6 DISCUSSION

Selenium is thought to have protective effects in neurodegenerative diseases. Microglial cells play a primary role as brain-specific macrophages. It is not yet known whether there is a relationship between microglia activation and the protection by selenium. To address this issue, the present study investigated the effect of selenium on microglial cells under stress conditions.

Traces of selenium are needed in the expression of essential selenoproteins. Higher concentrations are, however, toxic to the cells. The tolerable levels differ among cell types. Cells with a high rate of proliferation are generally more sensitive (129).

In the initial experiments of this work, the amount of selenium in the cell medium and serum was measured to determine the basal level of selenium in the cell culture environment. This was a crucial point, because it is well known that the serum-free media, especially those for immune cells and neurons, contain sodium selenite. Without selenium, cells can neither proliferate nor survive. Thus, before the start of studies on the effects of selenium it was important to define the basal selenium level.

It was found that selenium was present in very low concentrations (< 15 nM), and this allowed to explore the effects of selenium by adding different amounts of the element to the cells. Selenium was administered in form of sodium selenite, which can be incorporated into the selenoproteins.

The BV2 microglial cell system has frequently been used to study the oxidative cell death. By means of the MTT assay, which allowed the determination of the cell respiration as an indicator of the cell viability, a concentration of selenite above 1 μM was shown to be toxic for BV2 cells. The mechanism by which selenite induces cytotoxic effects is not yet clear, but it can react with intracellular thiol groups and thus, by disrupting normal processes, may lead to DNA-fragmentation and cell death.

Microglia are the macrophages of the brain. Activation of the microglial cells occurs during the pathogenesis of various neurological diseases. Activated microglial cells are characterized by an amoeboid morphology, which is associated with an enhanced phagocytotic activity, up-regulation of various immune effector molecules, and the production of inflammatory and cytotoxic factors.

In the resting state, the microglia are present in a ramified form as is illustrated in Figure 56. After activation, the ramified microglia change their morphology, retract their processes and change into an amoeboid form. The microglial activation is thought to be protective via the destruction of pathogens, the removal of debris, and the promotion of tissue repair. However, excess activation can be deleterious, in particular for neurons. Oxidative brain damage, such as excitotoxicity and stroke, leads to primary neuronal destruction. The primary damage is further potentiated by microglial cells which are attracted, invade the zone of damage and cause secondary neuronal death.

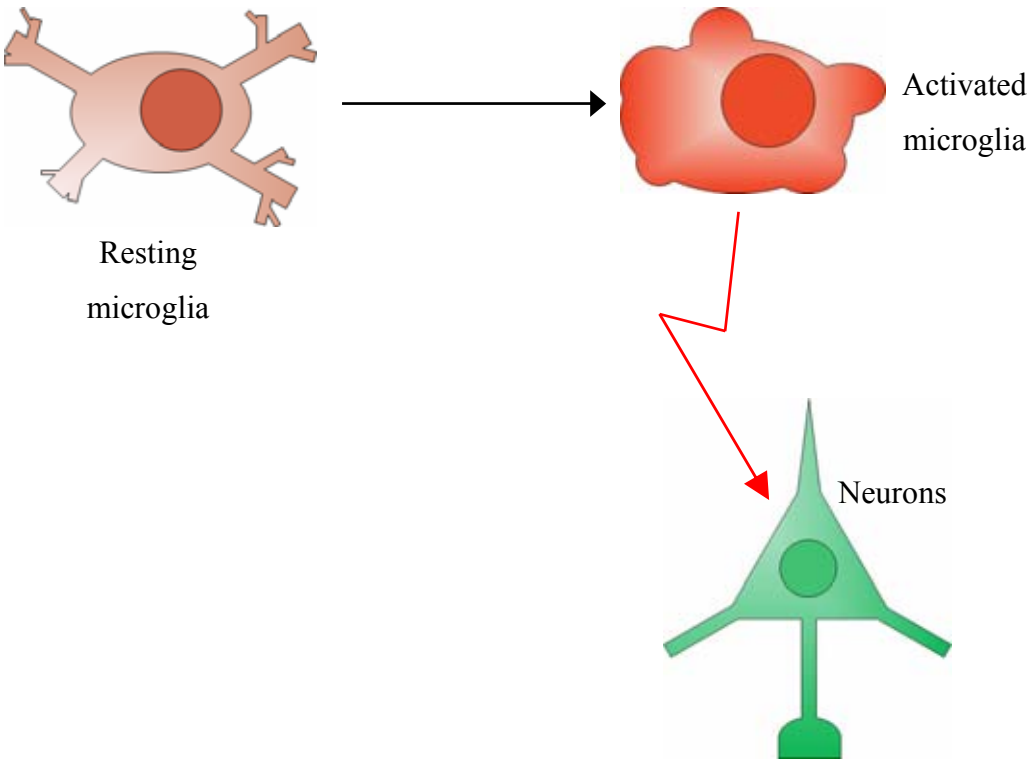

Figure 56 Morphological change of microglia during activation. The activated cells are dangerous for the neurons. See text for details.

