CHAPTER V

DISCUSSION AND CONCLUSION

P19 mouse embryonal carcinoma cells are sensitive to aggregation and RA, differentiating to an endoderm phenotype in response to low concentrations of RA (10 nM) and to a neuroectodermal-like phenotype in response to high concentration of RA (100 nM). Neuroectodermal differentiation can be induced by transiently treating floating aggregates of cells with RA and accompanied by upregulation of neurogenic genes a temporally appropriate manner. Neurogenesis is only induced once aggregated cells that have begun to express neuronal markers are plated onto substrate (Jones-Villeneuve et al., 1983). Late events such as neurite outgrowth and glial differentiation can be then stimulated by plating cells onto a permissive substrate (Jones-Villeneuve et al., 1982). Although RA is used to induce neuroectodermal differentiation of P19 cells, successful of P19 cells only occurs in cells that are aggregated in suspension, otherwise endoderm derivatives are produced. In monolayer culture, addition of RA induces differentiation into cells with endodermal and mesodermal characteristics (Roguska and Gudas, 1985).

Two aspects of this differentiation protocol bore special attention. To avoid this, firstly cells should be seeded at the minimal density. Spontaneous differentiation may occur if cells are plated at lower densities. Second, P19 cells did not regulate their replication in a density-dependent manner; at high densities they continued to divide and the overcrowded cultures became filled with lysed cells. It was imperative to passage cells before this occurs.

In the next step, we examined the differences in the expression of AChE, a cholinergic marker enzyme (Hoshi et al., 1997; Hayashi et al., 2000) and neurogenesis enzyme (Moreno et al., 1998; Torrao et al., 2000; Keller et al., 2001) in differentiated cells. AChE, an acetylcholine-hydrolyzing enzyme that was recently shown to enhance neurite outgrowth (Sternfeld et al., 1998). Besides its function at cholinergic synapses, AChE exerts structural differentiation, independent of its enzymatic activity. The P19-

derived neurons contain high level of AChE, indicating that at least some of neurons are cholinergic (Fukuchi et al., 1992). Aggregation of P19 cells following RA treatment initially involves cell surface carbohydrate. However, subsequent compaction of aggregates, largely mediated by cadherins, is required as a direct source of signals to initiate neurogenesis (Boubelik et al., 1996). During neuronal development, AChE is most likely involved in regulating neuritic growhth in various cellular systems. In mammal, AChE shows a high degree of sequence homology to the cell surface adhesion protein, neuroligin. Neuroligin binds to neurexin, which is a neuron surface protein involved in neuronal differentiation and axogenesis. Therefore, AChE is likely involved in neuronal differentiation processes by acting as an adhesion molecule. The present finding that differentiation and morphogenesis in P19 cells were synchronized with the expression of AChE protein. Furthermore, we demonstrated that the treatment of P19 cells with RA, which induced neuronal differentiation of P19 cells, increased AChE activity (Figure 3). It has been previously shown in P19 cells that RA increases AChE activity (Coleman and Taylor, 1996). Cells can respond to RA in two steps. Binding of RA to the intranuclear receptors, thereby inducing the transcription of specific genes, usually occurs within 30 min (early primary response). The proteins translated by the first response activate other genes and produce secondary-response proteins. In our experiment, after 4 days of RA treatment the AChE activity in EBs reached its maximum peak, which support the likelihood that AChE is a secondary response protein. These features were supported by many recent reports about the role of AChE on neurogenesis and development of neuron. The combination of AChE effects on early differentiation and initiation of axon extension may explain both the reduced number of cortical neurons, and the inability of the cortical barrel map to form in those AChE knockout mice that survive past birth (Duysen et al., 2001). Neuroectodermal differentiation in the pluripotent P19 EC line is highly reproducible and represents a useful model system in which to investigate functions in neuronal cells (Sberna et al., 1997; Woodgate et al., 1999).

Aβ-induced toxicity of neuronal cell lines and primary cultures is well established. P19 cell line was chosen for the present study because of its cholinergic

phenotype that vulnerable to Aβ insult and was used to study β-amyloid neurotoxicity (Finley et al., 1996; Sberna et al., 1997). It has been reported that inhibition of cell redox activity, as examined with MTT reduction assay, is an early indicator of the mechanism of cell death induced by Aβ. This effect is followed by a delayed reduction in cell survival (Shearman et al., 1994). The in vitro degenerative effect of Aβ appears to correlate with its age and its fibrillar morphology. Confirming this observation, we found that NLCs reacted to aged Aβ with a delayed dose and time-dependent reduction in cell viability as quantified by XTT reduction assay, trypan blue staining, and LDH release (Figures 5-7). There was a decrease in the XTT reduction and the cell viability determined by the trypan blue exclusion assay but an increase in the LDH release leading to cell death, which was proportional to the concentration of $A\beta_{1-42}$ added.

