Erfolgsbericht:

Projekt: **Chemoprophylaxe und Chemotherapie bei Prion-Infektionen**

[R:PRIONBMS]

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Wie schon in unserem Projektantrag aus dem Jahre 1994 festgestellt, so gilt auch noch heute, daß keine effektive Therapie der Prionerkrankungen vorhanden ist. Uns ist es erstmals gelungen zu zeigen, daß bei der Induktion des Nerventodes bei Prionerkrankungen ein erhöhter Einstrom von Ca^{2+} in die Zellen eine entscheidende Rolle spielt. Deshalb war es auch folgerichtig, daß wir als erste Gruppe einen Weg für eine Therapie aufzeigen konnten. Basierend auf diesen Vorarbeiten und entsprechend den förderpolitischen Zielen, wurde von uns der o.g. Antrag gestellt, mit der Zielsetzung eine effektive Chemoprophylaxe bei Prionerkrankungen auszuarbeiten.

Zwei Substanzen, die klinisch einsetzbar sein könnten, wurden von uns bearbeitet: • Memantin (ein NMDA-Antagonist) und • Flupirtin (ein neuartiges neuroprotektives Medikament).

Bezüglich Memantin war eine vorklinische Tieruntersuchung aufgrund ungünstiger pharmakokinetischer Daten nicht möglich.

Flupirtin wurde in seiner Wirkung molekular aufgeklärt. Es wurde ein neuer Angriffspunkt gefunden [Modulation der *Bcl-2*-Expression], der auch von anderen Arbeitsgruppen im Tierversuch bestätigt wurde.

Hauptsächlich aufgrund unserer Untersuchungen wurde eine klinische Phase 3-Studie bei Creutzfeldt-Jakob-Erkrankungen mit Flupirtin begonnen. Dies ist weltweit die erste klinische Studie bei diesem Krankheitsbild.

Ingesamt gesehen erachten wir die Ergebnisse, die bisher im Rahmen dieser Untersuchungen erarbeitet wurden, als international herausragend; diese Ergebnisse sind in international hervorragenden Journalen publiziert worden.

Ein weiterer Antrag auf finanzielle Förderung der von uns betriebenen Arbeiten mit dem Projekttitel "Entwicklung neuroprotektiver Substanzen zur Behandlung von Prion-Erkrankungen: Zweite Generation" wurde nicht genehmigt.

Eine ausführliche Beschreibung des wissenschaftlichen Erfolgs dieses Projekts ist im Schlußbericht zusammengefaßt, der dem Projektträger vorliegt. Eine

Zusammenfassung ist - entsprechend den Vorgaben zu dem damaligen Aufruf - in Englisch gehalten.

1.1. Summary: aims, methods, results, conclusion

- Goal 1. Elucidation of the interaction of the prion protein with neurons and its consequence
- Goal 2. Screening for and subsequent development of chemotherapeutics potentially applicable in the treatment of prion diseases in humans

• ***Methods:*** Cellular- [neurons *in vitro*], subcellular techniques.

• ***Results:*** **Goal 1:** One major RNA-binding brain protein, the ß-galactoside-specific CBP35, binds to PrP mRNA; to this complex PrP can associate. Small amounts of CBP35 are also present at the surface of cells, which apparently interact with extracellular PrP^{Sc}. Both PrP^{Sc} and PrP106-126 induce apoptosis in neuronal cells by sensitization of the NMDA receptor. **Goal 2: Memantine,** an NMDA receptor antagonist, previously found to protect neurons *in vitro* against PrP^{Sc}-mediated apoptosis, is in the progress to be tested in animal experiments [scrapie-infected hamsters]. As a pragmatic choice the animals will be treated with **memantine**, applied in food pellets after a tolerance study. **Flupirtine,** a drug used in clinics as a centrally acting, non-opiate analgesic agent, was found to display potent cytoprotective activity on neurons *in vitro*, treated with the prion protein fragment PrP106-126. It was demonstrated that flupirtine prevents the apoptotic process by two mechanisms in *in vitro* cultures: by normalization of the GSH level and by induction of *Bcl-2*.

• ***Conclusion:*** **Goal 1:** PrP^{Sc} may bind to neurons via a lectin interaction. **Goal 2:** The animal study with **memantine** is under way. **Flupirtine, is the candidate for clinical trials in humans** due to (i) our *in vitro* data on the neuroprotective activity against PrP106-126, and (ii) the already existing *in vivo* results in further neuroprotective models [e. g., focal cerebral ischemia (mouse), global cerebral ischemia (rat) and retinal ischemia (rat and rabbit)] as well as (iii) the favourable pharmacokinetic properties**; one trial is planned to be started soon**. Until now, no clinical studies on prion diseases in humans by other groups have been conducted.

• ***Future strategies:*** Special emphasis is laid on **new approaches in screening for chemotherapeutics potentially applicable in the treatment of prion diseases in humans.** The ***facilities*** to perform the planned experiments [e.g. P2- and P3-laboratories] are available.

