Up-regulation of cathepsin B and cathepsin L activities in scrapie-infected mouse Neuro2a cells

Yonghua Zhang,1,2 Eberhard Spiess,3 Martin H. Groschup4 and Alexander Bürkle1,2†

1Department of Gerontology, Institute for Ageing and Health, University of Newcastle upon Tyne, Newcastle upon Tyne, UK
2,3Abteilung Tumorvirologie2 and Arbeitsgruppe Biomedizinische Struktursforschung3, Deutsches Krebsforschungszentrum, Heidelberg, Germany
4Institute for Novel and Emerging Infectious Diseases, Federal Research Centre for Virus Diseases, Insel Riems, Germany

Correspondence
Alexander Bürkle
Alexander.Buerkle@uni-konstanz.de

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Prion diseases are characterized by the accumulation of an abnormal, proteinase K-resistant isoform of the prion protein, PrPSc, which is generated by a post-translational conversion of the protease-sensitive normal cell-surface glycoprotein PrPc involving major conformational changes. The conversion is thought to occur at the plasma membrane or along the endocytic pathway towards the lysosome. PrPSc aggregates have been found to accumulate in secondary lysosomes. In our study, the activities of two major lysosomal cysteine proteases, cathepsins B and L, were found to be significantly increased in scrapie-infected Neuro2a cells compared with uninfected cells using biochemical and cytochemical methods. We hypothesize that lysosomal proteases may be involved in a ‘second autocatalytic loop’ of PrPSc formation, acting in concert with the well-known autocatalytic enhancement of PrP conversion in the presence of PrPSc.

Prion diseases are characterized by the accumulation of an abnormal, proteinase K-resistant isoform of the prion protein, PrPSc, which is generated by a post-translational conversion of the protease-sensitive normal cell-surface glycoprotein PrPc involving major conformational changes (Aguzzi et al., 2001; Caughey, 1994; Collinge, 2001; Prusiner, 2001). The conversion from PrPc to PrPSc is thought to occur at the plasma membrane or along the endocytic pathway towards the lysosome (Caughey & Raymond, 1993). PrPSc aggregates have been found to accumulate in secondary lysosomes (McKinley et al., 1991). In scrapie-infected mouse brain up-regulation of several genes encoding lysosomal proteases has been described (Dandoy-Dron et al., 1998; Diedrich et al., 1991; Kopacek et al., 2000). However, it is unclear (i) whether the observed changes occur in neuronal or non-neuronal brain cells or in both; (ii) whether such changes are direct or indirect consequences of prion infection; and (iii) whether they are mirrored by similar changes in enzyme activity. We have measured the activities of two major lysosomal cysteine proteases, cathepsins B and L, following prion infection of Neuro2a cells, a purely neuronal cell type.

Neuro2a cells (ATCC), persistently scrapie-infected mouse neuroblastoma cells (ScNB; Caughey & Raymond, 1993) and the highly susceptible Neuro2a subline D11, either uninfected (‘D11’) or newly infected with the Chandler strain of mouse-adapted scrapie agent (‘ScD11’) (Zhang et al., 2002), were all maintained at 37°C in 5 % CO2 in OPTI-MEM (Life Technologies) supplemented with 10 % heat-inactivated foetal calf serum and penicillin/streptomycin. Briefly, D11 cells were isolated by cloning uninfected Neuro2a cells by limiting dilution and infecting aliquots of the resulting clonal populations with scrapie inocula (Chandler strain) according to the protocol developed by Bosque & Prusiner (2000). The inocula for the infection of cells used in the present work were homogenates from ScNB cells, diluted 1 : 30 in culture medium (Zhang et al., 2002). Cells were grown in the presence of inoculum for 4 days. Thereafter, cells were trypsinized, transferred to 24-well plates and grown in normal medium. Cells were used 7 weeks post-infection, by which time PrPSc production was confirmed by cell ELISA (Zhang et al., 2002). Cell clone ScD11 was selected for its high efficiency of PrPSc production and the uninfected D11 parental cells were used as controls. Morphology and proliferation rate was very similar between infected and uninfected cells.

All chemicals were from Bachem Biochemica or Serva (both in Heidelberg, Germany). Cathepsin B activity was detected in a cytochemical assay using the specific synthetic substrate N-benzoyloxycarbonyl-arginy1-arginine-4-methoxy-β-naphthyamine (1 mM) as described previously (Dolbeare &
Smith, 1977). The fluorescent reaction product 4-methoxy-
β-naphthylamine was precipitated by 5-nitro-salicylaldehyde
followed by microscopic evaluation.

