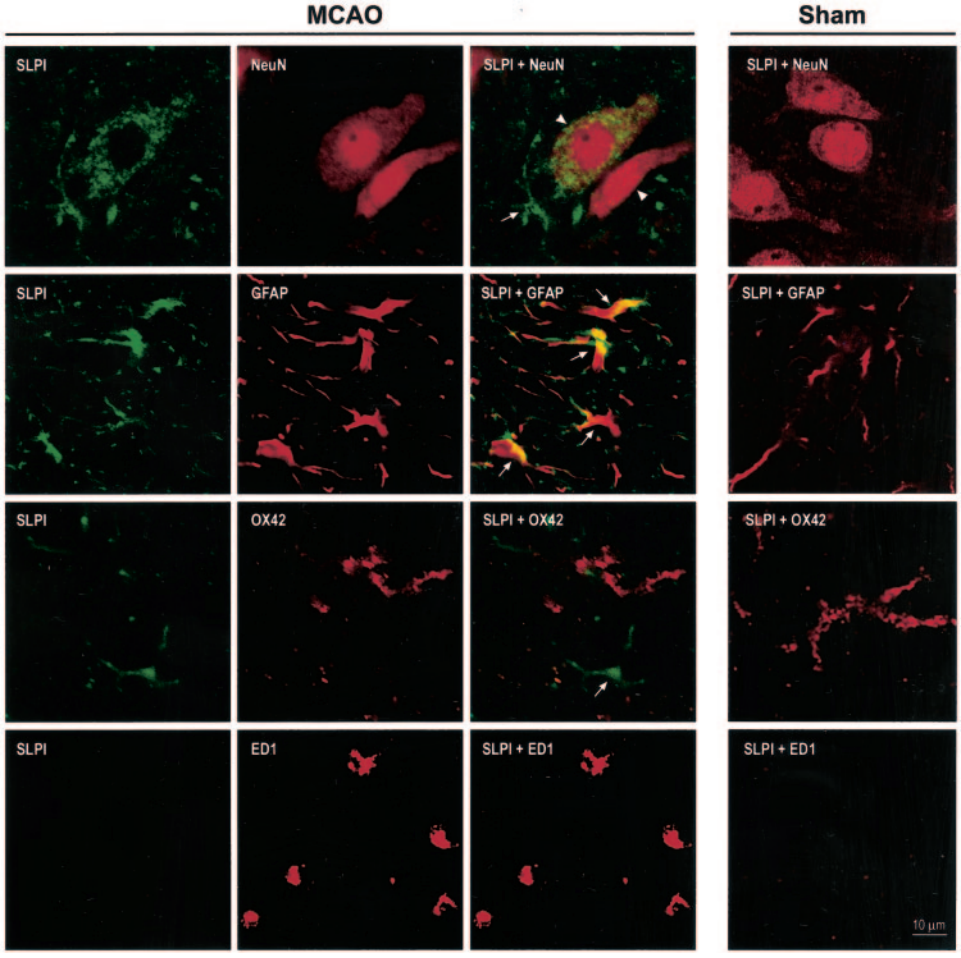

Fig. 4. Confocal immunohistochemical analysis of SLPI induction in rat brain after MCAO. SLPI immunoreactivity was detected with rabbit anti-human SLPI followed by biotinylated anti-rabbit IgG incubated with fluorescein streptavidin (green). NeuN, GFAP, OX42, or ED1 immunoreactivity was detected with mouse anti-NeuN, anti-GFAP, anti-OX42, or anti-ED1 antibodies, repsectively, followed by Texas Red dye-conjugated anti-mouse IgG (Fab)2 (red). The merged images (SLPI + NeuN, SLPI + GFAP, SLPI + OX42, and SLPI + ED1) illustrate regions of colocalization (yellow), demonstrating that SLPI immunopositive cells are astrocytes and a subset of neurons. The images illustrated for NeuN, GFAP, and OX42 are the peri-infarct areas 24 h after MCAO, and the image shown for ED1 staining is the ischemic zone 72 h after MCAO. Neurons immunoreactive to NeuN are indicated with arrowheads, and GFAP-positive astrocytes are illustrated with arrows. Note the SLPI immunoreactive astrocytes (shown with arrows) in the colocalization fields with NeuN and OX42. Right, control immunohistochemical study of SLPI expression in the brain after sham operation (24 h); no immunoreactive signal was detected for SLPI. Scale bar, 10 μm.

