TRANSCRIPTION OF "SLOW VIRUS" DEMENTIA PRION PROTEIN GENE IN LYMPHOCYTES OF HUMAN BLOOD

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ABSTRACT

We have analysed illegitimate transcription of brain specific genes in normal human blood lymphocytes. Total RNA was extracted from lymphocytes and after a first step of reverse transcription, the prion cDNA sequence was amplified by polymerase chain reaction (PCR) in the presence of specific primers. Amplification of a fragment of the prion gene sequence was observed, indicating that the corresponding gene was transcribed in blood lymphocytes.

INTRODUCTION

The "Slow virus" dementias which include Creuzfeldt-Jacob disease (CJD), Gerstmann Straussler syndrome (GSS), kuru in man, scrapie and similar disease in animals, are characterized by spongiform degeneration and, usually, by congophilic plaques in the brain parenchyma (1). These diseases are transmissible through the injection of infected brain tissue into the brain tissue of other animals (2). The characteristic plaques are made up of a protein, called the prion protein (1,3), which is coded for in man by a gene on chromosome 20 (4,5). However, while prion protein is expressed at similar levels in normal and affected brains, the prion protein which is deposited in the congophilic plaques appears to have been abnormally post-translationally modified (3). Prusiner et al. reported that purified fractions of prions were stained with Congo red dye (1). By light microscopy, numerous amorphous structures measuring 1-20 microns were observed. These structures exhibited a red color indicating that they were binding the Congo red dye when examined under bright field microscopy. Examination of these same amorphous structures with crossed polarizers demonstrated a green color and birefringence. These staining characteristics were similar to those observed for amyloid isolated from human spleen. They were also similar to amyloid purified from the cerebral blood vessels of patients with Alzheimer's disease (AD)(6).

Scrapie is a naturally occurring slow infection of sheep caused by a class of unconventional infectious agents that have unusual properties. These properties, and the co-purification of a 27- to 30- kilodalton (kD) protein and infectively, have led to speculation that the enigmatic informational molecule in scrapie is not a nucleic acid . It is now clear, however,that the 27- to 30-kD "prion" protein is the product of a normal cellular gene that is expressed to the same extent in infected and uninfected animals (7). The distinctive

pathological changes of scrapie are neuronal vacuolation and the formation of abnormal fibrils [8-12],but in some animal models of scrapie and cognate disease (kuru, Creutzfeldt-Jacob disease) there are other pathological alterations that resemble those seen in senile dementia of Alzheimer's type [8,9,10].

Recently, Chelly and co-workers suggested "illegitimate transcription" as a new tool for the investigation of abnormal highly tissue-specific genes. Whatever the mechanisms of illegitimate transcription, this phenomenon provides a powerful tool for investigating pathological transcripts by using easily accessible cells (e.g., fibroblasts, lymphoblasts, and even peripheral blood cells). The coding sequence being more compact in mRNA than in DNA, it is much easier to search for an unknown mutation by studying the messenger rather than the gene itself. This approach is particularly warranted for huge genes, like the DMD and the factor VIIIc genes. However, until now, this approach had been limited by the difficulty of obtaining pertinent mRNAs when they are located in inaccessible cells (e.g., brain, liver, pancreas, and heart), sometimes at a very low level. The finding that such mRNAs are virtually present in all cell types and the possibility of amplifying faithful cDNA fragments by starting from easily accessible cells greatly enlarges the perspectives for this approach. Starting from lymphoblast RNAs, one could amplify cDNA fragments approximately 2 kb-long encompassing the sequence of any mRNA by using four or five pairs of oligonucleotide primers(11). The present unequivocal diagnosis of Alzheimer's disease is only possible postmortem by histological examination of brain tissue. There also is no effective treatment for AD, so in order to develop effective therapies, it is essential that its causes be elucidated. The central nervous system (CNS) neuropathological and neurochemical hallmarks of AD include selective neuronal cell death, a decrease in markers for certain neurotransmitters and the presence of abnormal proteinaceous deposits in neurons (neurofibrillary tangles) as well as in the extracellular space (cerebrovascular, diffuse and neuritic plaques) (12). In this study, we have attempted to amplify prion protein gene from human lymphocyte RNA by using a polymerase chain reaction (PCR) in the presence of specific primers. The results clearly indicated that prion sequence amplification from human lymphocytes RNA was possible. This experiment may provide a basis for the development of tools for early diagnosis of AD.

