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Molecular analysis of prion protein (PrP) and glial fibrillary acidic protein (GFAP) transcripts in experimental Creutzfeldt-Jakob disease in mice

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Abstract. Prion protein (PrP^{sc}) which accumulates in the brains affected with subacute spongiform encephalopathies (SSE) is altered isoform of normal, cellular isoform (PrP^{c}), and PrP deposition is accompanied with spongiosis and astrogliosis. To find the amounts of PrP and GFAP transcripts during progression of experimental Creutzfeldt-Jakob disease we performed comparative RT-PCR on the terminally sick mice brains, 22 weeks following inoculation with Fujisaki strain of CJD agent, and on control brains. The intensity of bands for PrP-mRNA and control β-actin were similar for infected and uninfected brains, while amounts of transcripts for GFAP increased as for cytokines released by glial cells - TNF-α and IL-1α. This study supports thesis that PrP^{c} to PrP^{sc} conversion is post-translational process not related to PrP overproduction. Increased amounts of GFAP-mRNA during the course of the disease correlated with astrocytosis estimated by immunohistochemistry with anti-GFAP antibody.

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INTRODUCTION

Prion protein (PrP^{sc}) which accumulates in brains affected with transmissible spongiform encephalopathies (TSE) derives from its normal cellular isoform designated PrP^{c}, achieving a β-sheet conformation during post-translational modifications (Pan et al. 1993), possibly with the operation of another molecule designated "protein x" (Telling et al. 1995). Mice overexpressing a mutant transgene of PrP spontaneously develop neurologic disease and produce prions what was apparently demonstrated by a serial transmission in transgenic mice and hamsters but not normal mice (Hsiao et al. 1990, Hsiao et al. 1994). These experiments strongly support a notion that PrP^{sc} is the only molecule necessary to develop scrapie, and may even entirely form prions (the scrapie agent)(Prusiner 1982), while there are a few data that prions may still contain nucleic acids and even have a viral structure (Ozel et al. 1994, Manuelidis et al. 1995). Recently published successful conversion of PrP^{c} into PrP^{sc} in cell-free environment supports the protein-only prion hypothesis (Bessen et al. 1995, Kocisko et al. 1995). In experimental scrapie, PrP mRNA levels remain stable irrespective whether in uninfected or infected brains (Chesebro et al. 1985, Lazarini et al. 1992, Manson et al. 1992), and this observation may also be used against the viral theory.

Astrocytosis is one of the hallmarks of the TSE, but the majority of astrocytes neither interfere with PrP accumulation nor play any role in the development of the TSE. Knock-out mice devoid of GFAP-gene develop normally and are susceptible to scrapie agent (Gomi et al. 1995). It is not clear, however, if the astrogliosis in this disease results from merely GFAP overproduction by reactive cells (true hypertrophy), or it is caused by their proliferation (Eng and Ghirnikar 1994). Furthermore, GFAP-positive reactive glial cells may result from the GFAP overproduction by S-100 protein-immunopositive precursor glial cells. Biernat et al. (1995) using anti-PCNA antibodies found no immunopositivity in CJD-affected human brains and, in experimental CJD in mice, only approximately 5% of glial cells were positive. Unfortunately, PCNA was proved not to be a completely specific marker of proliferation and its immunohistochemical overexpression in not cycling cells has been reported (Hall et al. 1990, Kordek et al. 1996a). Careful search for mitotic figures in numerous brain specimens during experimental TSE may provide alternative insight into this problem.

We report here an analysis of the amounts of PrP and GFAP transcripts using comparative RT-PCR in the mice brains affected with experimental CJD. As controls were run -actin (transcripts for "house-keeping" gene) and cytokines: TNF-α and IL-1α, produced in the central nervous system by glial cells and overexpressed in experimental CJD (Kordek et al. 1996)

METHODS

CJD agent

The Fujisaki strain of CJD-agent, isolated from the brain of a 56-year old Japanese man (K.F.) with progressive dementia, presenting kuru-like plaques and severe white matter degeneration (Tateishi et al. 1978, 1979) is characterized by an incubation period of approximately 16 to 18 weeks following intracerebral inoculation in mice (Liberski et al. 1989). This agent is commonly designated the CJD-agent, althought the neuropathological features of K.F. patient met the criteria of GSS (Tateishi et al. 1979). Weanling,4 to 5-weeks old NIH-Swiss mice (Animal Production Area, Frederick Cancer Research and Development Centre, Frederick, MD) were lightly anaesthetised with metoxyflurane and injected intracerebrally (approximately into left thalamic area) with 0.03 ml of a 10% clarified brain suspension prepared from mice terminally sick with the Fujisaki strain of CJD prion (infectivity titter, 3.1×10^4 LD_{50} per 0.03 ml, by the intracerebral route). Control animals were injected with 0.03 ml of 10% clarified normal NIH-Swiss mouse brain suspension. All procedures were carried out in accordance with the National Institutes of Health (NIH) Animal Care and Use Committee Guidelines. Each week 4 mice infected and 2 controls were sacrificed till they were moribund in 22 weeks, four CJO - infected and two control mice were sacrificied and subjected to the present study. First clinical symptoms in a few animals occurred at 14 week, but to the study only animals not presenting clinical symptoms were regarded.