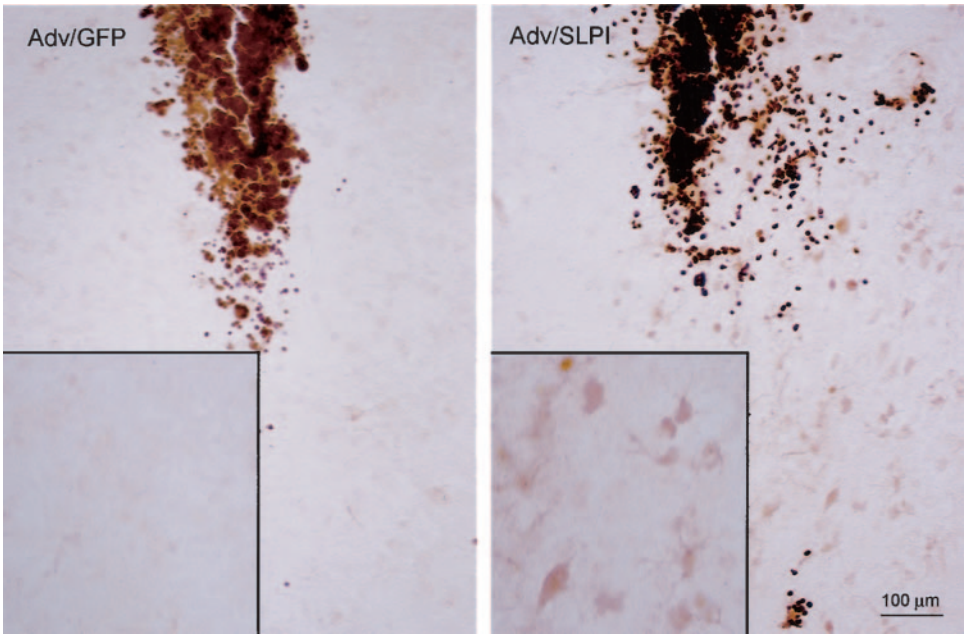

Fig. 5. Immunohistochemical study showing the expression of Adv/SLPI in the infected cortical region. Recombinant Adv/SLPI or Adv/GFP was injected into dorsal cortical region in rats as described in detail under *Materials and Methods*. Immunohistochemical analysis was used to evaluate the expression of SLPI protein by the recombinant adenovirus using rabbit anti-human SLPI antibodies. Brain tissues were collected 2 days after adenovirus injection; area including a needle trace was localized and illustrated for Adv/GFP- (left) or Adv/SLPI-injected (right) rats. Insets are high-power observations of brain tissue adjacent to the needle-traces after recombinant virus injection. Whereas the precise cellular source of adenoviral infection was not determined by coimmunostaining with a specific marker, SLPI immunoreactive cells (stained in brown) seem to include neurons, astrocytes, and microgia-like cells.

strated in cultured keratinocytes (Tanaka et al., 2000); or in turn, SLPI might suppress inflammatory cytokine gene expression as shown after hepatic ischemic reperfusion injury (Lentsch et al., 1999) or affect cytokine activity (such as TGF-β) as shown in cutaneous wound healing in SLPI-null mice (Ashcroft et al., 2000). In addition, the expression of SLPI preceded that of TGF-β1 after ischemic brain injury (Wang et al., 1995), and TGF-β1 was shown to inhibit SLPI expression in cultured bronchial epithelial cells (Jaumann et al., 2000). The temporal expression profile of SLPI with inflammatory mediators and leukocyte accumulation in the brain after ischemic injury again suggests that SLPI may be associated with regulation of inflammatory response. On the other hand, the delayed induction of SLPI in the ischemic process (peaking 2 days after ischemia), as well as its functions in suppression of TGF-β1 (which is also known to be involved in tissue remodeling) and serine protease activity (Ashcroft et al., 2000), suggests that ischemia-induced expression of SLPI may play a role in tissue remodeling rather than in the mechanisms of acute damage.

