American Journal of Infectious Diseases 3 (3): 165-168, 2007
ISSN 1553-6203

Accumulation of Cholesterol Esters in e*x vivo* Lymphocytes from Scrapie-susceptible Sheep and in Scrapie-infected Mouse Neuroblastoma Cell Lines

[1]Alessandra Pani, [2]Claudia Norfo, [2]Claudia Abete, [2]Claudia Mulas, [2]Marirosa Putzolu, [2]Sergio Laconi, [1]Christina Doriana Orrù, [1]M. Dolores Cannas, [1]Sarah Vascellari, [1]Paolo La Colla, and [2]Sandra Dessì
[1]General Microbiology and Virology and Microbial Biotechnologies Section,
[2]Experimental Pathology Section,
Department of Biomedical Sciences and Technologies,
University of Cagliari, Italy

Abstract: Our studies on the role of cholesterol homeostasis in the pathogenesis of scrapie in sheep, revealed abnormal accumulation of cholesterol esters in brains and in *ex vivo* skin fibroblasts from genetically scrapie-susceptible, as compared to sheep with resistant genotype. We now report that PBMCs isolated from scrapie-susceptible sheep, as well as mouse neuroblastoma cell lines persistently infected with two different mouse-adapted strains of scrapie, showed similar alterations with up to 3-fold higher cholesterol ester levels than their resistant or uninfected counterparts. Treatments with drugs that interfere with intracellular cholesterol metabolism strongly reduced accumulation of cholesterol esters in scrapie-infected cell lines, whereas had significantly lower, or no effect, in uninfected cell line. These data add support to our hypothesis that accumulation of cholesterol esters may represent a biological marker of susceptibility to prion infection and a potential molecular target for prion inhibitors.

Key words: scrapie, prion protein, cholesterol esters, inhibitors

INTRODUCTION

All prion diseases are invariably fatal and neither proven treatments for the underlying pathologic process nor conclusive means for diagnosis or prevention are currently available [1,2]. Given the present lack of molecular markers allowing for certain early diagnosis, any potentially effective treatment can only be administered after the actual onset of clinical disease, while the long incubation period (up to a decade in some prion diseases) indicates a considerable time period of clinically silent disease development [3]. Likely, during this period metabolic processes, in concert with genetic factor(s) [4], may promote or interfere, sustain or hamper, prion generation and accumulation, thus influencing the time length preceding clinical signs or, possibly, even preventing the irreversible cascade of pathogenic events.

Our recent studies on the role of alterations of intracellular cholesterol homeostasis in the pathogenesis of scrapie in sheep, indicated abnormal intracellular accumulation of cholesterol esters (CE) as a distinctive trait in both cerebral and peripheral tissues of animals carrying scrapie-susceptible genotype [5]. With respect to genetically scrapie-resistant (ARR) animals, increased levels of CE were observed in brains and *ex vivo* skin fibroblasts from both healthy and scrapie-affected sheep carrying the susceptible ARQ genotype.

We here report on the different levels of CE in *ex vivo* PBMCs from sheep with genotypes affecting scrapie-susceptibility, and in mouse neuroblastoma cell lines, either uninfected and persistently infected with two mouse-adapted strains of scrapie.

METHODS

Chemicals: Congo red (91%) was purchased from Sigma-Aldrich (Italy). Everolimus was kindly provided by Novartis (Switzerland), and Pioglitazone by Takeda (Japan). Everolimus was solubilized in 100% ethanol and stored at +4°C. Pioglitazone was solubilized in 100% ethanol and stored at room temperature.

Ex vivo sheep PBMCs: Sheep blood samples were a generous gift by Dr. Ciriaco Ligios and were collected at the Istituto Zooprofilattico Sperimentale of Sardinia, Italy. Samples were collected from the anterior vena cava of a total of 14 Sarda breed sheep; 4 carried the scrapie-resistant ARR/ARR genotype, and 10 the scrapie-susceptible ARQ/ARQ genotype. Of these, 2 were mock-infected; 1 had natural scrapie; and 7 developed clinical disease following experimental inoculation of scrapie. The 4 scrapie-resistant sheep, that were scrapie-infected in parallel with susceptible animals, as well as the 2 mock-infected scrapie-