1.2. Publications during funding period

1. S. Perovic, G. Pergande, H. Ushijima, M. Kelve, J. Forrest and W.E.G. Müller: Flupirtine Partially Prevents Neuronal Injury Induced by Prion Protein Fragment and Lead Acetate. Neurodegeneration 4, 369-374 (1995).
2. S. Perovic, P. Pialoglou, F.J. Romero, G. Pergande and W.E.G. Müller: Flupirtine Increases the Levels of Glutathione and *Bcl-2* in NT2 (human Ntera/d1) Neurons: Mode of Action of the Drug-mediated Anti-apoptotic Effect. Europ. J. Pharmacol., 317, 157-164 (1996).
3. W.E.G. Müller, F.J. Romero, S. Perovic, G. Pergande and P. Pialoglou: Protection of Flupirtine on ß-Amyloid-induced Apoptosis in Neuronal Cells in vitro: Prevention of Amyloid-induced Glutathione Depletion. J. Neurochem., 68, 2371-2377 (1997).
4. W.E.G. Müller, G. Pergande, C. Schleger, H. Ushijima and S. Perovic: Neurotoxicity in Rat Cortical Cells Caused by N-Methyl-D-Aspartate (NMDA) and Gp120 of HIV-1: Induction and Pharmacological Intervention. Progr. Molec. Subcell. Biol. 16, 44-71 (1996).
5. H.C. Schröder, U. Scheffer, J. Leuck, T. Sklaviadis, S. Perovic, A.-P. Sève, J.M. Leitão and W.E.G. Müller: Glycoprotein Lectin Interactions of Prion Protein. Possible Roles in Pathogenesis of the Disease Process Caused by Scrapie Prion Protein. In: (E. van Driessche, P. Rougé, S. Beeckmans and T.C. Bøg-Hansen; eds.) Lectins: Biology, Biochemistry, Clinical Biochemistry; vol. 11; Textop, Helerup Ltd. (Denmark), pp. 293-306 (1996).
6. U. Scheffer, T. Okamoto, J.M.S. Forrest, P.G. Rytik, W.E.G. Müller and H.C. Schröder: Interaction of 68-kDa TAR RNA-binding Protein and Other Cellular Proteins with Prion Protein-RNA Stem-Loop. J. NeuroVirology 1, 391-398 (1995).
7. U. Scheffer: Prionen. Medizinische Klinik. 90, 653-657 (1995).
8. W.E.G. Müller, U. Scheffer, S. Perovic, J. Forrest and H.C. Schröder: Interaction of Prion Protein mRNA with CBP35 and other Cellular Proteins: Possible Implications for Prion Replication and Age-dependent Changes. Arch. Gerontol. Geriatrics; in press.
9. W.E.G. Müller, F.J. Romero, S. Perovic, G. Pergande and P. Pialoglou: Protective Effect of the Drug Flupirtine on ß-Amyloid-Induced Apoptosis in Primary Neuronal Cells *in vitro*. J. Brain Res. 37, 575-577 (1996).
10. H.C. Schröder, H. Ushijima, C. Theis, A.-P. Sève, J. Hubert and W.E.G. Müller: Expression of Nuclear Lectin Carbohydrate-binding Protein 35 in Human Immunodeficiency Virus Type 1-infected Molt-3 Cells. J. Acqu. Imm. Def. Syndr. Human Retrovir. 9, 340-348 (1995).

11. A. Kuusksalu, E. Truve, A. Aaspollu, M. Kelve, U. Scheffer, W.E.G. Müller and H.C. Schröder: Impairment of Intracellular Antiviral Defense with Age: Age-dependent Changes in Expression of Interferon-induced and Double-stranded RNA-activated 2-5A Synthetase in Rat. Mech. Ageing Develop. 78, 103-115 (1995).
12. S. Perovic, H.C. Schröder, G. Pergande, H. Ushijima and W.E.G. Müller: Downregulation of *Bcl-2* and Glutathione Level in Neuronal Cells *in vitro* Treated with the Prion Protein Fragment (PrP106-126): Prevention by Flupirtine. Neurodegeneration; submitted.
13. H. Ushijima, O. Nishio, R. Klöcking, S. Perovic and W.E.G. Müller: Exposure to gp120 of HIV-1 Induces an Increased Release of Arachidonic Acid in Rat Primary Neuronal Cell Culture Followed by NMDA Receptor Mediated Neurotoxicity. Europ. J. Neurosci. 7, 1353-1359 (1995).
14. W.E.G. Müller, J.M. Dobmeyer•, Th.S. Dobmeyer•, G. Pergande S. Perovic, J. Leuck and R. Rossol: Flupirtine Protects Both Neuronal Cells and Lymphocytes to Undergo Induced Apoptotic Death *in vitro*: Implications for Treatment of AIDS patients. Death & Differentiation 4, 51-58 (1997).
15. S. Perovic, M. Böhm, H.C. Schröder, G. Pergande and W.E.G. Müller: Pharmacological Intervention in Age-associated Disorders: Alzheimer's- and Prion Diseases. Flupirtine Reduces Apoptosis of Neurons *in vitro.* Mech. Ageing Develop.; submitted.
16. W.E.G. Müller, S. Perovic, J. Leuck, G. Pergande und H.C. Schröder: Neuronaler Zelltod: Schutz durch Flupirtin. Forschungsmagazin [der Joh. Gutenberg Universität; Mainz]; Sonderheft BioRegio 12, 24-37 (1996).