The activation of the microglial cells was induced by treatment with hydrogen peroxide. H_2O_2 treatment was shown to affect the morphology and ultimately the viability of the mouse BV2 cells. It was found that the activation of the microglial cells led to cell death and that the activated state of the cells inversely correlated with

the cell viability. The changes in the microglial morphology indicated a higher state of activation in response to hydrogen peroxide addition.

The morphological transformation induced by treatment with 250 µM H_2O_2 was prevented by selenite supplementation. The inhibition of microglial activation and migration could be achieved by selenite application within the physiological range. In fact, in the concentration range between 100 nM and 1 µM selenite, selenium functions as an important protective factor in the BV2 cells.

Recent evidence indicates that the microglial migration is strongly controlled by the expression of the integrin CD11a, which is upregulated following microglial activation (130). Integrins comprise a large family of cell adhesion molecules that mediate interactions between the extracellular environment and the cytoplasm. Activated microglia show an up-regulation of integrins.

The expression of the surface integrin subunits CD11a (LFA-1 α chain) on BV2 cells was quantified by means of flow cytometry. It was found that hydrogen peroxide induced the over-expression of CD11a and pre-treatment with sodium selenite could reduce this H_2O_2- induced activation.

$ß_2$-Integrins are also located on the surface of neutrophils and are an important factor in the cell migration to an inflammatory site. Clinical studies described an upregulation of the CD11a expression in leucocytes in patients with ischemic stroke. In many clinical conditions, for instance after ischemic stroke, the accumulation of inflammatory cells would be deleterious for the patients. Thus, efforts to prevent migration to the site of injury could be advantageous. The influence of selenite on the modulation of the adhesion molecule expression, and therefore in the prevention of the microglial migration, indicated a beneficial role of selenium in the secondary neuronal cell death.

Many studies have reported that activated microglia are associated with the progression of pathological states by producing deleterious features (114, 117). In fact, the microglia can secrete a variety of damaging factors, such as reactive oxygen species (ROS). The results of this work showed that the BV2 cells exposed to H_2O_2 had a dramatic increase in their levels of ROS. Since it was found that microglia generate a great amount of free radicals when exposed to H_2O_2, the protective potential of selenium was examined. Selenium was able to reduce drastically the release of ROS by the BV2 cells, suggesting an important effect of this element in reducing the response of the microglia after stimulation.

ROS are closely involved in the pathogenesis of several neurodegenerative diseases. They can activate redox-sensitive transcription factors, such as NF-kB, which may control the expression of death genes.

The NF-kB transcription factor is ubiquitously expressed in mammalian cells, including the microglial cells. NF-kB is a member of the Rel transcription family that participates in the regulation of a broad spectrum of genes. It is a pro-apoptotic triggering molecule. The release of this factor from the cytosolic domain, where it normally is bound to its cytosolic binding protein lkB-α, may be caused by different pathogenic stimuli, including oxidative stress. These agents are thought to act intracellularly by distinct signalling pathways. After its passive diffusion through the cell membrane, H_2O_2 can be converted into more reactive oxygen compounds such as the superoxide anion, O_2^-, and the hydroxyl radical $OH^{\cdot}$. H_2O_2 alone was shown to be unable to activate the NF-kB- lkB complex. NF-kB was directly activated by reactive oxygen intermediates (131). In which way ROS modulate the activation of this factor is still an open question.

Experiments carried out to investigate the effect of selenium on the nuclear translocation of NF-kB revealed that selenium inhibits the process that leads to the activation of this factor by oxidative stress. Schmidt and collaborators (132) showed that the over-expression of an important detoxificant enzyme (Cu/Zn-dependent superoxide dismutase) was able to attenuate the ROS-induced activation of NF-kB. It is therefore possible that selenium acted via the increased-expression of important antioxidative selenoproteins, such as the glutathione peroxidase.

