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Research Report

Apoptosis signal-regulating kinase 1 (Ask1) targeted small interfering RNA on ischemic neuronal cell death

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ABSTRACT

Apoptosis signal-regulating kinase 1 (Ask1) is one of mitogen-activated protein kinase kinase kinase (MAPKKK) for cell differentiation and apoptosis. The aim of the present study is to evaluate whether RNA interference against Ask1 (Ask1-siRNA) down-regulates the expression of Ask1 and prevents apoptotic neuronal cell death after ischemia/reperfusion (I/R) in mice. Mice were subjected to intraluminal suture occlusion of the middle cerebral artery for 1 h, followed by reperfusion. The Ask1-siRNA or a control-siRNA was introduced using osmotic pump intracerebroventricularly at 3 days before I/R. The expression and mRNA of Ask1 were evaluated by Western blot and RT-PCR after I/R with time. Immunohistochemistry and TUNEL assay were also investigated to evaluate the effect of Ask1 on cerebral infarction by Ask1-siRNA treatment. The expression of Ask1 was increased significantly at 8 h after I/R. The level of mRNA and protein of Ask1 down-regulated after treatment of Ask1-siRNA and subsequently cerebral infarction volume was reduced. Our results suggest the increased Ask1 expression induce apoptotic cell death after I/R. And we also demonstrated that Ask1-siRNA attenuates upregulation of Ask1, which was followed by the reduction of infarction in ischemic brain after I/R. Ask1-siRNA could represent a molecular target for prevention of ischemic stroke.

1. Introduction

Numerous studies have reported that reactive oxygen species (ROS) can cause cell injury, directly or indirectly, through the signal transduction system. ROS plays an important role in the pathophysiology of cerebral ischemia through apoptosis (Chan, 2001; Fujimura et al., 1999, 2000; Murakami et al., 1997; Saito et al., 2004). The mitogen-activated protein kinase (MAPK) cascade is activated in response to diverse external stimulation, ROS, ultraviolet light (UV), and growth factors. MAPK has been shown to be involved in cell differentiation, growth, and apoptosis (Errede and Levin, 1993; Widmann et al., 1999). Apoptosis signal-regulating kinase 1 (Ask1), a member of the mitogen-activated protein kinase kinase kinase (MAPKKK) family, is widely distributed in various cells and is thought to be essential for cell differentiation and apoptosis (Chang et al., 1998; Hayakawa et al., 2006; Nishitoh et al., 1998; Saitoh et al., 1998; Takeda et al., 2003; Tobiume et al., 1997). In addition, Ask1 has been reported to activate under the state of stress and induce apoptosis through the MAPK cascade, SEK1–JNK signal pathway (c-Jun NH2-terminal kinase), and MAPKK3/MAPKK6-p38 signal pathway (Ichijo et al., 1997). Several studies have indicated that the JNK signaling (MAPK) is involved in neuronal cell death after cerebral ischemia (Gu et al., 2001; Ozawa et al.,

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1999). However, the role of Ask1 (MAPKKK), which is an upstream signal transduction participant in the MAPK cascade, is not well understood in brain.

In this study, we investigated the association between the temporal expression profile of Ask1 and apoptotic cell death in a murine model of transient focal cerebral ischemia. In addition, the effectiveness of Ask1 down-regulation in preventing ischemic cell death was evaluated.

2. Results

2.1. *Change in middle cerebral artery (MCA) blood flow during I/R*

The amount of blood flow in the middle cerebral artery was measured 10 min prior to ischemia, 10 min after ischemia, and 10 min after reperfusion. There were no statistically significant differences in regional cerebral blood flow before occlusion versus after reperfusion (%: 10 min prior to ischemia, 100±0, 10 min after ischemia, 14.4±2.8, 10 min after reperfusion, 97.3±1.8, mean±SEM, n=5).

2.2. *Expression pattern of Ask1 and phosphorylated Ask1 (pAsk1)*

Western blot analysis was used to assess changes in expression patterns of Ask1 and pAsk1 after I/R with time. Ask1 and pAsk1 immunoreactivity was detected at molecular weight 155 kDa (Fig. 1A). Ask1 expression was slightly increased and maintained for 4 h, but became significantly elevated at 8 h. Levels of pAsk1 also rapidly increased at 8 h after I/R, compared to normal controls (Ask1 O.D.: Nor., 127.66±16.01; 1 h, 272.38±28.47; 2 h, 197.42±19.81; 4 h, 286.99±35.41; 8 h, 596.63±106.88; 24 h 572.53±91.86; pAsk1 O.D.: Nor., 113.36±34.38; 1 h, 157.34±39.55; 2 h, 219.06±41.07; 4 h, 181.25±43.54; 8 h, 465.75±130.68; 24 h 323.37±323.37; mean±SEM, n=5, Nor., Normal control; *P<0.05; Fig. 1).