Our present study demonstrated for the first time that SLPI was expressed in neurons and astrocytes in the ischemic brain tissue. The significance of SLPI expression in these cells is unknown. In particular, because SLPI is a secreted protein, its function is not limited to the cells that produce the protein. Despite the presence of a large number of macrophages in the ischemic brain tissue (Clark et al., 1993; Garcia et al., 1994) and their capability (along with astrocytes and neurons) to produce various substrates of SLPI, neither macrophages nor activated microglia were found to express SLPI (Fig. 4). These data suggest that cells endogenous to the brain are actively participating in SLPI induction, and SLPI-mediated neuroprotection may be not limited to its anti-inflammatory actions; in particular, the area of its expression was mainly located in the peri-infarct zone, where liable brain tissue could still be rescued. Therefore, it is reasonable to speculate that ischemia-induced SLPI expression (especially in neurons) might be associated with its role in rescuing neurons from apoptosis. To test this hypothesis, we applied a well characterized apoptosis model

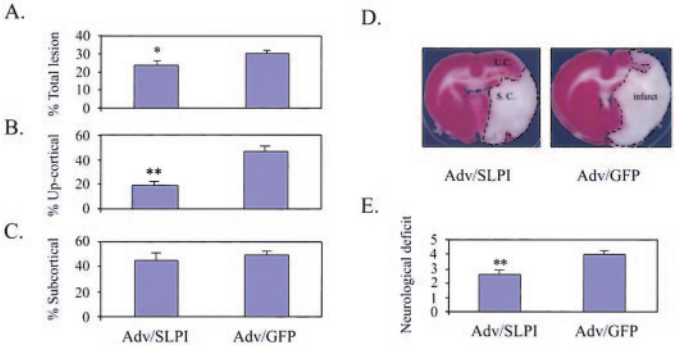

Fig. 6. Effect of cortical Adv/SLPI injection on ischemic brain injury. Recombinant adenoviruses Adv/SLPI and Adv/GFP were injected into four up-cortical positions in the ipsilateral hemisphere as described in detail under *Materials and Methods*. Two days after adenovirus injection, rats were subjected 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.

in PC12 cells induced by UV irradiation and/or staurosporine (Erhardt et al., 2000) and tested its outcome after Adv/SLPI or Adv/GFP infection. No difference was observed between Adv/SLPI and control virus groups in either UV irradiation-or staurosporine-induced apoptosis in PC12 cells (data not shown). This result suggests that it is unlikely that the expression of SLPI plays a direct role in apoptosis.

It is of interest to note that although SLPI sequences, particularly those of critical residues for its function and protein structure, are highly conserved among three species (rat, mouse, and human), variability of the protein sequences exists in rats (Fig. 2). Although the possible cause of different cellular sources could not be excluded (i.e., the clone by Song et al. (1999) from macrophages, the one by Gipson et al. (1999) from lung, and ours from brain), our present study showed that the rat SLPI cDNA sequence from spleen and ischemic brain tissue of the same rat strand (SHR) is exactly identical. Because different rat strands have been used for these studies [Long-Evans rat by Gipson et al. (1999), unspecified strand by Song et al. (1999) and SHR in ours], the sequence variability of rat SLPI might be caused by different rat strands or different genes that encode SLPI (because the copy number of this gene in rats has not been determined).

In summary, our present study illustrates the molecular cloning of a rat SLPI isoform in the ischemic brain tissue. Its unique temporal and spatial distribution, as well as the demonstration of its neuroprotective capacity in stroke using adenoviral transfection, suggests its potential utility as a neuroprotective agent.

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