Description of the project

1.3. Specific aims

The general aim of this project is the outline and the experimental elucidation of strategies to interfere with prion infections, especially in humans. *In this regard our project is the only one in this BMBF "Verbund".*

A therapeutic intervention against prion diseases does not exist. Thus, an urgent need exists for compounds displaying therapeutical potential, especially at present.

To achieve this goal the following strategies have been formulated and experimentally followed:

a. Elucidation of the interaction of the prion protein with neurons and its consequence

b. Screening for and subsequent development of chemotherapeutics potentially applicable in the treatment of prion diseases in humans

This program is an integrated approach, combining data resulting from basic research activities with applicable oriented efforts. Special emphasis is given to the development of (a) chemotherapeutic agent(s) potentially suitable for the treatment of prion diseases. *Hence, this individual project fulfills the demands of the BMBF program for an applied research activity in the field of infectious diseases, here of "prion diseases".*

1.4. Introduction and *own work* prior the establishment of the "Verbund"

(The references cited from our group are marked in italics)

The proteinaceous infectious particles, termed prions (PrP), have been implicated in the pathogenesis of a number of both animal and human neurodegenerative disorders. Two isoforms of PrP exist, the normal host protein, designated PrP^{C}, and the modified PrP, PrP^{Sc}, which is present in the infectious scrapie particles. In humans both PrP^{Sc} and PrP^{C} are encoded in a single exon of a single-copy gene. The function of PrP^{C} is not known; it is found predominantly on the surface of neurons, attached by a glycoinositol phospholipid anchor, while PrP^{Sc} has been described as accumulating intracellularly in cytoplasmic vesicles.

1.4.1. Elucidation of the interaction of the prion protein with neurons and its consequence

1.4.1.1. Interaction of prion protein with intracellular proteins

In the sequence of the PrP mRNA three sets of stem-loop structures are found which have the pentanucleotide CUGGG in the loop and the Ura- and Ade bulge in the stem; these features are also characteristic for the TAR sequence of HIV-1. The TAR element of HIV-1 mRNA is the target sequence for Tat. The TAR stem-loop is also recognized by certain host cell proteins (Gatignol et al., 1989; *Schröder et al., 1990; Müller et al., 1990*).

Evidence has been presented that certain nuclear lectins, carbohydrate-binding proteins (CBPs), including the ß-galactoside-specific CBP35 and the glucose-specific CBP67, are associated with or are constituents of nuclear ribonucleoprotein (RNP) complexes (*Schröder et al., 1992*; Wang et al., 1992). Nuclear lectins have been found to associate with RNA directly or indirectly via an RNA-binding protein: protein interaction (*Lauc et al., 1993*).

During the course of our studies, we described that PrP mRNA stem-loops associate with a set of proteins. CBP35, which was among the PrP mRNA-binding

proteins, could also be detected in purified prions from scrapie-infected hamster brains.

1.4.1.2. Proposed interaction of prion protein with the outer surface of cells

CBP35 is not only an intracellular, nuclear protein, but it occurs also in smaller amounts on the surface of cells (Wang et al., 1992). Hence, this lectin might interact not only with the normal PrP^{C} but also at the external cell surface with the extracellular PrP^{Sc}. In a first approach to study this question, PrP^{Sc} was labelled and binding studies were performed.

1.4.1.3. Induction of apoptosis of neuronal cells by prion protein

In 1993 we could demonstrate that PrP^{Sc} is able to induce apoptosis in cortical cells (*Müller et al., 1993*). When rat cortical cells were exposed to low concentrations of PrP^{Sc} (3 ng/ml; embedded into phosphatidylcholine-liposomes) the cell viability significantly decreased from 91% (controls) to 65% during a 12 h incubation period. Increasing the PrP^{Sc} to 30 ng/ml the viability dropped even to 41%. Simultaneous with the decrease in viability the cortical cells underwent DNA fragmentation.

In parallel, Forloni *et al.* (1993) reported that the PrP peptide 106-126, which corresponds to residues 106-126 of the aa sequence deduced from the human PrP cDNA, also acts neurotoxically. Like in our studies, evidence was presented that cell death is caused by apoptosis. DNA cleavage in cells dying by apoptosis has also been detected *in vivo* in scrapie-infected sheep brain using the single cell gel assay (Fairbain et al., 1994).

1.4.1.4. Effect of Ca^{2+} channel antagonists on PrP^{Sc}-induced toxicity

Three Ca^{2+} channel antagonists, memantine, MD-Ada and MK-801, were tested for their potential protective effect on cortical cells against the PrP^{Sc}-induced cytotoxicity. These compounds, which are potent blockers of NMDA receptor channels, were found to abolish the PrP^{Sc}-induced neurotoxicity (*Müller et al. 1993*).

1.4.2. Screening for and subsequent development of chemotherapeutics potentially applicable in the treatment of prion diseases in humans

At the beginning of the grant period within the frame of this BMBF "Verbund" it was already known in outlines, that NMDA receptor antagonists act cytoprotectively (*Müller et al., 1993*). These data were confirmed (Brown, Schmidt, Kretzschmar, 1995: The neurotoxicity of a prion protein fragment requires microglia and neuronal expression of PrP. Int Symp Prion Diseases; Göttingen, October 26-27, Abstract no. 43).