Corresponding Author: Alessandra Pani, Università di Cagliari, Dipartimento di Scienze e Tecnologie Biomediche, Cittadella Universitaria, 09042-Monserrato (CA), Italia. Phone: +390706754209 Fax: +390706754210

susceptible sheep, did not develop any clinical signs and are alive and healthy at the time of present report. All samples were collected at the time of terminal clinical stage of the ill animals, and mononuclear cells (PBMCs) were separated by Ficoll-Hypaque density gradient. After extensive washings, cells were suspended ($1x10^6$ cells/ml) in RPMI-1640 with 10% FCS and incubated overnight. For determinations, $5x10^5$ cells/ml non-adherent cells were incubated with PHA (10 μg/ml, Sigma-Aldrich) at 37°C in RPMI-1640 supplemented with 10% FCS. Number of viable cells was evaluated during time courses by counting trypan blue-excluding cells.

Cell lines: The mouse neuroblastoma N2a cell line, and the 22L-N2a and RML-N2a sublines, respectively infected with the mouse-adapted 22L or RML (Rocky Mountain Laboratory) strain of scrapie, were a generous gift by Dr. Byron Caughey, NIH/NIAID Rocky Mountain Laboratories, USA. Cells were grown and maintained at 37°C, 5% CO_2, in OptiMEM supplemented with 10% FBS (Gibco, Invitrogen; Italy), 2mM L-glutamine, 50 U/ml penicillin G sodium and 50 μg/ml streptomycin sulphate (Gibco, Invitrogen; Italy). Experiments were carried out in cell cultures during exponential growth.

Lipid staining: Neutral lipid content was determined at the indicated time points by oil red O (ORO) staining as previously described.[5] Stained cells were examined by light microscopy and digital images were recorded. Red color intensity in single cells, indicating neutral lipid-bound oil red O, was measured by the NIH Image 1.63 Analysis Software program (Scion Image). Values are expressed as the mean colour intensity per cell calculated on at least 30 single cells in 6 different microscopic fields.

Statistical analysis: All values are presented as the mean ± standard error (SE). Statistical comparisons were performed with the Student *t* test. Significance was set at $P < 0.05$.

RESULTS

Cholesterol esters in *ex vivo* PBMCs from sheep genetically susceptible or resistant to Scrapie. The comparative analysis of CE content in brains and *ex vivo* skin fibroblasts from sheep genetically susceptible or resistant to scrapie, had revealed in resistant (*PNRP* genotype: ARR/ARR) sheep lower CE levels than in scrapie-susceptible (*PNRP* genotype: ARQ/ARQ) animals, either scrapie-affected (ARQ/ARQ+) or uninfected (ARQ/ARQ-).[5] Therefore, we deemed interesting to investigate whether other peripheral cells from the sheep genetically susceptible or resistant to scrapie, showed similar alterations in cholesterol metabolism. To this end, purified sheep PBMCs were stained for intracellular neutral lipids with the ORO method, before and after mitogen stimulation with PHA. Similarly to what previously observed in skin fibroblasts, PBMCs from scrapie-susceptible sheep showed levels of CE markedly higher than those from sheep with resistant genotype (Table 1). Following mitogenic stimulus, CE levels increased in all cultures, but in PBMCs from scrapie-susceptible scrapie-affected sheep increased the most.

Table 1: Neutral lipid content in PBMCs from sheep genetically susceptible and resistant to scrapie

Sheep genotype	Mean red stain/ cell ± SE	
	Quiescent PBMCs	PHA-stimulated PBMCs
ARR/ARR	0.7 ±0.1	1.1 ±0.2
ARQ/ARQ -	1.5 ±0.2	2.4 ±0.3
ARQ/ARQ +	2.2 ±0.2	3.3 ±0.3

PBMCs ($1x10^6$/ml) were stained for intracellular neutral lipids with the ORO method, before or after 24-hour incubation with PHA. Values represent the mean ± standard error of red colour intensity per cell. Experiments were performed in duplicate and repeated at least three times. ARR/ARR: scrapie-resistant; ARQ/ARQ-: scrapie-susceptible; ARQ/ARQ+: scrapie-susceptible, scrapie-affected.

