relatively abundant collagen fibers were found in and around the adventitia of the blood vessels, which resulted from the invasion of the neoplastic astrocytes into the perivascular tissue. These findings indicated that the small nodules were astrocytoma.

The present studies clarified that so-called fowl glioma is a viral disease caused by an ALV and that nonsuppurative myocarditis concomitantly occurs in birds affected with the disease. Ultrastructural study indicated that gliomatous nodules at the initial stage of the proliferation should be considered as astrocytoma with reactive perivascular fibrosis.

Original papers of this thesis appeared in *Avian Pathology*, 31 : 197-203 (2002) and *The Journal of Veterinary Medical Science* (2002) (in press).

Vitrification of mouse preantral follicles using a mixture of ethylene glycol and raffinose

Eufrocina dela Peña Atabay

Laboratory of Theriogenology, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

---

Original papers of this thesis appeared in *Cryobiology*, 42 : 103-111 (2001) and *Reproduction*, 123 : 593-600 (2002).

Study of the neuroprotective mechanisms by the spin trap α-phenyl-N-tert-butylnitrone

Masahisa Tsuji

Laboratory of Radiation Biology, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

α-Phenyl-N-tert-butylnitrone (PBN) is an agent used most widely for investigating free radicals in biological systems and is known to be able to prevent oxidative injury without significant toxicity. PBN attenuates the age-related protein oxidation, decrease of enzyme activity and loss of memory in gerbils, and protects hippocampal cells from ischemia-reperfusion injury. It also protects LEC rats from copper-induced fulminant hepatitis. PBN is thought to exert these biological effects by blocking oxidative stress-induced free radical reactions. Recently, PBN has the ability to interfere with inflammatory cytokines, to increase anti-inflammatory cytokines and to inhibit the induction of inducible nitric oxide synthase in lipopolysaccharide-administrated rats or mice. These effects may be considered as the interruption of the redox-sensitive inflammatory signaling pathways such as NFκ-B by PBN. Furthermore, PBN attenuated the phosphorylation of p38 mitogen-activated protein kinase (p38) and increased the phosphatase activity in primary rat glia

and primary rat astrocyte by augmenting oxidant-sensitive phosphatase activity. These reports suggest that the protective effects of PBN are derived not only from its inherent ability for radical trapping but also from alteration of intracellular redox state. In this study, to clarify the effects of PBN on neuroprotective signal transduction pathways, *in vitro* and *in vivo* experiments were performed using rat pheochromocytoma cell line PC12 and stroke model in gerbil suffered from ischemia-reperfusion, respectively.

In *in vitro* experiments using PC12 cells, we found that PBN induced neurite outgrowth in a dose-dependent manner. The treatment of PC12 cells with 10 mM of PBN for 3 days induced it in about 70% of treated cells. Morphologically, the majority of neurites induced by PBN were bipolar in contrast to the multi-polar neurites observed in NGF-treated cells. We further examined the involvement of the TrkA-Ras-ERK cascade in PBN induced neurite outgrowth. PBN activated ERK within 5 min while the activation of SAPK and p38 was not observed, whereas NGF activated all MAPKs. The induction of neurite outgrowth was inhibited significantly not only by the transient transfection of PC12 cells with dominant negative Ras, but also by the treatment with MEK inhibitor PD 98059. These results suggest that the Ras-ERK pathway is necessary for PBN-induced neurite outgrowth. However, different from neurite outgrowth induced by NGF, the activation of receptor tyrosine kinase TrkA, subsequent activation of Shc and its association with Grb2, the activation of Akt resulting from that of phosphatidylinositol 3-kinase and that of phospholipase Cγ were not involved in PBN-induced neurite outgrowth. Moreover, protein kinase C (PKC) was shown to be necessary for neurite outgrowth by PBN and to regulate the activation of ERK partly. The PBN-induced neurite outgrowth and ERK-activation were counteracted by the thiol-based antioxidant N-acetylcysteine (NAC) and ERK-activation was also inhibited by antioxidants, DTT and 2-mercaptoethanol. From these results, it can be inferred that the target of PBN is the cysteine residue in signal proteins and the interaction between PBN or its metabolites such as nitric oxide (NO) and reactive thiol-group in the cysteine residue causes conformational changes to activate signaling proteins.

