CHARACTERISTICS OF PRION, BOVINE SPONGIFORM ENCEPHALOPATHY PATHOGEN, GENE KNOCKOUT COWS

Noboru MANABE[1], Ichiro ONOYAMA, Jun-You LI, Chun-Xiang PIAO, Hong-Mei GAO, Yasufumi GOTO, Takashi ONODERA, Yasuhiro YOSHIKAWA, Yutaka SENDAI* and Yoshito AOYAGI*

Animal Resource Science Center and Research Center for Food Safety, The University of Tokyo and * Central Research Institute for Feed and Livestock, and Embryo Transfer Center, Zen-Noh, Japan

Abstract: Prion (Pr) protein plays a pivotal role in the infection of bovine spongiform encephalopathy (BSE). The aim of the present research was to make Pr gene knockout (KO) cows, which lack Pr mRNA or protein, and to reveal their characteristics for estimating the usefulness. We were successful in production of Pr gene homo KO Japanese Black cows using leading-edge genetic engineering techniques, cell culture techniques and animal reproductive techniques (somatic cell nuclear transfer: SCNT and so on) as follows: Firstly, hetero Pr gene was knockouted in fibroblastic cells derived from bovine embryos. Each somatic cell nucleus with hetero Pr gene KO was transferred into oocyte without germinal vesicle (GV). After activation and *in vitro* culture, somatic cell nucleus of the somatic cell nuclear cloning embryo at blastocyst stage was pulled out and transferred into oocyte without GV to make homo Pr gene KO embryo. The homo Pr gene KO embryos were transferred in to the uterus. In the present meeting, we would like to show and discuss the procedure to make Pr gene homo KO cows and their usefulness, and to propose the importance of animal biotechnological techniques on food safety and public health. Moreover, detailed data of Pr distribution in organs of somatic cell nuclear cloning Japanese Black cows (used as controls) will be demonstrated as follows: A sandwich enzyme linked immunosorbent assay (ELISA) revealed that high levels of Pr proteinrotein (9.0 - 11.0 μg/g wet tissue) was demonstrated in brain of somatic cell nuclear transfer-cows (wild-type control animals). Lower levels of Pr proteinrotein were shown in other organs as follows in decreasing order: skeletal muscles (longissimus, iliocostalis thoracis, splenius, femoris biceps, triceps brachii, longissimus thoracis and omotransversarius muscles), intestinal tracts (ileum, jejunum, duodenum, colon and cecum) and tongue (apex, posterior, anterior and radix areas) (approx. 5.0- to 31.0 - fold less Pr proteinrotein than the brain). Immunohistochemical analyses showed that Pr proteinrotein was obviously expressed in nerve cells in brain and intestinal tissues. The presence of Pr proteinrotein in the bovine tongue, skeletal muscles and intestines raises the possibility of Pr proteinrotein accumulation in these tissues, indicating that these organs may serve as potential sources of BSE infection. No positive reaction for RT-PCR for Pr mRNA or Western blottingting, ELISA or immunohistochemistry for Pr proteinrotein was noted in Pr gene homo KO cows, indicating that Pr gene homo KO cows have no possibility of BSE infection. Thus, to produce the Pr gene homo KO cattle is the best and the only way to make safe bovine derived foods (beef and milk), drugs and medical materials and equipments.

Keywords: Bovine spongiform encephalopathy (BSE), Japanese black cow, Prion (Pr) protein, Pr gene homo knockout (KO)

Introduction

Misfolding of the Pr proteinrotein is involved in the pathogenesis of BSE [1, 2], but the mechanisms of prion transmission remains unresolved. This point is very important for reveal the pathogenic mechanism of Pr deseases. Furthermore, accumulation of abnormal Pr proteinrotein and cellular Pr proteinrotein deficiency induced by prion infection in the brain are presumed to be causative factors of Pr disease. One emerging hypothesis advocates that the abovementioned conversion phenomenon occurs at the site where the infectious agent (abnormal Pr proteinrotein) binds with cellular Pr proteinrotein. In cattle, apart from accumulating preferentially in

[1] **Contact details of corresponding author:** Noboru MANABE, Animal Resource Science Center, The University of Tokyo, 3145 Ago, Kasama 319-0206, Japan. Tel: +(81)-299-45-2606, Mail: amanabe@mail.ecc.u-tokyo.ac.jp

specified-risk materials (SRM). In Japan, SRM were immediately removed at slaughterhouse.Abnormal Pr proteinrotein has the potential to accumulate in peripheral tissues as well as in SRM. Abnormal Pr proteinrotein was recently detected in the muscles of sheep [3-5] and humans infected with sCJD [6, 7]. To assess the risk of abnormal Pr proteinrotein accumulation in peripheral tissues, it is important to understand the distribution and expression levels of cellular Pr proteinrotein in these tissues. To date, although studies have been performed in sheep [8, 9] and humans [10], cellular Pr proteinrotein levels in the peripheral tissues of cattle, which are regarded as transmission carriers of vCJD in humans, remain to be determined. To perfectly take away the risk and hazard factor for BSE infection, we produced Pr gene homo knockout (KO) Japanese black beef cows using forefront gene engineering techniques and animal reproductive biotechnological methods including SCNT, embryo transfer and so on.