In addition, cathepsin B and L activities were measured by
a quantitative fluorometric assay as described previously
(Barrett & Kirschke, 1981; Ulbricht et al., 1995). Briefly,
confluent cultures in 50 cm² flasks were trypsinized, washed
and suspended in ice-cold lysis buffer (400 mM sodium
phosphate buffer, pH 6.0, 75 mM NaCl, 4 mM EDTA-Na₂,
0-25 % Triton X-100) followed by incubation on ice for 1 h.
Lysates were homogenized in a Transonic-Bath three times
for 15 s each at 4 °C and centrifuged at 20 000 g for 30 min
at 4 °C. The protein concentration of supernatants (cell
extracts) was determined by the Bio-Rad DC assay and cell
extracts were stored at −80 °C. Prior to starting the reaction,
reaction buffer (for cathepsin B assays: 88 mM KH₂PO₄,
12 mM Na₂HPO₄, 1.33 mM EDTA-Na₂, 2.7 mM L-
cysteine, pH 6.0; for cathepsin L assays 100 mM sodium
acetate buffer, 1 mM EDTA-Na₂, 2 mM dithiothreitol,
ph 5.5) was pre-warmed to 37 °C. Then 100 µl of cell
diluted in lysis buffer (maximally 70–100 μg protein)
was added and mixed, followed by incubation at 37 °C for
5 min. Finally 25 μl substrate solution was added. Fluores-
cence time scans of the reaction mixture in quantitative
assays showed a practically linear course over the first 1000 s
(data not shown). Based on these observations, a 15 min
assay time was chosen as a standard time for all experiments.
A range of protein concentrations was used in the assays in
order to ensure that the reaction did not reach saturation.
All reactions were run at 37 °C for 15 min in a total volume
of 1 ml, in the absence or presence of cathepsin inhibitors
(see below), the latter value defining the non-specific
background activity to be subtracted. For cathepsin B assays,
the cathepsin B-specific substrate benzyloxy carbonyl-L-
arginyl-L-arginyl-7-amino-4-methylcoumarine (Z-arg-arg-
AMC; 5 μM) was used in conjunction with the cysteine
protease inhibitor L-trans-epoxy-succinyl-leucylamido-(4-
guanidino)-butane (E64; 50 μM), or, alternatively, the
cathepsin B- and L-specific substrate benzyloxy carbonyl-
L-phenylalanyl-L-arginyl-4-methylcoumarine (Z-phenyl-
leucylamido-(4-guanidino)-butane (E64; 50 μM), or, alternatively, the
cathepsin B inhibitor L-trans-epoxy-succinyl-Ile-Pro-OH propylamide
(CA-074; 50 μM). To detect cathepsin L activity, the
substrate Z-phe-arg-AMC (5 μM) was used in conjunction
with the specific cathepsin L inhibitor benzyloxy carbonyl-L-
phenylalanly-l-phenylalanlyl-diazomethylene ketone (Z-phenyl-
leucylamido-(4-guanidino)-butane (E64; 50 μM), or, alternatively, in the presence of either
CA-074 or E64, the difference between the two values
defining cathepsin L activity. To eliminate prion infectivity
in the samples before fluorescence measurements, reactions
were stopped by adding 5 vols 6 M guanidine
hydrochloride, 50 mM Tris/HCl, pH 7.4, and incubating for
20 min at room temperature. The fluorescent product was
quantified in a fluorometer (Hitachi-2000; Ex 370 nm, Em
460 nm) and standardized with amino-methylcoumarine.
One unit of enzyme activity represents 1 µmol substrate
turned over min⁻¹.

In the cytochemical assay, fluorescence signals resulting
from cathepsin B activity became visible in uninfected
Neuro2a cells by 90 min of incubation with substrate,
whereas in the persistently infected cells they were already
detectable by 30 min, with a time-dependent further
increase in intensity (Fig. 1). In order to see whether or
not this apparent increase in cellular cathepsin B activity in
the infected cells was the result of generally increased protein
content per cell under conditions of infection, we next
performed quantitative fluorometric assays in cell lysates
and normalized the enzyme activity data for total protein
content. These fluorometric assays revealed significant
increases in cathepsin B and also in cathepsin L activity in
ScNB cells compared with uninfected Neuro2a cells (Fig. 2
A, B).

