[S8-1] [10/11/2003(Sat) 09:30-10:10/ Grand Ballroom 105]

Molecular Basis of Neuronal Cell Death Following Neonatal Hypoxic-Ischemic Brain Injury

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Hypoxic-ischemic (H-I) encephalopathy in the prenatal and perinatal period is a major cause of morbidity and mortality and often results in cognitive impairment, seizures, and motor impairment (cerebral palsy). Many studies of neonatal H-I brain injury have utilized the well characterized Levine model in which unilateral carotid ligation is followed by exposure to hypoxia. This model of H-I results in a reproducible pattern of hemispheric injury ipsilateral, but not contralateral, to the carotid ligation. We have previously shown that there are prominent features of both apoptosis and necrosis when this model is performed in neonatal rats and mice. Currently, there is no effective treatment to prevent the consequences of neonatal H-I in humans. Thus, understanding how specific molecules contribute to neonatal H-I brain injury may give new insights into pathogenesis and treatment.

We previously found that H-I insult results in predominantly neuronal death. There was a marked increase in caspase-3-like activation, which is a hallmark feature of apoptosis. Peptide-based pan-caspase inhibitors are neuroprotective against neonatal H-I brain injury, suggesting a central role of caspases in brain injury following H-I. However, currently available peptide-based caspase inhibitors lack selectivity and potency, which would be key factors to develop therapeutics. In this study, we explored the neuroprotective effects of small, non-peptide caspase-3 inhibitors MF826 and MF867. This compounds selectively and potently inhibited both caspase-3 enzymatic activity (IC_{50}: 1~5 nM) and apoptosis in cultured cells *in vitro* (IC_{50}: 30 ~ 120 nM). In a rat model of neonatal H-I, MF826 blocked caspase-3 activation and cleavage of its substrates, without affecting calpain activation in the cortex and hippocampus following H-I. Though MF826 significantly reduced DNA fragmentation and brain tissue loss, apoptotic-like cell death was still present in the brain of rat treated with MF826. We found that caspase-2 processing/activation occurred prior to caspase-3 activation in neurons with a nuclear feature of apoptosis following H-I. MF826 did not influence caspase-2 processing/activation following H-I. These data suggest that caspase-2 appears to contribute to neuronal cell death through the caspase-3-independent pathway.

Clusterin/apolipoprotein J is a ubiquitously expressed, secreted molecule postulated to influence a variety of processes including cell death. In the brain, it accumulates in dying neurons following seizures and hypoxic-ischemic injury. Despite this, *in vivo* evidence that clusterin directly influences cell death is lacking. We found that following neonatal H-I brain injury in mice, there was evidence of marked apoptotic-like cell death with neuronal caspase-3 activation as well as accumulation of clusterin in dying neurons. Clusterin

deficient (-/-) mice had significantly decreased (50% less) brain injury following neonatal H-I as compared to mice expressing clusterin. Surprisingly, the absence of clusterin had no effect on caspase-3 activation, and clusterin accumulation and caspase-3 activation did not co-localize to the same cells. Immuno-EM studies demonstrated that clusterin localized to neurons dying via necrosis. Studies with cultured cortical neurons demonstrated that exogenous, purified astrocyte-secreted clusterin exacerbated oxygen-glucose deprivation-induced necrotic death. These results suggest that clusterin may be a new therapeutic target to modulate non-caspase-dependent neuronal death following acute brain injury.