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Recombinant adenoviral expression of dominant negative IκBα protects brain from cerebral ischemic injury

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Abstract

Transcription factor NF-κB is associated with inflammatory response and cell survival. Under inactive condition, NF-κB is sequestered in the cytoplasm by an anchor protein, inhibitor of NF-κB (IκB). NF-κB was shown to be activated during ischemic brain injury. In the present study we have investigated the role of NF-κB in ischemic brain injury using a recombinant adenovirus expressing a dominant negative form of IκB (Adv/IκBdn) to specifically inhibit NF-κB activation. Our data demonstrated that cortical injection of Adv/IκBdn significantly reduced ischemic brain injury following permanent occlusion of the middle cerebral artery (MCAO) in rats, showing 55% reduction ($p < 0.01$, $n = 8$) in total ischemic lesion or 80% reduction ($p < 0.001$) in the cortical area with Adv/IκBdn expression. Similarly, Adv/IκBdn expression significantly decreased neurological deficits (37% reduction over controls, $p < 0.01$, $n = 8$). These data provide further evidence for the role of NF-κB/IκB in ischemic brain injury and suggest that inhibition of NF-κB is neuroprotective in focal stroke.
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NF-κB is a heterodimer transcription factor, consists of a 65 kDa protein (p65/RelA) and a 50 kDa protein (p50) that is sequestered in the cytoplasm by an anchor protein, inhibitor of NF-κB (IκB). Phosphorylation of NF-κB on serines 32 and 36 by IκB kinase leads to its ubiquitination and degradation by proteosomal enzymes, which allows NF-κB heterodimer to translocate to the nucleus and regulate gene expression [1]. There are several forms of IκB protein that have been identified, of which IκBα represents the predominant form in the brain [2].

Cerebral ischemia is a pathophysiological condition caused by decrease in blood supply to the brain and hence the deprivation of oxygen and glucose in the ischemic brain eventually leads to cell death (necrosis and apoptosis), inflammation, and tissue repair [3]. NF-κB is expressed in diverse cell types in the nervous systems [4]. NF-κB is activated and plays a crucial role in ischemic brain injury as evidenced by reduced ischemic injury and cell death in p50 NF-κB subunit deficient mice [5]. NF-κB is also a key transcription factor that regulates a number of inflammatory mediators such as inflammatory cytokines and adhesion molecules [1] that have been suggested to play an important role in ischemic brain damage [3]. On the other hand, inhibition of NF-κB by an inhibitor such as diethyldithiocarbamate (DDTC) was shown to increase cell death and infarct size following transient ischemic insult in rats [6], suggesting that NF-κB induces survival signaling in neuronal cells as well. The conflicting information derived from genetic models and the pharmacological studies are difficult to reconcile, since genetic models may represent developmental adaptation and compensatory situation that are not present in normal animals while pharmacological agents have not been proven to act selectively and their effects might be contaminated by multiple biochemical interactions. Therefore, our present study was designed to further validate the role of NF-κB in ischemic brain injury by using a recombinant

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adenovirus expressing a dominant negative form of IκB (Adv/IκBdn) as a specific inhibitor for NF-κB activation [7,8] in a rat model of focal stroke.

Materials and methods

Focal brain ischemia. Rats were housed and cared for in accordance with the Guide for the Care and Use of Laboratory Animals [DHEW (DHHS) Publication No. (NIH) 85-23, revised 1996, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205]. Procedures using laboratory animals were approved by the Institutional Animal Care and Use Committee of Bristol-Myers Squibb Company.

Focal cerebral ischemia was created by occlusion of the middle cerebral artery (MCAO) using a 3–0 mono-filament suture in male Sprague–Dawley rats weighing 250–330 g as described in detail previously [9]. Rats were anesthetized with gas inhalation that comprised of 30% oxygen (0.3 liter/min) to 70% nitrous oxide (0.7 liter/min) mixture. The gas was passed through an isoflurane vaporizer set to deliver 3–4% isoflurane for initial anesthesia induction and 1.5–2% during surgery. Sham-operation was performed using the same procedure except that no suture was inserted.