In *in vivo* experiment using stroke model of gerbil, the intraperitoneal administration of 200 mg/kg of PBN to gerbils before ischemia-reperfusion largely protected the delayed-neuronal death in hippocampal CA1. Immunoblot analysis revealed that ischemia-reperfusion, PBN administration and ischemia-reperfusion with PBN induced the different activation pattern of mitogen-activated protein kinase (MAPK) family at 6 hours after ischemia-reperfusion as following, respectively. (1) The ischemia-reperfusion enhanced the phosphorylation (activation) of extracellular response kinase (ERK) and stress-activated protein kinase (SAPK), (2) PBN administration enhanced the spontaneous phosphorylation of ERK and suppressed that of SAPK and p38, and (3) PBN-administration did not suppress the ischemia-reperfusion-enhanced phosphorylation of ERK but supressed that of SAPK and p38 at the resting level. Moreover, the increase of the expressions of two heat shock proteins (HSPs), HSP27 and HSP70, were also observed at 6 h after ischemia-reperfusion. Administration of PBN significantly enhanced this ischemia-reperfusion-induced expression of HSP27 and HSP70 in gerbil hippocampus. These results indicate that PBN, which has an inherent radical-scavenging property, protects against delayed neuronal death by the

regulation of the MAPK signaling pathway and the induction of a tolerant state by up-regulating HSPs in the brain.

In summary, we demonstrated that PBN induced neurite outgrowth via ERK and PKC pathways which was inhibited by thiol-based antioxidant in the *in vitro* studies using PC12 cells, suggesting that this PBN-induced formation of neurite in PC12 cells was associated with intracellular redox regulation. Besides this, in the *in vivo* studies using a model for ischemia-reperfusion injury in gerbil hippocampus, it was also shown that PBN attenuated neuronal cell death via the up-regulation of ERK and HSPs and down-regulation of SAPK and p38. These results suggested that the neuroprotective effects of PBN *in vitro* and *in vivo* were involved in not only scavenging activity against oxygen radical but also its redox-regulation of signal transduction

Original papers of this thesis appeared in *Neuroscience Letters,* 282 : 41-44 (1999), and *Journal of Biological Chemistry,* 276 : 32779-32785 (2001).

Fas-mediated signal transduction pathway in apoptosis induced by ionizing radiation

Kenji Takahashi

Laboratory of Radiation Biology, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

The studies of radiation-induced apoptosis are very important to understand the basic mechanisms of not only cell death but also the protection against it. To clarify signaling pathway concerning caspase family in radiation-induced apoptosis in lymphoblast cells, the effects of a protein synthesis inhibitor, cycloheximide, on the apoptotic signaling pathway including the activation of caspases and stress-activated protein kinase/ c-Jun N-terminal kinase (SAPK/JNK), the expression of Fas/CD 95 /APO-1 (Fas), the reduction of mitochondria membrane potential ($\Delta\Psi_m$) and the release of cytochrome c were examined in X-irradiated MOLT-4 cells.

MOLT-4 cells pretreated with 0.5 μg/ml cycloheximide (CHX) for 1 h were exposed to 7.5 Gy of X-rays. The appearance of apoptosis, expression of Fas, activation of caspases-3, -8, -9, SAPK/JNK and AP-1, the release of mitochondrial cytochrome c and the formation of death-induced signaling complex (DISC) between Fas and Fas-associated protein with death domain (FADD) were observed by fluorescence microscopy, Western blotting, flow cytometry, gel shift assay and immunoprecipitation methods, respectively. The ligation of Fas and Fas ligand was also examined.

When cells were exposed to 7.5 Gy of X-rays, the typical morphological alterations, characteristic of apoptosis, including nuclear fragmentation and chromatin condensation were observed 6 h after X irradiation and gradually increased up to 12 h. Apoptosis induction was significantly attenuated by CHX. Moreover, the activation of caspases-3 and-8,