RT-PCR

Brains from two CJD-infected mice and one brain from control animal were removed, snap frozen in liquid nitrogen and stored at -80°C. Approximately 100 mg of each brain (frontal part of right hemisphere) was used for RNA extraction by RNAzol (TEL-TEST, Inc. Friend-

swood, TX). RNA was diluted in diethylpyrocarbonate (DEPC)-treated water to obtain 400 μg/ml solution (checked twice on spectrophotometer), to decrease completely intensity of bands for TNF-α for control brains. Following primers programmed on the data from GeneBank were used: GFAP, product size: 394 bp: 5'-CAC AGG ACC TCG GCA CCC TG-3', 5'-GGA GGA GCT CTG CGT TGC GG-3'; PrP, product size: 329: 5'-TGG GGA CAA CCT CAT GGT GGT-3', 5'-GAT ATT GAC GCA GTC GTG CAC-3'; β-actin, product size 348 bp: 5'-TGG AAT CCT GTG GCA TCC ATG AAA-3', 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3'; TNF-α, product size: 264 bp: 5'-GAA TGG GTG TTC ATC CAT TCT-3', 5'-ACA TTC GAG GCT CCA GTG AAT TCG-3'; IL-1α, product size 491 bp (purchased from Clontech Lab., Inc., Palo Alto, CA, USA): 5'-AAG ATG TCC AAC TTC ACC TTC AAG GAG AGC CG-3', 5'-AGG TCG GTC TCA CTA CCT GTG ATG AGT TTT GG-3'. PCR reactions were performed under the same conditions. All samples with each pair of primers were run together. Master Mix was prepared as follow: 10X PCR Buffer II - 5 μl, 25 mM $MgCl_2$ sol - 3 μl, 10 mM dNTP - 4 μl, *AmpliTaq* DNA Polymerase 5 U/μl - 0.25 μl (all from GeneAmp PCR Core Reagents from Perkin Elmer - Roche Molecular Systems, Inc., Branchburg, NJ, USA.), RNasin (Ribonuclease inhibitor, 40 U/μl) - 0.5 μl, AMV Reverse Transcriptase (10 U/μl, both Promega, Madison, WI, USA) - 0.4 μl, 0.4 μg of sample RNA and DEPC treated water ad 50 μl. After incubation 60 min. at 42°C (reverse transcript step) samples were heated 1 min. at 96°C and then 35 cycles (45 s 96°C, 45 s 55°C, 60 s 72°C) in Perkin Elmer's thermocycler were performed. After 5 min anealing at 72°C samples were stored at 4°C. PCR products (7μl of each with 1μl of Stop Solution (USB, Cleveland, OH, USA) were run in 2% agarose gel (0.0015% Ethidium Bromide) together with 100 bp DNA Ladder (Gibco BRL, Gaithersburg, MD, USA).

Immunohistochemistry

Brains from two CJD-infected mice and one from control animal from each week post inoculation were immediately fixed in 10% buffered formalin, after one day of fixation kept for one hour at 96% formic acid to reduce the infectivity (Brown et al. 1990), washed, paraffin embedded and cut on the sialinized slides. GFAP (1:50) and S-100 protein (1:100) were stained with polyclonal rab-