Measurement of infarct volume. To measure the infarct volume, brains were removed after anesthesia 24 h post-MCAO and evaluated 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 (PBS). The image was captured using a Microtek ScanMaker 4 DUO Scanner (MicroWarehouse Lakewood, NJ) and quantitated using an Image Pro Plus 4.1 software (Media Cybernetics, Silver Spring, MD). Total ischemic lesion or the breakdown lesions in up-cortical (the adenovirus infected area) and subcortical (from caudate putamen to lat preoptic area) regions were measured.

Neurological deficits. Neurological deficits were also examined in the same groups prior to TTC staining using a 5-point scale as described elsewhere [10]. Specifically, 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.

Physiological measurements. Regional cerebral blood flow (CBF) was measured with a Laser Doppler Perfusion Monitor (Moor Instruments, Wilmington, DE). Under anesthesia, a small incision was made at the midpoint between the right orbit and the external auditory canal. The temporalis muscle was retracted and the underlying fascia was cleared. A small area of skull about 1 mm posterior and 5 mm lateral to the Bregma in the ipsilateral hemisphere was thinned to allow placement of the laser Doppler probe. CBF was monitored 15 min before and 2 h after MCAO.

The arterial blood pressure and heart rate were measured using an MP100 Workstation and analyzed using an AcqKnowledge software (BIOPAC Systems, Santa Barbara, CA) according to manufacturer's specification. Femoral arterial blood samples were analyzed for pH, oxygen (*p*O2), and carbon dioxide (*p*CO2) by direct collection through a PE-50 tubing into an i-STAT G3+ cartridge and processed with a portable clinical analyzer (Abbott Laboratories, Abbott Park, IL).

Recombinant adenovirus and cortical injection. A dominant negative mutant IκBα [7] was subcloned into the shuttle plasmid pAdv/CMV. The resulting plasmid, pAdv/CMV-IκBdn, was co-transfected with a helper plasmid, pJM17, into 293 cells to generate the recombinant adenovirus, Adv/IκBdn [8]. The recombinant adenoviruses were plaque purified and amplified in 293 cells. Concentrated adenoviruses were prepared by CsCl gradient centrifugation, followed by desalting with chromatography in 1 mM MgCl2 in phosphate-buffered saline (PBS). 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/GPF, containing the cDNA of green fluorescent protein (GFP), was generated by a similar strategy.

Cortical injection of Adv/IκBdn and Adv/GFP was carried out using a stereotaxic instrument. Each rat was subjected to 4 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 side of the brain, i.e., ipsilateral hemisphere to the MCAO. Two μl 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 adenovirus injection, rats were subjected to MCAO as described above.

Immunohistochemical analysis. Immunohistochemical analysis was used to confirm the expression of Adv/IκBdn at 24 and 48 h after cortical adenovirus injection in rats as described in detail previously [9] except that mouse anti-IκB antibody was used as the primary antibody in the present study, which detects both IκB wild type and dominant negative proteins.

Statistical analysis. Data in text and figures are means ± standard errors for the indicated number (n) of animals. Statistical comparisons were made by analysis of variance (ANOVA; Fisher's protected least squares difference) and values were considered to be significant when $p < 0.05$.

Results and discussion

Fig. 1 illustrates the detection of a strong IκB immunoreactive signal in brain tissues adjacent to the needle trace following Adv/IκBdn injection; in contrast, only a weak basal immunoreactive signal of IκB was detected in