Materials and Methods

Production of Pr protein KO cows: The cows lacking prion protein were produced at the experimental farm of Japan Agriculture Cooperation (Zen-Noh, Japan) using modified methods reported previously [11].

Generation of $PRNP^{-/-}$ fetal fibroblastic cell lines: Because these specialized techniques are applying for world patents, details are not able to write now. Briefly, sequential gene targeting was carried out. Two types of KO vectors were used [the first and second vectors were pBPr protein(H)KOneo (resistant to geneticin 418) and pBPr protein(H)KOpuro (resistant to puromycin)vectors, respectively] to sequentially disrupt both alleles of PRNP. To provide more specific information on the bovine PRNP genomic DNA, we used approx. 8.3 kb of BamHI-BamHI region (GenBank accession No.: AJ298878) for the 3' homologous arm and approx 1.2 kb of BglII-BamHI region for the 5' homologous arm.

The first KO procedure: Female Japanese black primary fetal fibroblastic cell lines were electroporated at 550 V and 50 muF with the first KO vector (pBPr protein(H)KOneo). We screened more than 100 colonies resistant to geneticin 418 (500 μg/ml), an aminoglycoside antibiotic by PCR to identify homologous recombinants and then homologous recombinants were identified. Based on their cell proliferation activity and morphology, we selected 1 colony and cloned embryos to generate fetuses. At approx. 50 days of gestation, 8 fetuses were collected and 4 of them were confirmed to be PRNP-/+ (hetero KO).

The second KO procedure: The heterozygous $PRNP^{-/+}$ cell line was electroporated with the second KO vector (pBPr protein(H)KOpuro), and more than 200 colonies resistant to puromycin (1 μg/ml) were screened by PCR to identify homozygously targeted colonies. Ten colonies were identified to be $PRNP^{-/-}$, 1 of which was used for embryonic cloning to generate recloned fetuses. At approx. 50 days of gestation, 12 fetuses were collected. The fibroblastic cell lines were established. All of them were confirmed to be homozygous $PRNP^{-/-}$ by the targeting event-specific and negative PCR analyses. We also performed Southern hybridization analysis on SphI and BamHI-double digested genomic DNA extracted from $PRNP^{-/-}$ fibroblast cell lines using the coding region of the neo or puro gene as a probe, which showed the expected band size and a single-site integration of the KO cassettes.

Embryonic cloning: The researchers of the Animal Disease Center used chromatin transfer procedure [11], but we used SCNT technique to produce the cloned fetuses and calves. Using embryonic fibroblastic cells without gene KO procedure, SCNT embryos were produced to use wild-type controls. All animal works described in this paper were done following a protocol approved by the Japanese Committee for Animal Gene Manipulation, Care and Use.

Gross and microscopic examinations: Six wild-type and six $PRNP^{-/-}$ KO (Pr KO) cows were used. Two calves (1 wild-type and 1 PR KO calves) at the birth (0 month), 4 calves (2 wild-type and 2 PR KO calves) at 3 months and 2 calves (1 wild-type and 1 PR KO cows) at 12 months were killed with deep pentobarbital anesthesia and were subjected to complete necropsy examinations. Representative samples of organs [whole brain, eyes with theiroptic nerves, sciatic nerve, both trigeminal nerves, ganglia, pituitary gland, spinal cord (cervical, thoracic and lumbar), skeletal muscles (longissimus, iliocostalis thoracis, splenius, femoris biceps, triceps brachii,

longissimus thoracis and omotransversarius muscles), gastro intestinal tracts (reticulum, rumen, omasum, abomasum, ileum, jejunum, duodenum, colon and cecum) and tongue (apex, posterior, anterior and radix areas), skin, nasal turbinate, lung, liver, kidney, spleen, salivary gland, thyroid gland, tonsils (pharyngeal and palatine), thymus, adrenal gland, pancreas, urinary bladder, lymph nodes (retropharyngeal, prescapular, mesenteric, popliteal), aorta, heart and ovaries] were evaluated by gross and microscopic examinations.