Metabolic integrity was assessed by the XTT reduction, which quantified cellular reducing potential. By this measure, neuronal viability begun to decline at 24 h of treatment and approached a minimum by 96 h. Membrane integrity was assessed by both the trypan blue exclusion assay and the LDH release, which were lost after metabolic integrity, We concluded that Aβ treatment for 72 h induced a delayed neuronal cell death within a decline in metabolic activity, which occurred before disruption of plasma membrane integrity. This temporal pattern of loss of metabolic and membrane integrity is consistent with the majority of cell death occurring by an apoptotic mechanism (Estus et al., 1997). Measurable toxicity of $A\beta_{1-42}$ could be achieved within 2-5 days post-peptide addition (Bozyczko-Coyne et al., 2001) which was corresponding to our finding. Additionally, altered cellular properties and degeneration after a prolonged incubation with Aβ may reflect a cascade of cellular responses, including altered gene expression and protein synthesis as well as the aging of the added Aβ. The experiments required hours to days of exposure to Aβ to produce cell death. The effect was not immediate. It suggested that the modest toxic effect of Aβ seen in this study compounded over many hours to be more deleterious to cell survival.

Aβ induces multiple events in culture, and a determination of the hierarchy of these events is important not only for understanding the nature of Aβ

toxicity, but also for considering appropriate protective approaches. On this basis, we determined the effects of VPA and VPU on P19 cells, which were differentiated into neuron-like cells, an established in vitro model of neural cells. VPU was found to be more potent than VPA, in preventing maximal electroshock-induced seizures, when the compound was administered into Wistar rat. When both compounds were given by intraperitoneal route, the protective index for VPU was greater than the protective index for VPA (Tantisira et al., 1997). Thus, these results together with our findings suggested that VPU might have some advantages as compared to VPA. The present investigation demonstrated that VPU protected neurons from Aβ-induced toxicity similar to VPA's survival promoting effects on cells (Yuan et al., 2001) and hippocampal neurons following Aβ treatment in vitro (Mark et al., 1995b).

We found that both VPA and VPU significantly attenuated the inhibition of the XTT reduction, the cell viability and the LDH release caused by $A\beta_{1-42}$ peptide at a concentration of 5 μM when added together. VPU but not VPA at a concentration of 50 μM significantly reduced LDH release-induced by $A\beta_{1-42}$ (Figure 9). The results indicated that VPU was more potent than VPA in the attenuation of the loss in metabolic function and membrane damage. Neuroprotective effects of VPA and VPU against $A\beta_{1-42}$-induced neurotoxicity using the in vitro culture system was similar to previous observation that the neuroprotective effects of VPA in hippocampal cultures against Aβ (Mark et al., 1995b). VPU might attenuate the Aβ-induced toxicity via a common VPA-sensitive pathway. The precise biochemical, cellular or molecular mechanisms of actions of VPA-mediated neuroprotection or attenuation remains largely unknown. It is possible that VPA might protect against neurotoxicity by binding directly to its own specific receptor, or it could possibly mediate its effect through regulation of the Aβ-induced neurotoxicity cascades. Several evidences showed the involvement a Ca^{2+}-mediated process in a toxic effect of Aβ. Application of Aβ elevated intracellular Ca^{2+} level, thereby rendering the neurons vulnerable to neurotoxicity and excitotoxicity (Huang et al., 2000b; Yallampalli et al., 1998). In addition, Aβ toxicity was found to be inhibited by nimodipine, a blocker for L-type Ca^{2+} channels (Weiss et al., 1994). Our cotreatment experiments suggested that both VPA and VPU might reduced Aβ toxicity

by normalized intracellular calcium levels via blocking L-type Ca^{2+} channels and NMDA receptor (Fueta et al., 1995). However, the pathway of NMDA receptor was more preferable. It was previously reported that VPA afforded significant protection against glutamate-induced excitotoxicity in cultured cerebral cortical neuron which was exclusively mediated by NMDA receptor (Hashimoto et al., 2002).

These findings point to beneficial effects of anticonvulsant compounds against ionotropic glutamate receptor-mediated injury. These results suggested that VPU and VPA attenuated $A\beta_{1-42}$ toxicity presumably by reducing Ca^{2+} uptake potentiated by $A\beta_{1-42}$ via glutamate response pathway. VPU significantly decreased cortical level of glutamate in a dose dependent manner and was more potent than VPA (Tantisira et al., 1997). This effect was therefore encountered by a stronger reduction of excitatory neurotransmitter, which might explain the stronger potency of VPU in the attenuation of the cell injury induced by Aβ as shown in Figures 9-10.