MATERIALS AND METHODS

1. Preparation of RNA samples by acid guanidium thiocyanate-phenol chloroform (AGPC) method

RNA was isolated according to a protocol modified from that of Chomczynski and Sacchi (13) as follows. Human whole blood was diluted (1:1,V/V) with phosphate buffer saline (PBS). Diluted blood was layered on the top of Ficoll solution (Pharmacia) and then centrifuged at 400 g for 1 hr at room temperature. After centrifugation, the sample separated into three layers. The top plasma layer was discarded and lymphocytes present in the white interface ring between the top plasma and the bottom Ficoll layers were collected into fresh plastic tubes and then were washed twice with PBS (1:3,V/V). Sequentially, 3-5 ml solution D (4 M Guanidine thiocyanate ,25 nM Sodium citrate ; pH 7.0, 0.5% Sacrosyl and

0.1M β-mercapto-ethanol) ,1 volume of phenol (water saturated), 0.1 volume of 2 M sodium acetate, pH 4.0, and 0.56 volume of chloroform-isoamyl alcohol mixture (24:1) were added to the isolated lymphocytes, with thorough mixing by inversion after addition of each reagent. The final suspension was shaken vigorously for 10s and cooled on ice for 15 min. Samples were centrifuged at 10,000 g for 20 min at 4°C. After centrifugation, RNA, which was present in the aqueous phase, was transfered to a fresh tube, mixed with 1 volume of isopropanol, and then left at -20°C at least 3 hr to precipitate RNA. Sedimentation at 10,000 g for 30 min at 4 °C was again performed and the resulting RNA pellet was dissolved in Tris-HCl-EDTA-Sacrosyl (TES) buffer (10 mM Tris-HCl, pH7.4,5mM EDTA and 0.5% Sacrosyl). RNA suspension was layered on the top of 1 volume of chloroform/butanol (4:1), with thorough mixing. Samples were centrifuged at 10,000 g for 15 min at 4°C. The supernatant phase was transferred to a fresh tube, mixed with 0.1 volume of 3M sodium acetate , pH 5.2, and 2.2 volumes of 95% ethanol, and then placed at -20°C at least 3 hr to precipitate RNA. After centrifugation in an Eppendorf centrifuge for 10 min at 4°C the RNA pellet was washed with 70% ethanol, sedimented, vacuum dried (15min) and dissolved in deionized water. At this point the RNA preparation could be used for cDNA-PCR amplification.

2. Oligonucleotide Primers

The synthetic oligonucleotide primers used to amplify a segment of the prion protein gene were the antisense primer (5'-CTGGGTGATACACATCTGCT) and the sense primer (5'-ACATGGCTGGTGCTGCAGCA).

3. Amplification by the cDNA Polymerase Chain Reaction (cDNA-PCR)

3.1.Reverse transcription

The first strand of cDNA generated by reverse transcription(RT) of mRNA can be used as a template for polymerase chain reaction as follows. One ug of total RNA was incubated at 42°C for 1 hr in 20 ul of reaction mixture containing 200 units of Moloney murine leukemia virus reverse transcriptase (BRL), 1mM of each dNTP (dATP, dCTP, dGTP, and dTTP), 30 units of placental ribonuclease inhibitor, and 25 pmol of the oligonucleotide primer complementary to the transcript and corresponding to the 3' part of the fragment (antisense primer)

in PCR buffer (20 mM Tris-HCl, pH 8.4 ,50 mM KCl, 2 mM $MgCl_2$, and 0.1 mg bovine serum albumin (Nuclease free)).