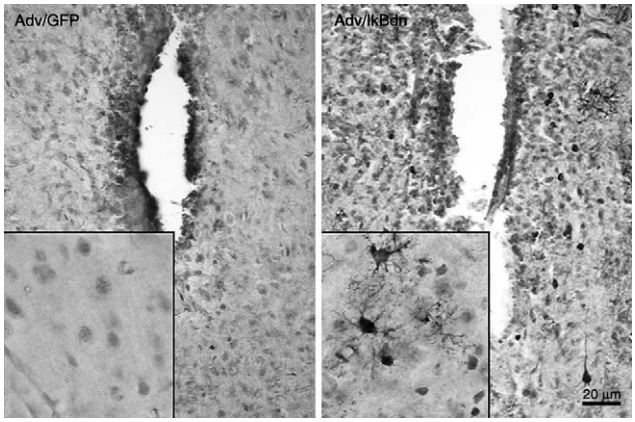

Fig. 1. Immunohistochemical study showing the expression of Adv/IκBdn in the infected cortical region. Recombinant Adv/IκBdn or Adv/GFP was injected into dorsal-cortical region in rats as described in detail in Materials and methods. Immunohistochemical analysis was used to evaluate the expression of IκBdn protein by the recombinant adenovirus using anti-IκB antibodies. Brain tissues were collected two days after adenovirus injection; area including a needle trace was localized and illustrated for Adv/GFP- (left) or Adv/IκBdn-injected (right) rats. Insets are high power observation of brain tissue adjacent to the needle-traces following recombinant virus injection. Note that IκB immunoreactive cells are illustrated.

the control, Adv/GFP injected rats, suggesting the active expression of IκBdn protein by Adv/IκBdn.

The biological function of the same Adv/IκBdn as a dominant negative inhibitor of NF-κB activation was demonstrated in fibroblasts by measuring NF-κB-dependent cytokine production in response to lipopolysaccharide (LPS) stimulation [8], and in rat aortic endothelial cells for NF-κB-dependent fractalkine induction in response to interleukin-1β (IL-1β), tumor necrosis factor-α (TNFα), and LPS stimulation [11].

The effect of IκBdn on ischemic brain injury was investigated by measuring infarct size and neurological function as end-points to compare the Adv/IκBdn- and Adv/GFP-treated rats. Total ischemic lesions, or the breakdown lesions in up-cortical (the adenovirus infected area) and subcortical (from caudate putamen to lat preoptic area) regions, are illustrated in Fig. 2. The expression of Adv/IκBdn in the cortex resulted in 55% reduction in total ischemic lesion ($n = 10$, $p < 0.01$ compared to controls) or 80% reduction in the up-cortical region (the site of Adv/IκBdn expression; $p < 0.001$) (Fig. 2). In contrast, there was no difference in the non-viral infected subcortical regions between the two groups (Fig. 2). Similar to the reduction in infarct size, neurological deficits were markedly reduced in the Adv/IκBdn infected rats (37% reduction over controls, $n = 10$, $p < 0.001$) (Fig. 2).

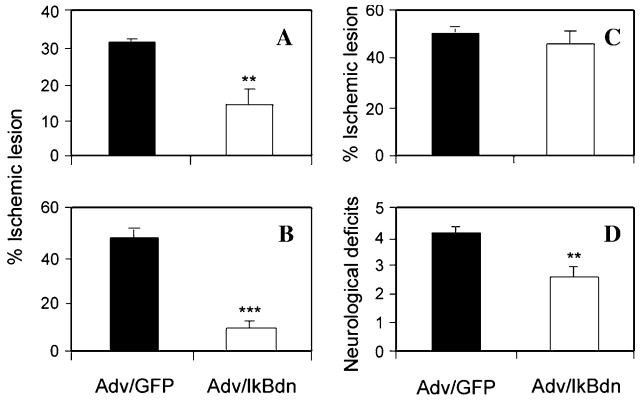

Fig. 2. Effect of cortical Adv/IκBdn injection on ischemic brain injury. Recombinant Adv/IκBdn and Adv/GFP were injected into four positions of dorsal-cortical region at the ipsilateral hemisphere. Two days after the adenovirus injection, rats were subjected to MCAO. Ischemic lesions are (A–C) and neurological deficits (D) were measured 24 h after MCAO. Total ischemic lesions depicted in (A) and the lesions at the dorsal-cortical region (the site of adenovirus infection) and subcortical region are illustrated in (B) and (C), respectively. Neurological deficits were determined as described in Materials and methods and depicted in (D). $^{**}p < 0.01$ and $^{***}p < 0.001$, compared with Adv/GFP-treated animals.