Furthermore the inhibition of NF-kB activation by selenium in microglial cells is associated with the suppression of iNOS transcription.

Activated microglia are thought to be involved in neuronal inflammation by over-producing various bioactive molecules such as nitric oxide (NO). Significant amounts of NO can be produced by inducible nitric oxide synthase (iNOS), the gene expression of which is mostly regulated at the transcription level. The use of agents aimed at the inhibition of iNOS expression is thus a potential therapeutic strategy to reduce neuronal injury in neurodegenerative diseases. The iNOS gene is induced in microglia by pro-inflammatory cytokines and hydrogen peroxide. Once iNOS has been induced, the formation of nitric oxide proceeds for hours. This leads to the formation of much larger amounts of NO than normally present. Nitrogen oxides, such as nitric oxide (NO), are highly reactive free radicals. Nitric oxide reacts with the

superoxide anion to produce peroxinitrite, which affects the SH groups of proteins and enhances DNA injury, finally resulting in cell death. NO and its oxidation products are much more toxic to neurons than to the glial cells which produce it. Thus the inhibition of iNOS expression by selenium may be one of the mechanisms responsible for its protective effects.

In the present work it was found that low concentrations of selenium, supplied as sodium selenite, improved microglial viability in oxidative stress conditions, which normally are associated with apoptotic cell death.

To further investigate in which way selenium affects oxidative stress-induced cell death, the occurrence of apoptosis in response to selenium pre-treatment was determined.

Several molecular changes occur during apoptosis. A characteristic feature of apoptosis is the externalization of phosphatidiyserine (PS), a lipid normally restricted to the inner leaflet of the plasma membrane. The externalization of PS can be detected by Annexin V binding. The apoptosis of BV2 cells was displayed by an increase of Annexin-V positive cells and the activation of caspase after hydrogen peroxide treatment.

To explore further the site of action of selenium in the apoptotic cascade, the activation of caspase 3 was investigated. Caspase plays a central role in the cellular apoptosis of the central nervous system. The apoptotic program launched by H_2O_2 could be blocked by pre-treatment with selenium. A variety of apoptotic stimuli lead to the activation of initiator caspases, which in turn trigger the caspase cascade, ultimately resulting in apoptotic cell death. The activation of caspase 3 occurs during pathological conditions, thus the resistance against induced apoptosis acquired by the selenium treatment can be interpreted as a beneficial effect.

The microglial cells were also stimulated with two important inflammatory agents, lipopolysaccharide (LPS) and interferon-gamma (IFN γ). After stimulation with LPS or IFN γ, the microglia produced a large amount of NO and died. Selenium treatment had no effect on these processes. Kim and collaborators (133) have recently described that the generation of NO by LPS and IFN γ may be mediated by independent pathways. However, the underlying activation mechanisms of these two immunostimulants are not well understood. Further studies are therefore needed to clarify the signalling pathways involved in the microglial activation induced by LPS and IFN γ.

The protective function of selenium in the primary oxidative destruction of neurons was shown to require de novo protein synthesis (113), and it was very likely that the effects of this essential element on the microglia were caused by the upregulation of specific selenoenzymes which abrogate microglial migration and activation.

In order to find out which selenoprotein is responsible for the effects of selenium supplementation, analytical SDS polyacrylamide gel electrophoresis was chosen for the protein separation because of its high resolution.

The possibility of labelling the cells with ^{75}Se was very helpful in determining the levels of selenoproteins expressed in the BV2 cells. Previous studies showed that the tracer is distributed in the same way as the native amounts of the element (134). Leonard et al. (135) who measured ^{75}Se-labelling of selenoproteins detected by immunoprecipitation, found that ^{75}Se-labelling correlated with antibody binding. Thus the level of a ^{75}Se-labelled protein can be used to quantitate its translation.

By means of the radioactive tracer more than 8 major protein subunits were found in the BV2 lysate after one-dimensional electrophoresis. After two-dimensional electrophoresis it was found that the microglial selenoproteome consisted of more than 25 selenoproteins. Some spots could be identified by comparison with the results of previous studies. It was shown that the microglia BV2 cells contain most of the known selenoproteins. In particular, they express high levels of anti-oxidative selenoproteins, such as thioredoxin reductase and glutathione peroxidase. These results agree with the data of other investigators who previously showed that the microglia represent a prominent glutathione system (136). As activated microglial cells are able to produce ROS and NO, these cells are in immediate contact with these radicals. Thus, microglial cells have to be equipped with sufficient antioxidative defence mechanisms to prevent damages that would endanger their functions.