To avoid any artefacts when comparing uninfected with
persistently infected cells due to the commonly performed
recloning of the latter to select for producer cells (Bosque &
Prusiner, 2000), we also performed cathepsin assays on
newly infected cells from a highly susceptible Neuro2a
subline (Zhang et al., 2002) that we had isolated according to
a published protocol (Bosque & Prusiner, 2000) and the
respective uninfected cells. In this system, very similar,
significant increases in cathepsin B and L activities were
found in the infected cells (Fig. 2C, D).

Furthermore, additional assays of cathepsin B were per-
formed using substrate Z-phe-arg-AMC in conjunction
with the inhibitor CA-074 (E) and also of cathepsin L using
the substrate Z-phe-arg-AMC in the presence of either CA-
074 or E64 (F). Again, the data yielded the same pattern, i.e.
significantly elevated cathepsin B and L activities in the

Fig. 1. Cytochemical detection of cathepsin B activity in unin-
fected Neuro2a (A–D) and ScNB (E–H) cells at assay times of
30 min (A, E), 60 min (B, F), 90 min (C, G) and 120 min
(D, H). For details see text.
infected cells, but the absolute cathepsin L activity values were lower than with the previous assay design, indicating superior specificity for cathepsin L.

The up-regulation of cathepsin activities in neuronal cells following prion infection described here may have important consequences for both pathogenesis and agent replication. Lysosomal hydrolases seem to play a role both in necrosis (Yamashima, 2000) and in the autophagic cell death described in neurons (Kegel et al., 2000). On the other hand, cathepsin B has been implicated in the caspase cascade in apoptosis (Guicciardi et al., 2000; Ishisaka et al., 1999; Kingham & Pocock 2001; Lotem & Sachs 1996; Vancompernolle et al., 1998), and apoptosis appears to be the predominant type of neuronal cell death in prion diseases (Fairbairn et al., 1994; Forloni et al., 1993; Giese et al., 1995; Gray et al., 1999). In view of the above, the possible role of up-regulation of cathepsin activity in the pathogenesis of prion disease merits further study.

Lysosomotropic agents and cysteine protease inhibitors are reported to block PrPSc accumulation in scrapie-infected...
neuroblastoma cells (Doh-ura et al., 2000). Viewing these results together with our present data, we hypothesize that lysosomal proteases might be actively involved in PrPc formation, which would represent a ‘second autocatalytic loop’ in prion replication, acting in concert with the first, i.e. the well-known autocatalytic enhancement of PrP conversion in the presence of PrPc (Fig. 3). A possible function for up-regulated cathepsin activities with respect to prion replication might be to disrupt large aggregates of PrPc, thus creating an expanded population of ‘converting units’, analogous to the sonication step in the ‘in vitro protein-misfolding cyclic amplification’ procedure (Saborio et al., 2001).

How the up-regulation of cathepsin B and L activities by prion infection is mediated is unknown. One intriguing possibility is the involvement of glycosaminoglycans. On the one hand, PrPc amyloid is known to contain sulfated glycosaminoglycans, which suggests that these may play an essential role in PrPc formation or stabilization (Snow et al., 1990). Depending on the circumstances, sulfated glycans can either be cofactors (Wong et al., 2001) or inhibitors (Caughey & Chesebro, 2001; Caughey et al., 1991) of PrPc formation. On the other hand, there is evidence that glycosaminoglycans are involved in the in vivo processing of lysosomal cysteine proteases (Rozman et al., 1999; Ishidoh & Kominnami, 1995) and in cathepsin B regulation (Almeida et al., 2001). Whether or not PrPc-associated glycosaminoglycans indeed play a role in the up-regulation of cathepsin activity in scrapie-infected cells shown in the present work requires further investigation.

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**Fig. 3.** Hypothetical model for the possible involvement of cathepsins in a ‘second autocatalytic loop’ (dashed and dash-dot arrows) involved in the formation of the scrapie-associated isoform of prion protein (PrPSc). The model is supported by the following data: (i) *in vitro* conversion studies as performed by many groups (continuous bent arrow representing the ‘first autocatalytic loop’ in prion replication); (ii) data by Doh-ura et al. (2000) (dash-dot arrow); (iii) the present paper (dashed arrow). GAG, glycosaminoglycans. Note that the possible involvement of GAG (dotted lines) in the proposed ‘second autocatalytic loop’ is speculative. For details see text.