Later we discovered that flupirtine, a drug used in clinics as a centrally acting, non-opiate analgesic agent, displays potent cytoprotective activity on neurons *in vitro*, treated with the prion fragment PrP106-126 (*Perovic et al., 1995 [#1]*).

1.4.2.1. Memantine: animal experiments

We described that memantine, a drug used in clinics in the therapy of spasticity, Parkinsonism and organic brain syndrome, acts neuroprotectively against PrP^{Sc} *in vitro* (*Müller et al., 1993*). Therefore, it was proposed to initiate an *in vivo* study using Scrapie infected hamsters.

1.4.2.2. Flupirtine

Flupirtine, a clinically safe drug, is a centrally acting, non-opiate analgesic agent (Szelenyi et al., 1989), which was identified as an anti-apoptotic agent for neurons *in vitro* (*Perovic et al., 1994*). It is in clinical use under the trademark Katadolon® (Bebenburg et al., 1981; Friedel and Fitton, 1993). Flupirtine was found to act like an NMDA receptor antagonist both *in vitro* and *in vivo* (*Perovic et al., 1994*; Osborne et al., 1994; Schwarz et al., 1994; Rupalla et al., 1995); however, the drug does not bind to the NMDA receptor (Osborne et al., 1996).

During the progress of this grant period we discovered the neuroprotective effect of flupirtine against PrP106-126 (*Perovic et al., 1995 [#1]*).

1.5. Results and discussion

1.5.1. Elucidation of the interaction of the prion protein with neurons and its consequence

1.5.1.1. Interaction of prion protein with cellular proteins

Results

In order to localize PrP in scrapie prion-infected ScN_2a cells a polyclonal PrP antiserum (R073) was used. It became evident that most of the immunoreactive material was present in the cytoplasm. A detailed analysis of the images of multinuclear ScN_2a cells revealed that PrP-positive material is also present in the nucleus. The antigen found in the nucleus caused either a homogenous staining, or appeared condensed in the nucleolus. Uninfected N_2a cells contained no immunoreactive material (reviewed in: *Schröder et al., 1996 [#5]*).

The steady-state levels of PrP mRNA in N_2a- and ScN_2a cells do not increase in N_2a cells after infection with PrP. Based on this result and the quantitation of the levels of transcripts in nuclear run-off experiments we conclude that regulation of PrP gene expression in N_2a- and ScN_2a cells is not altered at the level of transcription or posttranscription. Also the half-life of PrP-mRNA was not altered in the two cell strains.

The binding of proteins present in brain extracts to PrP mRNA was determined. The RNA•protein complexes were analyzed in UV cross-linking experiments as well as Northwestern blotting experiments. One major species, a 35-kDa RNA-binding protein, was detected. No RNA-binding protein could be detected that corresponded to the PrP^{C}. This result indicates that PrP^{C} is unable to bind to PrP mRNA. Western-blotting experiments additionally revealed that the level of PrP^{C} in brain extracts of rats did not change significantly during ageing. However, the amounts of the PrP-RNA-binding proteins strongly decreased in the course of ageing (*Schröder et al., 1996 [#5], Scheffer et al., 1995 [#6], Müller et al., in press [#8]*).

Binding of CBP35 to PrP mRNA was detected by determining the capability of the formed protein•RNA complexes to bind to an agarose column. From this finding we conclude that CBP35 associates with PrP RNA and is present in the PrP-RNA• protein complex formed with nuclear proteins.

Next it was examined whether PrP^{C} is associated with the RNP complexes formed between PrP RNA and proteins present in brain cell extracts. RNP/CBP35 complexes were assembled by incubating brain extracts with *in vitro* synthesized PrP RNA and covalently cross-linked by UV irradiation, and then isolated on a galactose affinity column. The results revealed that PrP^{C}, although incapable of binding to RNA directly, could be detected among the bound proteins by SDS-PAGE and immunoblotting. This result indicates that binding of PrP to immobilized galactose requires the presence of intact RNP complexes. In Western blotting experiments, we could detect the lectin, CBP35, also in purified prions from scrapie-infected hamster brains (*Schröder et al., 1996 [#5], Scheffer et al., 1995 [#6], Müller et al., in press [#8]*).

Discussion

CBP35 has been shown to be complexed with RNP both in the nucleus and in the cytoplasm (Wang et al., 1992). Because a small amount of scrapie prion protein is present also in the nucleus (*Schröder et al., 1996 [#5], Scheffer et al., 1995 [#6], Müller et al., in press [#8], Pfeifer et al., 1992*) it seems possible that PrP, although incapable of RNA binding, might be involved in regulation of its own mRNA at the posttranscriptional level. Interestingly, CBP35 could also be detected in isolated scrapie prions purified from scrapie-infected hamster brains. This finding might be important because this protein appears to be a docking element allowing an association of the prion protein to the cell surface.

At present it is unclear how PrP^{C} or PrP^{Sc} interact with CBP35. PrP^{C} and PrP^{Sc} are highly glycosylated; it remains to be established whether CBP35 binds to the oligosaccharide side chains of these molecules. If this would turn out to be true,

PrP molecules could attach to nucleic acids due to the bipartite nature of CBP35 (lectin and RNA-binding protein).