Immunohistochemistry for Pr protein: For immunohistochemistry, each tissue sample was fixed and embedded in resin as described previously [12]. Sections, 4-μm in thicknwss, were cut, mounted on silanized glass slides, subjected to immunohistochemistry. Briefly, to observe distribution in the peripheral nervous system and Pr protein in the bovine tissue sections were reacted with anti-Pr protein mAb T2 (1:100) or anti-PgP 9.5 pAb (1:100) for 2 hr at 37 °C, then further incubated with Alexa FluorR 488-coupled goat anti-mouse IgG (H+L) antibody or with goat anti-rabbit IgG (H+L) antibody coupled with Alexa FluorR 546 (5 μg/ml) at room temperature for 30 min. Sections were then counterstained with DAPI and observed using a fluorescence microscope.

Reverse transcription-polymerase chain reaction (RT-PCR) for Pr mRNA: Total RNA was extracted from $PRNP^{+/+}$ and $PRNP^{-/-}$ calf fibroblasts by using RNeasy mini kit, and first strand cDNA synthesis was done by using superscript first-strand synthesis system for RT-PCR according manufacturer's protocols. PCR was performed as previously described [12].

Western blotting for Pr protein: As previously described [12], protein was extracted from $PRNP^{+/+}$ and $PRNP^{-/-}$ calf fibroblasts, peripheral blood lymphocytes and brain stem. The protein content was quantified with a protein assay kit. Western blotting analysis was carried out by running approx. 75 μg of protein sample on a 12% (w/w) sodium dodecyl sulfate-polyacrylamide gel electrophoresis SDS-PAGE gel under nonreducing conditions. Recombinant bovine Pr protein was used as standard in this study. The proteins were transferred to a nitrocellulose membrane and the membrane was stained by the mouse anti-bovine prion protein monoclonal antibody as a primary antibody and second HRP-labeled affinity-purified antibody to mouse IgG (H+L). The stained membrane was developed by ECL plus Western blotting analysis detection system. After development, the stained gels were recorded with a digital fluorescence-recorder, and at each expression level, the fluorescence intensity of each band, was quantified using Image-Gauge on a Macintosh computer. The relative abundance of specific mRNA was normalized to the relative abundance of Recombinant bovine Pr protein. Detection limit for Pr protein protein in the Western blotting analysis was estimated to be approx. 1.2 μg of brain homogenate from the wild type, which was approx. 60-fold less than the protein amount (75 μg total brain protein).

Enzyme linked immunosorbent assay (ELISA) for Pr protein: Bovine tissues were homogenized in lysis buffer [phosphate-buffered saline (PBS) containing 0.5% (w/v) Nonidet P-40, 0.5% sodium deoxycholate and 2 mM phenylmethylsulfonyl fluoride] using a bead shocker for the preparation of 10% (w/v) tissue homogenates. The hamster brain was also homogenized in lysis buffer. Then, samples were subjected to centrifugation at 5,000 g for 5 min and stored at -80 °C until use. Purification and HRP-labeling of anti-Pr protein monoclonal antibodies T2 and T17 were performed as previously described [12]. Briefly, hybridomas producing T2 and T17 monoclonal antibodies were grown separately in hybridoma-SFM (serum-free medium) at 37 °C in a humidified 5% CO2 incubator. Cell suspensions were subjected to centrifugation at 1,500 g for 5 min before the supernatants were harvested. The monoclonal antibodies were isolated from hybridoma supernatants using HiTrap Protein-G and a PD-10 column in accordance with the manufacturer's instructions. Harvested monoclonal antibodies were labeled with HRP using a Peroxidase-labeling Kit-SH in accordance with the manufacturer's instructions. For sandwich ELISA, microtiter plates were coated with 100 μl of purified capture monoclonal antibodies (1 μg/ml) in 0.1 M carbonate buffer (pH 9.5) overnight at 4 °C and washed 3 times with PBS. The coated plates were then blocked with 200 μl of Block Ace (diluted 1:4 in PBS) for 1 hr at room temperature (22 - 25 °C), and subsequently rinsed 3 times with PBS. Diluted samples in PBS (100 μl)

dispersed in 96-well plates were incubated for 1 hr at room temperature. The plates were washed 5 times with PBS prior to treatment with 100 μl of HRP-labeled anti-Pr protein monoclonal antibodies (0.5 μg/ml) and PBS in the wells. After washing with PBS, o-phenylenediamine solution was dispensed at 100 μl/well. After incubation for 30 min in the dark, each well was treated with 20 μl of H2SO4 (6 N), then the absorbance of each well was measured at 490 nm on a microplate reader.