The present data also provided in vitro evidence that the neuroprotective efficacy of valproates in neuronal cell death after pretreatment with valproates for 3, 5, and 7 days prior to the $A\beta_{1-42}$ exposure. After 3 days of pretreatment prior to 72 h exposure to Aβ, both VPA and VPU could not prevent membrane damage induced by Aβ. When pretreatment of NLCs with VPA or VPU, both compounds time-dependently suppressed toxicity with maximal protection after 5 days of pretreatment (Figure 12). Our results were consistent with the current investigation that one-day VPA incubation did not produced significant increases in Bcl-2 levels, whereas 5-day VPA exposure produced >5-fold increase in Bcl-2 levels (Yuan et al., 2001). Taken together, $A\beta_{1-42}$ produced a decrease in neuroprotective protein, Bcl-2 level (Wei et al., 2000) suggesting that the protective effect of VPA and VPU against Aβ toxicity were time requirement to induce the expression of neuroprotective protein. The glucose regulated protein (GRP78) may play a role in the protection of cells from Aβ toxic insults. GRP78 possesses molecular chaperone activity and binds Ca^{2+} in the endoplasmic reticulum and might protect cells from the deleterious effects of damaged proteins, Aβ. The increase of GRP78 mRNA expression by VPA appeared to occur in part at the level of

transcription (Wang et al., 1999), suggesting why the pretreatment with valproates in the present finding required a lag period for onset of neuroprotective action.

Previous studies have shown that the neuroprotective effects of VPA required that cells be pre-treated with VPA for 3-5 days, a time frame consistent with transcription-based mechanism of action. The prevention of neuronal cell death by valproates in our findings is probably associated with transcriptional regulation. The precise mechanisms by which VPA brings about these effects are currently unknown, but likely involve the activation of intracellular signaling pathways since there are no known cell surface "VPA receptor". The possible mechanisms underlying VPU's protection might be similar to VPA.

In addition, effect of VPA at a concentration of 1 mM on untreated cells slightly increased the XTT reducing activity (Figure 4). The improved mitochondrial function of VPA-treated NLCs is likely to contribute to enhanced cell survival by stimulating secretion of a neurotrophic factor, which in turn activates the ERK pathway through cell surface receptor tyrosine kinase or other mechanism (Yaun et al., 2001).

The approach in the post-treatment experiment involved attempts to enhance regeneration of an injured cell induced by Aβ. Although it was once believed that adult CNS neurons intrinsically lacked any ability to regenerate, some retinal ganglion cells, a type of CNS neuron could regenerate their axons (Hauben et al., 2001). Brain and spinal cord have recently been established, raising the possibility that such molecules could also influence neural plasticity and nerve regeneration. Their findings provided hope that VPA and VPU might be able to promote neuronal regeneration. However, both VPA and VPU in our study did not reverse the toxic damage on mitochondria and plasma membrane induced by Aβ when treatment of NLCs with $A\beta_{1\text{-}42}$ at a concentration of 5 μM for 72 h (Figure 14). The simplest hypothesis for the failure to regenerate or rescue NLCs might be due to the severed damage and the atrophy of cell bodies. For further studies, the duration of post-exposure time and the concentration dependent experiment should be performed to clarify the neurorescuing effect of valproates.

In conclusion, both VPA and VPU significantly protect cultured NLCs from cell death induced by the AD-related insult Aβ. These results might support the future development of VPU as a potential treatment of Alzheimer's disease without neurological toxicity. It is interesting to note that the effect of VPU could be seen only when the compound was added together with the aggregated form of Aβ onto the cells. Pretreatment of the cells with VPU could also protect the cells from the Aβ-induced cell death. Moreover, the addition of VPU failed to rescue the cell when added after Aβ exposure.

The present study suggested that VPU might exert its neuroprotective effect on different targets and might represent an effective survival agent with multiple mechanisms of action. The neuroprotective mechanisms of VPU might involve in the stabilization of cellular calcium homeostasis and the enhancement of antioxidant defense systems. The present findings were consistent with previous reports that VPA blocked Aβ neurotoxicity to hippocampal neurons and VPA pretreatment could suppress calcium responses to glutamate, possibly by altering the expression of glutamate receptor proteins and calcium binding proteins. The possibility of the attenuation of the cytotoxicity of VPU was that VPU might interfere with the conformation of Aβ, thus prohibiting the protein from exerting its toxic effect. There are three main ways we hypothesized for further investigation that VPA and VPU might influence and protect neurologic pathways. One might be the action on the reduction of calcium influx induced by Aβ. Second might be mainly due to their interaction with the structural conformation of $A\beta_{1-42}$ to prevent the action of Aβ on neuronal cell. Finally might be through a family of proteins called Bcl-2, which might block in the apoptotic pathways.

Both VPA and VPU were themselves not toxic to NLCs and might protect neuron by blocking toxic influences of $A\beta_{1-42}$ and hence reduced the risk of AD. Both VPA and VPU were particularly effective when treated before $A\beta_{1-42}$ treatment. Surprisingly, late addition of VPA and VPU was not effective in reversing already advanced neurotoxic effect of $A\beta_{1-42}$. It would be of interest to determine whether VPU possesses clinical efficacy in the prevention and/or the treatment of vascular and neurodegenerative diseases associated with Aβ toxicity. Although both VPA and VPU

could protect neuron cells against Aβ-induced cell death, the cellular and molecular mechanisms of valproates mediated neuroprotective effects have not yet been determined. Thus, further investigations are required to understand molecular mechanisms underlying the modulation of neuroprotection of valproates against Aβ toxicity.

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