1.5.1.2. Proposed interaction of prion protein with the outer surface of cells

Results

Based on our finding that PrP [or a fraction of it] is present in a complex with CBP35 we asked if PrP^{Sc} binds to neuronal cells. PrP^{Sc}, isolated from Scrapie-infected hamster brain, was iodinated with ^{125}I. Binding assays using highly purified neurons (*Ushijima et al., 1995; [#13]*) were performed. The results revealed that 14% of the applied PrP^{Sc} bound to the cells with a K_d of 2×10^{-5} M. This association could be prevented by the highly glycosylated "bird nest protein". The latter result suggests that PrP^{Sc} binds to the surface of neurons involving at least one lectin - perhaps via CBP35 - interaction (to be published).

Discussion

It was conceivable but not yet experimentally established that PrP^{Sc} causes apoptosis to neurons. In our studies during this project period we could present experimental data indicating that "particles" of PrP^{Sc}, very likely associated with CBP35, cause neuronal death by binding to the surface of neurons.

Both PrP isoforms contain asparagine-linked complex-type oligosaccharides, which are located near their *C*-termini (Oesch et al., 1985). Prion proteins are able to bind to certain lectins.

Our results indicate that infection of cells with PrP^{Sc} involves the participation of a cellular receptor - very likely CBP35. The identification of cell surface proteins that bind to PrP is an important task not only for the understanding of PrP^{Sc} propagation but also for the development of strategies for prevention and therapy of PrP^{Sc} infection.

1.5.1.3. Induction of apoptosis of neuronal cells by prion protein

Results

In a subsequent series of experiments PrP^{Sc} was introduced into liposomes (*Müller et al., 1993*). Neurons were incubated with increasing concentrations of PrP^{Sc} [3 to 30 ng/ml]. The resulting apoptotic fragmentation of DNA could be abolished by pretreatment of the cells with bird nest protein. From these results we conclude that PrP^{Sc}-induced apoptosis is prevented by highly glycosylated proteins.

In addition we found that the prion protein fragment (PrP106-126) likewise caused apoptosis in neurons (*Perovic et al., (1995) [#1]*) as published first by Forloni et al., (1993).

Discussion

Our data indicate that PrP^{Sc} associates with neurons via binding to cellular receptors, a process which results in the induction of apoptosis, as already suggested earlier (*Müller et al., 1993*).

1.5.1.4. Effect of Ca^{2+} channel antagonists on PrP^{Sc}-induced toxicity

Results

Based on our findings that PrP^{Sc}-induced apoptosis can be almost totally prevented by Ca^{2+} channel antagonists, e.g. memantine (*Müller et al., 1993*), we asked, if it is indeed the NMDA receptor channel complex which is affected by PrP^{Sc}. Therefore, the increase of the intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, in neurons was determined in the presence of PrP^{Sc}. The results show that after 5 min incubation in the presence of PrP^{Sc}, the $[Ca^{2+}]_i$ rises from 0.2 to 1.3 μM. This rise could be blocked by 10 μM of memantine by 75%. The same effect is seen if the neurons were treated with bird nest protein, prior to addition of PrP^{Sc}. From these data we conclude that PrP^{Sc} induces apoptosis via a sensitizion of the NMDA receptor channel complex (*Müller et al., 1996 [#4]*; and to be published).

Discussion

The PrP^{Sc}-induced cytotoxicity in neurons can be blocked by antagonists of the NMDA receptor channel, such as memantine, MD-ADA or MK-801. We conclude that the cytoprotective effects of the antagonists of the NMDA receptor-channel complex towards PrP^{Sc} can be attributed mainly to an interaction with the channel but not with the synthesis and/or the processing of PrP^{Sc}.

1.5.2. Screening for and subsequent development of chemotherapeutics potentially applicable in the treatment of prion diseases in humans

1.5.2.1. Memantine: animal experiments

Results

According to our proposal *in vivo* studies were performed with memantine to clarify if the approach to antagonize the NMDA receptor activity by a specific blocker might be a direction to develop agents suitable for treatment of prion diseases in human.

The animal studies have been and will also in future be performed in collaboration with Dr. Groschup [Bundesforschungsanstalt für Viruskrankheiten der Tiere - Tübingen].

For the animal trials, hamsters are used, which are infected intracerebrally with brain extracts from Scrapie-infected animals. In the first trial, the animals were treated during and after infection with 30 mg of memantine/kg for the entire observation time. The drug was administered *per os*, in food pellets. Unexpectedly,

no increase in survival of drug-treated animals was observed. This failure was attributed to a suboptimal application route and a too low dosage of the drug.

A new protocol for the animal studies was formulated on 17. July 1996. After discussions it became overt that a *per os* administration via a stomach tube, an intraperitoneal application or application via an osmosis pump would be most suitable. An intraperitoneal application had been chosen in previous studies with Amphothericin B (Pocchiari et al., J. Infect. Dis. 160: 759-802). These routes are, however, presently too labour-intensive.