3.2.PCR amplification

The polymerase chain reaction was performed as follows. Twenty ul of RT amplification solution were diluted to 100 ul of PCR buffer contain of twenty five picomoles of each pair of oligonucleotide primers, 1 uCi of ^{32}P dCTP (Pharmacia) and 5 uits of Thermus aquaticus DNA polymerase. The mixture was sealed under 50 ul of mineral oil and incubated first at 94 °C for 5 min, 55 °C for 2 min, and 72°C for 2 min, and then subjected to 35 cycles of thermal cycling (94 °C for 1 min , 55°C for 2 min, and 72 °C for 2 min) before reducing the temperature to 4 °C for storage.

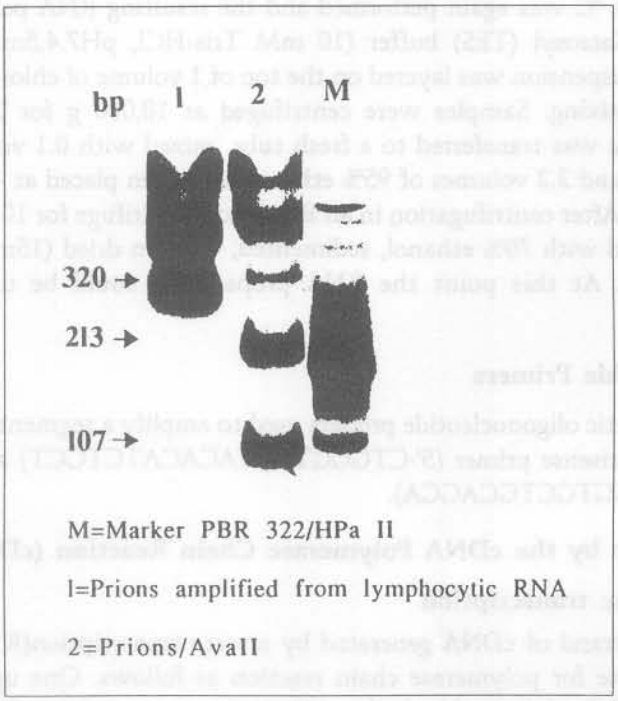

Fig. 1. Autoradiography of prion protein amplification product from human lymphocytes. The amplification products were observed by electrophoretic separation of restriction enzyme (AvaII) digestion.

4.Analysis of amplified samples

Typically, 20 ul of amplified samples were digested with the appropriate amount of AvaII restriction enzyme for two hr, and subjected to electrophoresis in 10 % polyacrylamide gels which were then dried and exposed to a Kodak X-Omat AR film for 12 hr.

RESULTS AND DISCUSSION

Agarosegel resolution patterns of total RNA isolated from human lymphocytes by AGPC and standard RNA gave similar patterns (data not show). RNA lymphocyte isolations yielded concentrations of about 1.0 - 2.0 ug/ul. RNA isolated was then used for expression studies of prion protein gene by PCR in the presence of specific primers. The AGPC extraction method provided efficient yield of undegraded RNA. Due to its simplicity and the elimination of ultracentrifugation,the AGPC method allows simultaneous processing of a large number of samples. Degradation and loss of RNA is minimized by the limited handling involved in this technique . The AGPC method may therefore be useful for clinical investigations that employ gene expression such as proto-oncogene expression in malignancy and tumor progression[13].

Using the preparation of human lymphocyte RNA we were able to amplify a 320 bp segment of human prion protein gene employing cDNA-PCR with a pair of prion gene specific oligonucleotides (Fig.1). In order to verify that the PCR product was indeed generated from prion mRNA, an aliquot of the PCR reaction mixture was digested with the restriction endonuclease AVa II to yield the expected fragments of 107 and 213 bp (Fig.1) (14,15). Thus, by this simple method, it can be readily demonstrated that the prion protein gene is expressed, albeit at a very low level, in non neuronal peripheral cells. We are currently applying this technique to develop tools for early diagnosis of Alzheimer's disease in Thai subjects.