Accumulation of cholesterol esters in scrapie-infected mouse neuroblastoma cell lines: Mouse neuroblastoma cell lines and sublines infected with different mouse-adapted strains of scrapie, represent *in vitro* cell models largely used to study prion infection and replication, as well as to identify novel prion inhibitors. The results obtained in sheep skin fibroblast and PBMC cultures, promped us to compare CE levels in such cell systems to investigate whether establishment of scrapie infection was accompanied by modifications in the CE levels. The comparison of neutral lipid content in uninfected mouse neuroblastoma N2a cell line and in two persistently scrapie-infected sublines, 22L-N2a and RML-N2a, revealed markedly higher CE levels in the infected cells as compared to the uninfected cell line (Table 2).

Table 2: Neutral lipid content in uninfected and prion-infected mouse neuroblastoma cell lines

Cell line	Mean red stain/ cell ± SE	
	24 hours	48 hours
N2a	3.1 ±1.0	4.2 ±1.5
22L-N2a	8.4 ±1.8	12.3 ±1.8
RML-N2a	7.9 ±1.2	9.8 ±1.7

Cells seeded at $2x10^5$/ml were stained for intracellular neutral lipids with the ORO method at the indicated times. Values represent the mean ± standard error of red colour intensity per cell. Experiments were performed in duplicate and repeated at least three times. N2a: parental cells; 22L-N2a: N2a subline infected with 22L prion strain; RML-N2a: N2a subline infected with 22L prion strain.

CE accumulation was greater in 22L-N2a than in RML-N2a cells, with 3-fold and 2-fold higher CE levels than N2a cells, respectively. Treatments with two cholesterol

modulators, Everolimus and Pioglitazone, known to affect cholesterol esterification [6,7,8], strongly reduced CE levels in scrapie-infected 22L-N2a cells (Fig. 1), whereas had no significant inhibitory effect in the uninfected N2a cultures (not shown). In 22L-N2a cells, CE content was reduced by 60% with 50nM Everolimus, and by 35% with 40μM Pioglitazone. A somehow unexpected result was obtained with Congo red, used as anti-prion reference compound which, although at high concentrations (10μM), showed the ability to decrease CE levels by almost 70% in the scrapie-infected 22L-N2a cell line (Fig. 1). Similar results were obtained in the RML-N2a subline (not shown)

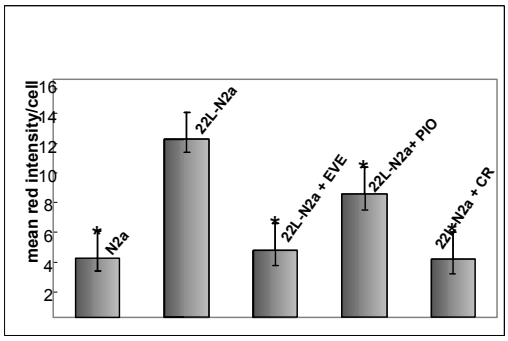

Fig. 1: Neutral lipid content in N2a and 22L-N2a mouse neuroblastoma cell lines. Intracellular neutral lipids in N2a and 22L-N2a cells were stained by the ORO method after 48-hour incubation in the absence or in the presence of everolimus (EVE, 50 nM), pioglitazone (PIO, 40 μM), and congo red (CR, 10 μM). Quantification of the intensity of the lipid-bound stain color was determined by densitometric analysis through the Scion Image software (NIH). Values represent means ± SE of quadruplicate determinations from triplicate experiments. *P<0.05 vs 22L-N2a cells.

DISCUSSION

The data presented herein confirm our previous evidence [5] of a relationship between abnormal accumulation of cholesterol esters and cell susceptibility to scrapie infection/replication. Similarly to sheep brain tissue and skin fibroblasts i) *ex vivo* PBMC cultures from scrapie-susceptible sheep, either or not scrapie-affected, showed abnormally high cholesterol ester levels as compared to cells from scrapie-resistant animals; ii) two scrapie-infected cell lines showed up to 3-fold higher levels of cholesterol esters than parental uninfected cell line; and iii) drugs modulating intracellular cholesterol metabolism reduced cholesterol esters in the scrapie-infected cell lines, whereas showed significantly lower or no effects in their uninfected counterpart.