2.3. *Suppression of Ask1 and pAsk1 protein and mRNA by treatment with Ask1-siRNA*

To assess outcome at 24 h after I/R, ask1 inhibition by Ask1-siRNA treatment was tested by Western blot analysis and RT-PCR in both the Ask1-siRNA and Cont.-siRNA treatment groups (Fig. 2). RT-PCR analysis showed that Ask1 gene expression in the Ask1-siRNA treatment group was significantly suppressed 24 h after I/R (O.D.: Cont.-siRNA, 100±0; Ask1-siRNA, 11.1±6.8; mean±SEM, n=5, *P<0.05; Fig. 2A). Western blot analysis demonstrated that the expression of Ask1 protein was reduced in the Ask1-siRNA treatment group compared with the Con.-siRNA treatment group (O.D.: Cont.-siRNA, 100±0; Ask1-siRNA, 26.8±6.0; mean±SEM, n=5, *P<0.05; Fig. 2B). Furthermore, pAsk1 was barely expressed (O.D.: Cont.-siRNA, 100±0; Ask1-siRNA, 24.1±8.7; mean±SEM, n=5, *P<0.05; Fig. 2C). These results suggest that our synthetic Ask1-siRNA suppresses Ask1 expression, and subsequently pAsk1, after I/R.

2.4. *Reduction of cell death and infarct size by Ask1-siRNA treatment*

To examine whether suppression of Ask1 expression protects the brain from ischemic injury, apoptotic cell death and lesion size were compared between the two groups, using immunohistochemistry and TTC staining.

To determine the relationship between Ask1 expression and DNA fragmentation in neuronal cells, double/triple fluorescence labeling was performed for Ask1 (green/red), TUNEL (green), nuclei (blue) and NeuN (red) staining in mouse brain (Fig. 3A and B). In the Ask1-siRNA group, there was a significant decrease in Ask1 immunopositive cells. No remarkable TUNEL-positive cells were detected at 24 h after I/R (Ask1; Cont.-siRNA, striatum, 680.0±191.1, cortex, 600.4±157.5; Ask1-siRNA, striatum, 22.1±1.9, cortex, 7.4±5.7; mean±SEM, n=5, *P<0.05; Fig. 3Ab) (TUNEL; Cont.-siRNA, striatum, 789.9±179.8, cortex, 981.8±206.7; Ask1-siRNA, striatum, 97.8±39.2, cortex, 39.4±18.3; mean±SEM, n=5, *P<0.05; Fig. 3Ad). TTC staining showed results consistent with

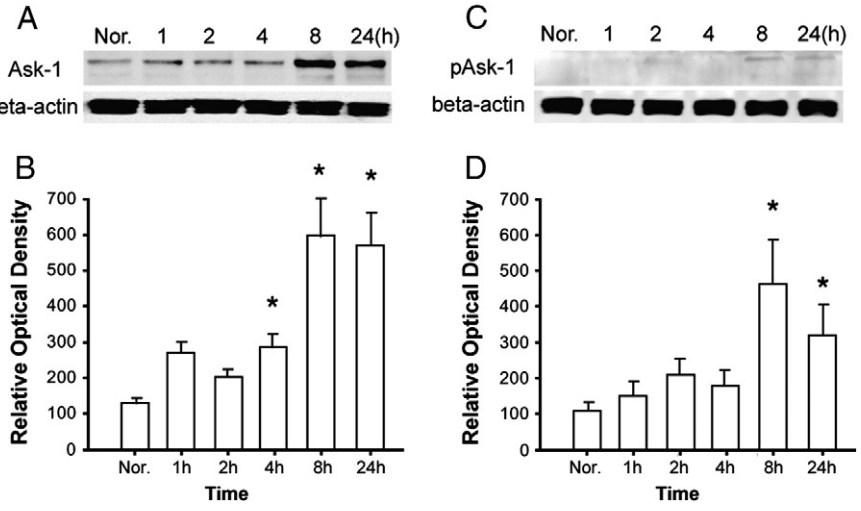

Fig. 1 – Western blot analyses for Ask1 and pAsk1. Expression of Ask1 and pAsk1 protein was quantified at times after Ischemia/Reperfusion. Nor, normal control; mean±SEM; *P<0.05.