The protein misfolding cyclic amplification (PMCA) procedure: A sensitive detection of pathological Pr protein by cyclic amplification of protein misfolding was performed as previously described [12]. Briefly, 10% (w/v) brain homogenates were prepared from the cortex or hypothalamus of wild-type or PRNP-/- cows. The homogenates were prepared in conversion buffer [PBS containing 150 mM NaCl, 1.0% (v/v) Triton X-100, 4 mM ethylenediaminetetraacetic acid disodium salt (EDTA) and the complete protease inhibitor cocktail]. The samples were clarified by a brief, low-speed centrifugation (1,500 rpm for 30 sec) using a entrifuge. Dilutions of this brain homogenate were done in conversion buffer and they are expressed in relation to the brain. Aliquots of wild-type and $PRNP^{-/-}$ cow brain homo genate prepared in conversion buffer were mixed and either immediately subjected to 48 cycles of PMCA. For PMCA, each tube was positioned on an adaptor placed on the plate holder of a microsonicator and programmed to perform cycles of 30 min incubation at 37 °C. Each sample was incubated with 50μg/ml of proteinase K for 60 min at 45 °C. The digestion was stopped by adding electrophoresis sample buffer. Proteins were fractionated by SDS-PAGE under reducing conditions, electroblotted into nitrocellulose membrane, and probed with an antibody (diluted 1:5,000 in PBS). The immunoreactive bands were visualized by an enhanced chemiluminescence assay.

Statistical analysis: Using StatView-4.5 program on a Macintosh computer, statistical analysis was performed by Student's t-test using both confidence interval estimate analysis and t score probability hypothesis testing method for two independent sample groups. Differences at $P < 0.05$ were considered significant. Both methods of analysis showed that there were no significant differences between PRNP-/- and wild type control cattle groups.

Results and Discussion

Pr distribution in bovine organs: Histochemical stainings showed that Pr was expressed in nerve cells in brain and intestinal tissues. The presence of Pr in the bovine tongue, skeletal muscles and intestines raises the possibility of Pr accumulation in these tissues, indicating that these organs/tissues may serve as potential sources of BSE infection.

Pr levels in bovine organs/tissues: ELISA revealed that high levels of Pr (9.0-11.0 μg/g wet tissue) was demonstrated in brain of SCNT cows (wild-type control animals). Lower levels of Pr were shown in other organs [in decreasing order as follows: skeletal muscles (longissimus, iliocostalis thoracis, splenius, femoris biceps, triceps brachii, longissimus thoracis and omotransversarius muscles), intestinal tracts (ileum, jejunum, duodenum, colon and cecum) and tongue (apex, posterior, anterior and radix areas) (5.0 - to 31.0-fold less Pr than the brain).

Conclusion: We success to produce Pr gene homo KO Japanese Black cows, which lack Pr mRNA or protein. The Pr gene homo KO cows have no possibility of abnormal Pr proteinrotein infection. Thus, to produce the KO cattle is the best and the only way to make safe bovine-derived foods (beef and milk), drugs and medical materials and equipments.

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References

1. Prusiner SB: Prions. Proc Natl Acad Sci USA 95: 13363-13383, 1998.
2. Jackson GS and Clarke AR: Mammalian prion proteins. Current Opini Struct Biol 10: 69-74, 2000.
3. Masujin K *et al.*: Prions in the peripheral nerves of bovine spongiform encephalopathy-affected cattle. J Gen Virol 88: 1850-1858, 2007.
4. Sakudo A *et al.*: Recent advances in clarifying prion protein functions using knockout mice and derived cell lines. Mini Rev Med Chem 87: 589-601, 2006.
5. Andreoletti O *et al.*: Protease-resistant form Pr accumulation in myocytes from sheep incubating natural scrapie. Nat Med 10: 591-593, 2004.
6. Glatzel M *et al.*: Extraneural pathologic prion protein in sporadic Creutzfeldt-Jakob disease. N Engl J Med 349: 1812-1820, 2003.
7. Kovacs GG *et al.*: Creutzfeldt-Jakob disease and inclusion body myositis: abundant disease-associated prion protein in muscle. Ann Neurol 55: 121-125, 2004.
8. Horiuchi M *et al.*: A cellular form of prion protein (Cellular Pr) exists in many non-neuronal tissues of sheep. J Gen Virol 76: 2583-2587, 1995.
9. Moudjou M *et al.*: Cellular prion protein status in sheep: tissue-specific biochemical signatures. J Gen Virol 82: 2017-2024, 2001.
10. Pammer J, Suchy A, Rendl M and Tschachler E: Cellular prion protein expressed by bovine squamous epithelia of skin and upper gastrointestinal tract. Lancet 354: 1702-1703, 1999.
11. Richt JA *et al.*: Production of cattle lacking prion protein. Nat Biotechnol 25: 132-138, 2006.
12. Ano Y, *et al.*: Incorporation of s-amyloid protein through the ileal epithelium before and after weaning: Model for orally transmitted amyloidoses. Microbiol Immunol 52: 429-434, 2008.