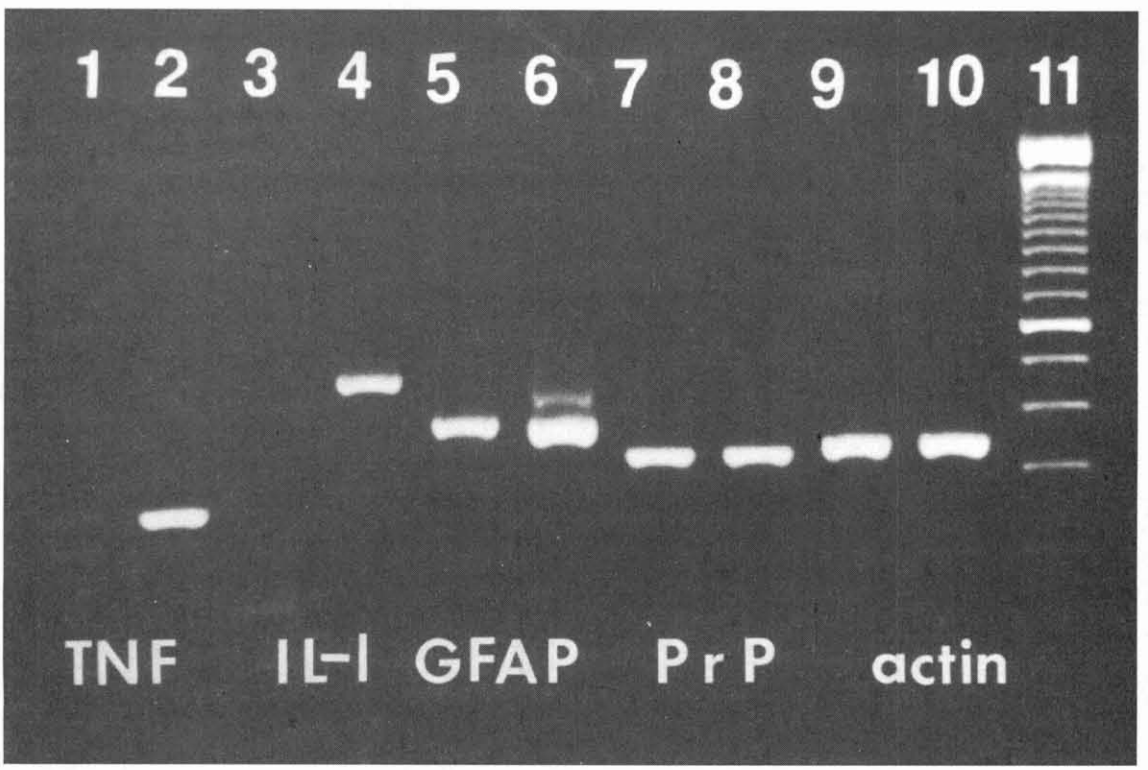

Fig. 1. Ethidium bromide stained RT-PCR products for TNF-α, IL-1α, GFAP, PrP and -actin from control brain (lanes 1,3,5,7,9) and CJD infected brain, 22 weeks after inoculation (lanes 2,4,6,8,10).

bit antibodies purchased from DAKO. Streptavidin-biotin-peroxidase system (DAKO) was used for visualisation. All sections stained with hematoxylin-eosin (H-E) and immunohistochemically, were carefully revised,in search for mitotic figures.

RESULTS

RT-PCR

Intensity of bands for GFAP, TNF-α and IL-1α for material from terminally sick animals at 22nd week following inoculation were much stronger when compared to those from control animals. To the contrary, intensity of bands for PrP and control -actin were virtually the same for both - CJD-affected and control animals (Fig. 1).

Histology and immunohistochemistry

Spongiform change in the gray and white matter progressed during development of the disease and these features correlated well with the level of astrocytosis as estimated by the GFAP and S-100 protein immunohistochemistry (not shown).

In the brains in terminal stages of the disease, we observed mitotic figures in cells which diameter was similar to glial cells, particulary within gray matter (Fig.2). These cells however, were immunopositive neither for GFAP nor for S-100 protein (Fig. 3). In all of each brain we could find 1-2 dividing cells, and the number of observed mitoses correlated with the degree of CJD-specific neuropathology: astrogliosis and spongiform change.

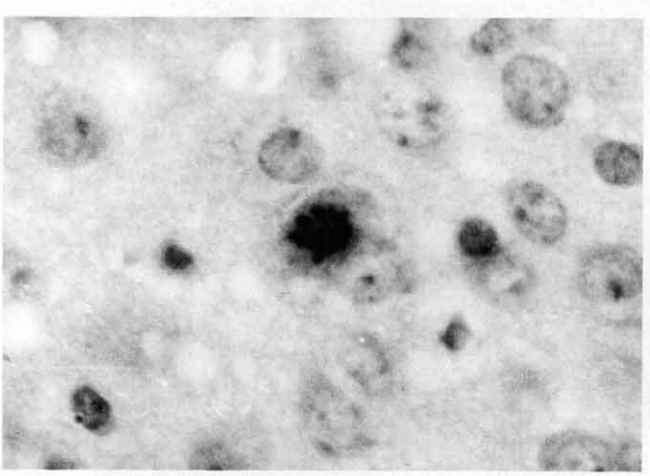

Fig. 2. Mitosis at cerebral cortex of CJF infected mouse, 22 weeks after inoculation. Hematoxylin-eosin staining, x 1000.