As an alternative, the administration in drinking water had been proposed at a daily dose of 30 mg/kg to maintain the memantine level constant in the body. However, the commission did not accept this application form (27. September 1996), in consideration that the animals come from arid regions and might cover their fluid needs from the food. They underline that a suitable application of the compound is via minipumps or by intraperitoneal injections. These routes have been considered to be not practicable at the present time in Tübingen.

Therefore, a protocol was submitted (17. October 1996), which proposed the application in food pellets at a daily dose of 60 to 70 mg/kg in consideration of the half-life of the compound.

Again the commission had objections (11. November 1996). They considered the new route and the dosage as potentially suitable, but proposed the performance of pharmacokinetic studies - which are not available in hamsters - prior to the start of the therapeutic animal trial.

We responded and proposed to conduct a 4-week study with the proposed dosage to estimate the tolerance of the compound (2. December 1996). Now, it appears that the animal trial can be started.

Discussion

The animal studies have been stopped because of pharmacokinetical difficulties.

1.5.2.2. Flupirtine: a new direction

Results

1.5.2.2.1. Toxicity of PrP106-126 and time course of its effect on cortical cells

The toxic fragment of PrP, PrP106-126, was added for 5 days *in vitro*. PrP106-126 significantly reduced cell viability by 32% after an incubation period of 3 days at 50 µM. At 75 µM to 125 µM the toxicity reached values between 60% and 70%.

1.5.2.2.2. Neuroprotective effect of flupirtine on PrP106-126-mediated cell toxicity

As shown in the Figure, concentrations above 1 µg/ml of flupirtine caused a significant reduction of the toxic effect of the peptide. The cytoprotective effect of flupirtine was still significant at a concentration of 10 µg/ml if compared to assays

without compound; however, this cytoprotective effect was lower than observed in the presence of 1 or 3 µg/ml of flupirtine (79.4%; 83.2%).

1.5.2.2.3. Effect of PrP106-126 with or without flupirtine on intracellular glutathione level in cortical cells

In the absence of flupirtine PrP106-126 caused a significant drop of intracellular glutathione [GSH] level in neuronal cells. Untreated cells at day 0 contained 29.2 nmoles of GSH/10^6 cells; this value was set to 100%. One day incubation with PrP106-126 at 100 µM resulted in a decrease in the intracellular GSH level to 28%. This reduced level of GSH decreased further during the subsequent incubation and reached a value of 12% at day 9.

Coadministration of PrP106-126 and flupirtine significantly prevented the reduction of the GSH level caused by PrP106-126. Addition of 1 µg/ml of flupirtine to PrP106-126-treated cultures normalized the GSH level during the 9 days of incubation; values of 80% (with respect to the controls) were measured at day 1 and 48% at day 9.

1.5.2.2.4. Expression of Bcl-2 in cells treated with PrP106-126 and flupirtine

The level of *Bcl-2* protein in cortical cells was determined quantitatively by immunoprecipitation. Cells were metabolically labelled with [^{35}S]methionine/cysteine. Immunoprecipitates were analyzed by Western blotting using polyclonal antibodies against rat *Bcl-2* or by determination of the radioactivity. As determined, samples obtained from cells treated with flupirtine or PrP106-126 alone showed a weak band at 26 kDa, corresponding to *Bcl-2* protein. In contrast, a sample from cells treated with flupirtine and PrP106-126 showed a much stronger 26-kDa band.

In a quantitative approach the immunoprecipitates obtained from 3 x 10^6 cells were analyzed for radioactivity [metabolically labelled *Bcl-2*]. Samples from cells treated with flupirtine alone (1 µg/ml) remained almost constant during the entire incubation period; at time zero the amount of radioactivity was found to be 7.2 $\pm$ 1.6 dpm/10^6 cells (this value was set to 100%). The level of metabolically labelled *Bcl-2* increased in samples, treated with 100 µM PrP106-126 after 3 and 6 days of incubation; after 9 days the *Bcl-2* level droped to 82%. A slight and nonsignificant increase of *Bcl-2* expression is measured in samples treated with flupirtine alone. In contrast, samples from cells treated with flupirtine (1 µg/ml) and PrP106-126 (100 µM) showed a strong increase in the amount of metabolically labelled *Bcl-2*. Maximal levels were measured at day 3 (242%) and 6 (266%) after addition of the two components (*Perovic et al., 1995 [#1], Perovic et al., 1996 [#2], Müller et al., 1996 [#4], Perovic et al., submitted [#12], Perovic et al., submitted [#15]*).

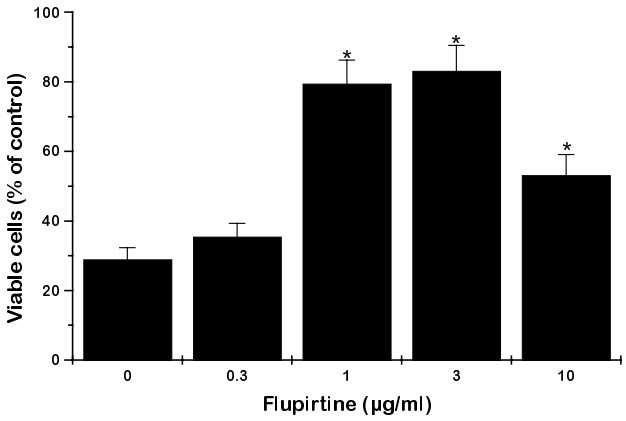

Figure. Neuroprotective effect of flupirtine on PrP106-126-induced cell toxicity. The cortical cells were incubated for 9 days in the presence of 100 µM PrP106-126. The cultures remained either in the absence of the drug or in the presence of 0.3 to 10 µg/ml of flupirtine.