Besides our investigations, other studies had pointed out to the potential role of cellular cholesterol in prion generation and replication [9,10], and drugs known to affect the *de novo* cholesterol biosynthesis (i.e. statins) have been reported to inhibit scrapie-like prion protein *in vitro*.[11] The ability shown by cholesterol modulators to effectively inhibit cholesterol esterification only in scrapie-infected cell lines, add support to our hypothesis [5] that a cell phenotype characterized by abnormally high levels of cholesterol esters, could increase, in cooperation with the *PRNP* genotype, susceptibility to prion infection or, alternatively, better sustain prion replication. Although with present knowledge we are unable to explain the unexpected ability of the prion inhibitor congo red to affect cholesterol ester content in the scrapie-infected cell lines, this result further underlines the likewise of a biological link between intracellular level of cholesterol esters and generation/accumulation of prion protein.

Our previous [5] and present findings have been object of US patent applications [12,13], and further studies are already in progress to establish whether cholesterol esters may truly represent a target of clinical interest and a biological marker of susceptibility to prions.

ACKNOWLEDGEMENTS

The Authors are indebted with Ciriaco Ligios (Istituto Zooprofilattico Sperimentale, Sassari) and with Byron Caughey (NIH/NIAID Rocky Mountain Laboratories, USA) for providing sheep samples and mouse neuroblastoma cell lines.

REFERENCES

1. Cashman N.R., B. Coughey, 2005. Prion diseases-Close to effective therapy? Nat. Rev. Drug Disc., 3: 874-84.
2. Trevitt, CR., J Collinge, 2006. A systemtic review of prion therapeutics in experimental models. Brain, 129 (9): 2241-65
3. Collinge J., J. Whitfield, E. McKintosh, J. Beck, S. Mead, D.J. Thomas, M.P. Alpers, 2006. Kuru in the 21st century-an acquired human prion disease with very long incubation periods. Lancet, 367 (9528):2068-74
4. Mead S., S.P. Mahal, J. Beck, T. Campbell, M. Farral, E. Fisher, J. Collinge, 2001. Sporadic- but not variant-Creutzfeldt-Jakob disease is associated with polymorphisms upstream of PRNP exon 1. Am. J. Hum. Genet., 69(6): 1225-35

5. Pani A., C. Abete, C. Norfo, C. Mulas, M. Putzolu, S. Laconi, M.D. Cannas, C.D. Orrù, P. La Colla, and S. Dessì, 2007. Cholesterol metabolism in brain and skin fibroblasts from Sarda breed sheep with scrapie resistant or susceptible genotype. Am. J. Infect. Dis. (present issue)
6. Batetta, B., M.F. Mulas, F. Sanna, M. Putzolu, R.R. Bonatesta, A. Casperi-Campani, L. Roncuzzi, D. Baiocchi, S. Dessì, 2003. Role of cholesterol ester pathway in the control of cell cycle in human aortic smooth muscle cells. FASEB J., 17(6):746-8
7. Freeman, D.A., A. Romero, 2003. Effects of troglitazone on intracellular cholesterol distribution and cholesterol-dependent cell functions in MA-10 Leydig tumor cells. Biochem. Pharmacol., 66 (2): 307-13
8. Galantuomo, M., M.F. Mulas, P. Baire, C. Abete, E. Peiretti, S. Dessì, M. Fossarello, 2005. Proliferative Activity and Cholesterol Ester Metabolism in Primary Culture of Human Pterygium Fibroblasts. Invest. Ophthalmol. Vis. Sci., 46 (11): 3982-5
9. Taraboulos A., M. Scott, A. Semenov, D. Avrahami, L. Laszlo, S.B. Prusiner, 1995. Cholesterol depletion and modification of COOH-terminal targeting sequence of the prion protein inhibit formation of the scrapie isoform. J. Cell. Biol., 129(1):121-32
10. Gilch, S., C. Kehler, H.M. Schatzl, 2006. The prion protein requires cholesterol for cell surface localization. Mol. Cell Neurosci., 31(2):346-53
11. Bate, C., M. Salmona, L. Diomede, A. Williams, 2004. Squalestatin cures prion-infected neurons and protects against prion neurotoxicity. J. Biol. Chem., 279(15):14983-90
12. Dessì, S., P. La Colla, A. Pani, 2007. Methods for the diagnosis of proliferative and/or conformational diseases. PCT/IT2007/000110
13. Anchisi, C., S. Dessì, P. La Colla, A. Pani, 2007. Prophylactic and/or therapeutic treatment of proliferative and conformational diseases. PCT/IT2007/000109.