The results of tracer experiments, in which the selenium concentrations were increased, showed that the expression of the selenoproteins depends on the selenium status. It was shown that the expression of the glutathione peroxidase 1 (Gpx1) protein in the microglial cells increased during selenium supplementation. The levels of the other selenoproteins did not change to such a degree, as was also shown by Western blot analysis. This may be a reflection of the hierarchy of the selenoproteins with regard to their expression in periods of insufficient supply (66). Due to this hierarchy, the expression of Gpx1 decreases to a greater degree during selenium deficiency than that of the other selenoproteins so that during selenium

supplementation the increase in the concentration of Gpx1 back to normal levels is more distinct than that of the other selenium compounds.

To investigate possible mechanisms of protection in which Gpx1 is involved, a molecular biological approach was chosen. Here Gpx1 was overexpressed or underexpressed in BV2 microglial cells, in which the Gpx1 functions were studied. A reliable protocol for the over- and under-expression of Gpx1 was developed.

As a specific antibody against the Gpx1 was not available, a plasmid was designed and produced which could induce the expression of a GFP-recombinant protein. The BV2 cell lines transfected with the Gpx1-EGFP-C1 construct were successfully established. The cells expressed the Gpx1-EGFP encoded protein. In this way the Gpx1 could be imaged by fluorescence or by Western blot using an antibody against GFP. The recombinant protein had an apparent molecular mass of 52 kDa in SDS-PAGE.

RNA interference (RNAi) technology was applied for the silencing of the Gpx1 gene. The first difficulty was the successful selection of a good target sequence within the Gpx1 gene. The selection of an effective siRNA is mostly a matter of trial and error. Three target sequences were selected; two of them were successfully ligated into the pSUPER vector, but only one construct was able to repress the Gpx1 expression. Another problem in the use of an RNAi system is the possibility of unspecific silencing effects. The labelling of the cells with ^{75}Se was extremely useful to check simultaneously the expression levels of the selenoproteins in the BV2 cells after transfection. It was found that the expression of Gpx1 was indeed silenced and that its under-expression did not affect the synthesis of the other selenoproteins, as was shown by the tracer experiments.

The over-expression of the recombinant Gpx1 enzyme did not lead to more resistance against H_2O_2-induced cell death. This may be due to the fact that the normal microglial cells supplied with adequate amounts of selenium had already a sufficiently high Gpx1 level. In microglial cultures, the specific activity of Gpx1 is similar to that in cultured astrocytes and significantly higher than in cultures of neurons (136).

The best way of elucidating the function of an enzyme is to knock it out and study the consequences. It was found that Gpx1 under-expressing cells were less resistant to hydrogen peroxide treatment than the control cells. The treated cells did not regain the normal resistance after pre-incubation with selenite. These data offer the first

experimental evidence for a protective role of Gpx1 in microglial cells against the H_2O_2-induced cell death. This confirmed the hypothesis that selenium (added as sodium selenite) protects BV2 microglial cells against oxidative stress by induction of selenoproteins, in particular Gpx1.

The created vectors are also of great value for future explorations of the other biological functions of the Gpx1 protein.

The influence of the selenium status was also investigated in primary microglial cells isolated from rats fed a selenium-deficient or selenium-adequate diet. The results of these experiments likewise showed that the cells with the normal selenium status were more resistant against oxidative stress and confirmed the essentiality of this element for the microglial cells in the living organism.

The activity and concentrations of selenoproteins depends on the selenium content of the diet. With limited selenium supply, there are differences in the effects on the selenoenzymes. It has been reported that selenium deficiency enhances brain susceptibility to oxidative damage. In degenerative pathologies with oxidative components such as Parkinson's disease, decreases in Gpx1 activity have been reported (137). In this work, the effects of selenium deficiency were specifically investigated in isolated microglia. These cells have recently received attention in a class of diseases characterized by chronic inflammation of the CNS (e.g. Alzheimer's and Parkinson's diseases).

Several groups investigated the functions of Gpx1 in oxidative stress conditions by using Gpx1-knockout mice. However, studies on microglial cells have still been missing. The findings of this work indicate that selenium, by acting via the functions of selenoproteins (Gpx1 in particular), can reduce the microglial activation and the subsequent cell death against H_2O_2 toxicity.

Overall, these results suggest that selenium may be useful as a therapeutic agent in the protection of neurons against the microglia-induced secondary damage, which occurs in many neurodegenerative diseases.