Discussion

Recent reports indicate that prion- and ß-amyloid-mediated apoptosis of neuronal cells can be prevented by compounds which lower the load of reactive oxygen species (ROS) (Brown et al., 1996; *Müller et al., 1997* [#14]). The following two strategies have been developed to intervene therapeutically with the ROS-mediated apoptosis, by scavenging of the destructive oxidants by antioxidants, e.g. N-acetyl-cysteine or vitamin E, or (ii) by stimulation of the GSH-dependent clearance pathway. It is known that the ROS species superoxide anion [$O_2^{\bullet -}$] is converted by superoxide dismutase to another reactive ROS, hydrogen peroxide. This compound is effectively eliminated by either catalase or glutathione peroxidase. The latter enzyme requires GSH as substrate which is converted during the reaction to glutathione disulfide (GSSG). Separate from lowering of the ROS load, the induction of the proto-oncogene *Bcl-2* also prevents induction of apoptosis.

In the studies performed during the past granting period we could show that the prion toxic fragment PrP106-126 reduces viability of neuronal cells in parallel with a drastic drop of the GSH level by more than 70%. Coincubation of the cells with flupirtine at concentrations of 1 µg/ml significantly lowers the toxic effect of PrP106-126 and increases the GSH level close to physiological concentrations. Hence we have to conclude that one molecular mode of the neuroprotective effect displayed by flupirtine is the normalization of the GSH level in cells. This property of the drug provides the cells with the required defense potential against ROS. Further studies will be performed to clarify if flupirtine induces the GSH reductase which converts the oxidized GSH (GSSG) into the reduced GSH form.

A further important outcome of our studies is the finding that flupirtine induces the level of *Bcl-2* in neuronal cells as well. A 3 days incubation with the drug in the presence of PrP106-126 even causes a more than 2.5-fold induction of this anti-apoptotically acting proto-oncogene was observed. The mode of action by which *Bcl-2* prevents apoptosis remains controversial; while some experiments indicate that *Bcl-2* does not prevent apoptosis by lowering the load of the cells with ROS (Jacobson and Raff, 1995), others strongly suggest an inhibition of radical generation by *Bcl-2* (Wiedau-Pazos et al., 1996).

The data presented in this report demonstrate, that flupirtine acts cytoprotective against a series of inducers of apoptosis in neurons, e.g., neurotransmitters (*Perovic et al., 1994*), prion protein (*Perovic et al., 1995 [#3]*), viral proteins (*Perovic et al., 1994*; *Ushijima et al., 1995 [#13]; Müller et al., 1992, 1996*). In addition to prion protein, Flupirtine was found to prevent ß-amyloid-induced apoptosis (*Müller et al., 1996 [#9]*) by two, perhaps interrelated, mechanisms the apoptotic process in *in vitro* cultures of neurons. Firstly, by normalization of the GSH level with the consequence of lowering the ROS load of the cells and secondly, by induction of the anti-apoptotically acting proto-oncogene *Bcl-2*.

1.5.3. Potential clinical application of flupirtine

The potential usefulness of flupirtine in clinical trials in patients with prion diseases is strongly indicated in view of the presented data. This conclusion is also supported by *in vivo* data which showed that flupirtine acts neuroprotective at doses of 1-10 mg/kg in animal models, e. g. focal cerebral ischemia (mouse) (Rupalla et al., 1995) and global cerebral ischemia (rat) (Block et al., 1995) as well as retinal ischemia (rat and rabbit) (Osborne et al., 1996). Furthermore, the drug is clinically safe (McMahon et al., 1987) and shows a favourable pharmacokinetic profile. Administration of 200 mg of flupirtine given orally or the therapeutic daily dose of 600 mg (3 x 200 mg), results in a peak concentration (c_{max}) of 2.4 µg/ml (6 µM) in the plasma after 90 min (Friedel and Fitton, 1993). Similar concentrations are reached in the cerebrospinal fluid *in vivo*, too (Obermeier et al., 1985). The level of ≥ 1 µg/ml of flupirtine which was found here to protect PrP106-126-mediated cell death in vitro remains in plasma for 12 h (Hlavica et al., 1985).

The *in vitro* data, elaborated by us, additional *in vivo* results in further neuroprotective models [e. g., focal cerebral ischemia (mouse), global cerebral ischemia (rat) and retinal ischemia (rat and rabbit)] as well as the favourable pharmacokinetic properties of flupirtine, are the basis for clinical trials in humans which will start soon. Until now, no clinical studies on prion diseases in humans have been conducted.

Additional publications from our group, related to the subject

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Müller WEG, Ushijima H, Schröder HC, Forrest J, Schatton WFH, Rytik PG, Heffner-Lauc M (1993) Eur J Pharmacol 246, 261-267.

Perovic S, Schleger C, Pergande G, Iskric S, Ushijima H, Rytik P, Müller WEG (1994) Eur J Pharmacol 288, 27-33.