Cerebral blood flow, heart rate, arterial blood pressure, pH, blood oxygen (pO_2), and carbon dioxide (pCO_2) were measured in Adv/IκBdn- and Adv/GFP-treated rats before and after MCAO. No significant difference was observed in all these parameters between these two experimental groups (Table 1).

Taken together, our present study demonstrated that the adenoviral expression of IκBdn protected the brain from ischemic injury, as evidenced by reduction in infarct size and neurological deficits; yet, no difference was observed in CBF and several key hemodynamic and hematologic parameters as compared to the control group under normal conditions or post-stroke. Since IκBdn protein expressed by Adv/IκBdn was shown to be the specific inhibitor of NF-κB activation in vitro [7,8,11], IκBdn is likely to have the same effect in ischemic brain injury. These data are also in agreement with a previous report that mice deficient in NF-κB p50 subunit significantly reduced ischemic brain injury, possibly by suppression of programmed cell death [5]. It is also of interest to note that selective up-regulation of neuronal nuclear IκBα was observed in ischemic penumbra following focal cerebral ischemia [12], suggesting a correlation between IκBα and neuronal survival. However, inhibition of NF-κB by means of the NF-κB inhibitor, DDTC, showed increase in cell death and infarct size in a rat model of transient MCAO [6], suggesting a protective mechanism of NF-κB activation in focal stroke. It should be pointed out that while DDTC has been used as a NF-κB inhibitor, additional roles of this drug were identified as antioxidants or inhibitor of superoxide dismutase and zinc ionophores [13]. In addition to its role in brain ischemia, activation of NF-κB was shown to be crucial in brain tolerance in rats [14,15].

Table 1
Physiological conditions in rats with cortical administration of Adv/IκBdn or Adv/GFP before and after MCAO

Treatment	MCAO	CBF	HR (min^{-1})	MABP (mmHg)	pCO_2 (mmHg)	pO_2 (mmHg)	pH
Adv/GFP		$n = 9$	$n = 9$	$n = 9$	$n = 5$	$n = 5$	$n = 5$
	Before	100	351 ± 19	112 ± 4	47 ± 3	131 ± 8	7.42
	2 h	14 ± 3	365 ± 11	97 ± 5	44 ± 2	137 ± 10	7.41
Adv/IκBdn		$n = 9$	$n = 9$	$n = 9$	$n = 5$	$n = 5$	$n = 5$
	Before	100	339 ± 8	115 ± 2	48 ± 2	135 ± 9	7.43
	2 h	13 ± 3	370 ± 10	100 ± 4	43 ± 4	139 ± 11	7.42

Adv/IkBdn or saline was injected in the cortical regions 48 prior to MCAO as described in Materials and methods. Physiological data were measured at 15 min before and 2 h after MCAO. CBF, cerebral blood flow (illustrated as percentage relative to pre-MCAO levels); HR, heart rate (per min); MABP, mean artery blood pressure (mmHg). No statistical difference was observed in any of these parameters between the two groups.

Thus, it is possible that NF-κB might play a role in both cell death and survival in different conditions.

In addition, NF-κB is one of the key regulators of a number of inflammatory mediators including inflammatory cytokines, leukocyte adhesion molecules, inducible nitric oxide synthase, cyclooxygenase-2, and matrix metalloproteinase-9 [16,17]. These inflammatory mediators are upregulated and suspected to have contributed to ischemic brain injury [3]. Therefore, it is likely that the effect of Adv/IκBdn on ischemic injury may involve regulation of inflammatory mechanisms through NF-κB. Likewise, ischemia/reperfusion injury has also been associated with inflammatory reaction and reported to be modulated by the activation of NF-κB in the heart [18], lung [19], and liver [20].

In summary, the data provided in this study suggest that acute inhibition of NF-κB activation protects the brain from ischemic insult. Whether acute intervention after onset of ischemic brain injury may provide equal neuroprotection remains to be further investigated.

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