DISCUSSION

The intensity of bands for PrP mRNA irrespective whether in infected or in uninfected brains were virtually the same, what it is in concordance with results obtained in experimental scrapie (Chesebro et al. 1985, Lazarini et al. 1992, Manson et al. 1992). In neuroblastoma cells *in vitro* either uninfected or infected with scrapie, no differences between PrP-transcripts or in general transcriptional activity were found (Caughey et al. 1989). Furthemore, the half-time of PrP-mRNA was the same under both experimental conditions (Pfeifer et al. 1993). In transgenic mice expressing different steady-state levels of mRNA for PrP, the incubation time of experimental scrapie correlated well with the levels of PrP-transcripts, - mice which express higher level of PrP mRNA had shorter incubation time (Prusiner et al. 1990). Similar results were obtained by Manson et al. (Manson et al. 1994). Collectively, these data support a hypothesis that accumulation of PrP^{sc} is entirely caused by a postranslational modification of PrP^{c}, and strongly argue against classical viral-related overproduction caused by the transcriptional activity.

Astrogliosis may be caused by accumulation of PrP or, alternatively, by cytokines secreted by astroglial or by microglial cells (Giulian et al. 1985, 1986, 1988, Barna et al. 1990, Chung and Benvensite 1990, Forloni et al. 1994). Expression of GFAP seems to be merely the marker of astrocytic hypertrophy, and play no role in the development of PrP disease (Gomi et al. 1995). In experimental CJD in hamsters, levels of mRNA for GFAP

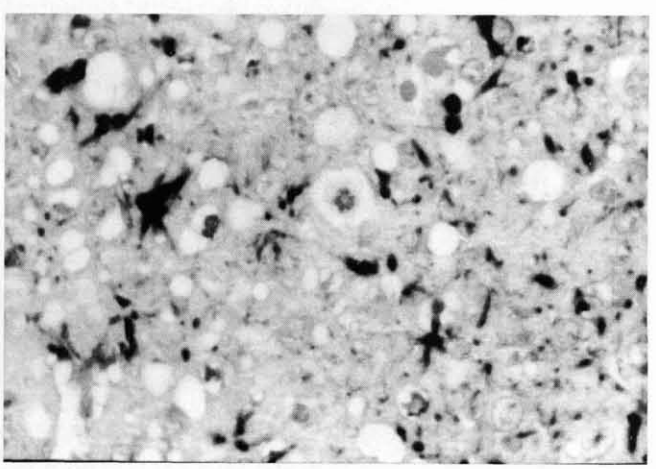

Fig. 3. Mitosis at cerebral cortex of CJF infected mouse, 22 weeks after inoculation (in the center). GFAP-immunostaining, H-E counterstain, x 400.

increased approximately 13-fold (Manuelidis et al. 1987). Similar results were obtained by Lazarini et al. in mice infected with scrapie (Lazarini et al. 1992). Campbell et al., obtained also significant (unestimated) increase of GFAP-mRNA in scrapie infected mice (Campbell et al. 1994). Althought it was suggested that the accumulation of PrP occurs first in astrocyte population (Diedrich et al. 1991), the highest levels of PrP mRNA were found in neurons (Kretzschmar et al. 1986) - cells being the target of pathology in prion diseases.

Astrogliosis is the halmark of SSE, although it is not clear whether is it the result of astrocytic proliferation or merely hypertrophy. Accumulation of GFAP which occurred in earlier GFAP-immunonegative but S-100p-immunopositive protoplasmic astrocytes may produce such an effect. We found mitotic figures in brains affected with CJD, but these cells were GFAP-negative. This observation does not exclude however that these mitoses were still in the glial cells - cycling glial cells may not produce GFAP, what is the halmark of differentiated astrocytes. In our study, similarly to other experiments, increase of GFAP-immunopositivity was related to intensity of astrogliosis (Hatten et al. 1991, Eddleston et al. 1993), and it was proportional to the increase of GFAP-transcripts level. Similar increase of GFAP transcripts was observed in experimental scrapie in mice (Campbell et al. 1994) and in experimental CJD in hamsters (Manuelidis et al. 1987, Lazarini et al. 1992). The study on TNF-α and IL-1α was discussed elsewhere (Kordek et al. 1996b).

In conclusion, we reported here, that PrP mRNA levels are not increased during development of experimental CJD, and that the astrogliosis in this disease may be caused also by cell proliferation.

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