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Schröder HC, Ugarkovic D, Wenger R, Reuter P, Okamoto T, Müller WEG (1990) AIDS Res Human Retrovir 6, 659-672.

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1.6. Future strategy

Even though our data gathered during the first period of financial support have contributed to a planned clinical trial to treat prion diseases, the following aspects, which should be seen as a continuation of the ongoing program, need further experimental studies. Basically, the topics are related to those investigated at present.

1.6.1. Elucidation of the interaction of the prion protein with cells and its consequence

1.6.1.1. Identification of the cellular [neuronal] receptor of PrP

The results gathered during this granting period indicate that infection of cells with PrP^{Sc} involves the participation of a cellular receptor. Therefore, binding studies will be performed with the purified PrP on neuroblastoma cells. The following preparations will be used, PrP peptide 106-126, PrP^{C} and PrP^{Sc} (prion rods, detergent-lipid-protein-complexes, or liposomes).

These studies will be performed both with intact cells and cell extracts (from metabolically labeled cells). The cells will then be solubilized with a non-ionic detergent. Crosslinking experiments will be performed. The proteins will be identified by SDS-PAGE and autoradiography.

1.6.1.2. Clarification if an association of the prion protein with its potential cellular PrP receptor is required for apoptosis

If it turns out that a cellular receptor is involved in infection of neuroblastoma cells, antibodies will be raised against its binding domain. Neurons will be pre-treated with those antibodies and subsequently treated with prion protein. As an endpoint marker for apoptosis, DNA fragmentation is measured by agarose gel electrophoresis and the TUNEL technique.

In addition neurons, obtained from $PrP^{0/0}$ mice, will be investigated with respect to the presence of a prion protein receptor and their response to PrP. Among the questions to be asked is if native PrP^{Sc} is ineffective in this cell system.

1.6.1.3. Elucidation of the effect of prion protein on non-neuronal cells

Even though these data from our group are compatible with the view that neurons are one main target for PrP^{Sc} it does not exclude the possibility that also other cells are affected by PrP^{Sc} as well. It was reported by Brown et al. (1996) that microglia participate in the induction of neurotoxicity, caused by the prion toxic fragment, PrP106-126. Our very recent data also show that PrP106-126 causes apoptosis in umbilical cord endothelial cells. A detailed study of the target cells of PrP^{Sc} remains to be performed in the future. Further cells, e.g. lymphocytes, will be studied for their sensitivity towards prion protein.

1.6.1.4. Role of the NMDA receptor in the prion protein-induced apoptosis

The role of NMDA receptor in pathogenesis of cell damage will now be studied in more detail. It remains to be clarified by which mechanisms the increase of the intracellular Ca^{2+} level cause apoptosis. Therefore, the relationships of the different pathways, activated by Ca^{2+}, e.g. xanthine/xanthine oxidase system, with the induction of apoptosis have to be studied.

1.6.2. Screening for and subsequent development of chemotherapeutics potentially applicable in the treatment of prion diseases in humans

1.6.2.1. New approaches

Based on the results to be expected from the above mentioned and proposed series of experiments, novel strategies for the development of new therapeutic approaches to protect neurons against prion protein-induced apoptosis can be outlined and tested experimentally. Examples: Scavengers for ROS, or modulators of intracellular GSH level.

1.6.2.2. Development of therapeutic antibodies for prevention of PrP mediated neurotoxicity by site-directed monoclonal antibodies

The goal will be: Inhibition of neurotoxic effects of prion amyloids on neural cell cultures via site-directed mAbs or other small compounds which bind to regions where pathological conformational alterations in these proteins occurred. These experiments will be performed with PrP peptides. The basis for the current proposal is the prevention of pathological conformational changes in the soluble prion proteins and inhibition of neurotoxic activity via site-directed mAbs or other small compounds which mimic antibody activity.

1.6.2.3. Memantine

Animal study: The protocol is written; the agreement of the ethic committee is expected soon.

Mode of action in suppressing apoptosis: Knowing that memantine - an antagonist of the NMDA-receptor - protects cells against prion protein-induced apoptosis, this drug will be used to study the role of intracellular Ca^{2+} and the subsequent ROS in the induction pathway leading to apoptosis.

1.6.2.4. Flupirtine

Kinetics of drug action: Flupirtine causes a decrease of intracellular Ca^{2+} level, a modulation of GSH level and an alteration of *Bcl-2* expression in neurons treated with prion protein. The proposed interrelations of these parameters will be studied. Kinetic data of these parameters, gathered in pre -and post-incubation studies will be elaborated.

Potential effect of flupirtine on gene expression: Genes involved in apoptosis are thought to be targets for therapeutic intervention (Thompson, 1995). Flupirtine was found to upregulate *Bcl-2* in PrP106-126-treated neurons (*Perovic et al., submitted [#12]*). Therefore the mode of action by which *Bcl-2* prevents apoptosis remains to be studied. Furthermore, it will be examined if expression of *Bcl-2* is mediated by a NF-κB-dependent gene expression mechanism which is known to be controlled by the redox state of the cells.

Special facilities available: P2 laboratories (for the gene technological work) and P3 laboratory (for the experiments with infectious prions).