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REFERENCES

1. Prusiner, S.B. (1987). Prions and neurodegenerative disease. *N. Engl. J. Med.* **17**,317(25),1571-1581.
2. Gibbs, C.J., Gajdusek, D.C., Asher, D.M. and Alpers, M.P. (1968). Cruetzfeldt-Jacobdisease (Spongiform Encephalopathy): Tramsmission to the chimpanzee. *Science* **161**,388-389.
3. Hope, J. and Kimberlin, R.H. (1987). The molecular biology of scrapie : the last two years. *TINS* **10**,149-151.
4. Oesch, B., *et al.* (1985). A cellular gene encodes scrapie PrP 27-30 Protein. *Cell* **40**, 735-746.
5. Chesebro, B.,*et al.* (1985). Identification of scrapie prion-specific mRNA in scrapie-infected and uninfected brain. *Nature* **315**, 331-333.
6. Prusiner, S.B., Mckinley M.P., Bowman, K.A., *et al.* (1983). Scrapie prions aggregate to form amyloid-like birefringentrods. *Cell* **35**, 349-358.
7. Wietgrefe, S., Zupancid, M., Haase, A., *et al.* (1985). Cloning of a gene whose expression is increased in scrapie and in senile plaques in human brain. *Science* **230(4730)**,1177-1179.
8. Wisniewski, H.M., Bruce, M.E. and Fraser, H. (1975). Infectious etiology of neuritic (Senile) plaques in mice. *Science* **190**,1108-1110.
9. Dickinson, A.G., Bruce, M.E. and Scott, J.R. (1983). *Banbury report* **15**,387.
10. Prusiner, S.B. (1984). Purification and structural studies of a major scrapie prion protein. *Cell* **38**,127-134.
11. Chelly, J., Concordet, J. P., Kaplan, J.C. and Kahn, A. (April 1989). Illegitimate transcription: Transcription of any gene in any cell type. *Proc. Natl. Acad. Sci. USA.* **86**, 2617-2621.
12. Holtzman D.M. and Mobley, W.C. (April 1991). Molecular studies in Alzheimer's disease. TIBS 16,140-144.
13. Chomczynski, P. and Sacchi N. (1987). Single-stepmethod of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* **169**,156-159.
14. Gibbs, R.A., and Chamberlain, J.S. (1989). The polymerase chain reaction: a meeting report. *Genes & Develop.* **3**, 1095-1098.
15. Hixson, J.E., and Vernier,D.T. (1990). Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with Hhal. *J. Lipid Res.* **31**,545-548.

บทคัดย่อ

การศึกษาหรือตรวจวินิจฉัยความผิดปกติของผู้ป่วย Alzheimer's ที่นิยมใช้กันในปัจจุบัน คือ การทดลองศึกษาเนื้อเยื่อของผู้ป่วยหลังจากเสียชีวิตแล้ว แต่จากการวิจัยศึกษาที่นำเสนอในครั้งนี้ พบว่าเราสามารถศึกษาถึงยีนที่เฉพาะเจาะจงของโปรตีนบางชนิด เช่น โปรตีน Prion ซึ่งเป็นโปรตีนที่มีรูปร่างลักษณะคล้ายกับโปรตีน Amyloid ที่พบในเนื้อเยื่อสมองของผู้ป่วย Alzheimer's โดยใช้ mRNA lymphocytes และอาศัยเทคนิค PCR เข้าช่วย ซึ่งผลจากการวิจัยน่าที่จะเป็นประโยชน์ต่อการนำเทคนิคและวิธีการดังกล่าวมาประยุกต์ใช้กับการศึกษา วิจัย และวินิจฉัยผู้ป่วย Alzheimer's โดยใช้เฉพาะส่วนประกอบของเลือดผู้ป่วย ซึ่งง่ายต่อการเก็บส่งตรวจและสามารถทำได้ในขณะที่ผู้ป่วยยังมีชีวิตอยู่