Measuring prions by bioluminescence imaging

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Prions are infectious proteins that cause fatal neurodegenerative diseases. Because astrocytic gliosis marked by the deposition of fibrils composed of GFAP is a prominent feature of prion disease, we asked whether GFAP might be used as a surrogate marker for prions. To interrogate this postulate, we inoculated prions into transgenic (Tg) mice expressing luciferase (luc) under the GFAP gene (Gfap) promoter, denoted Tg(Gfap-luc) mice. Weekly noninvasive, bioluminescence imaging (BLI) detected an increase in light emitted from the brains of Tg(Gfap-luc) mice at ∼55 d after inoculation and ∼62 d before neurologic deficits appeared. To determine whether BLI could be used as a proxy bioassay for prion infectivity, we performed endpoint titrations of prions in Tg(Gfap-luc) mice. BLI bioassays were as or more sensitive than those determined by the onset of neurologic dysfunction, and were completed in approximately half the time. While our studies were in progress, an article was published that reported increased BL in asymptomatic [Tg(Gfap-luc/SOD1,G93A)] mice, a model for amyotrophic lateral sclerosis (25); those findings as well as the data reported here demonstrate the potential applicability of BLI for other neurodegenerative disorders.

Results

Using an IVIS Spectrum imaging system, we quantified the BL emitted from the brains of Tg(Gfap-luc) mice (Fig. 1A) at various time points (dpi). BL was significantly elevated in prion-infected brains at 55 dpi (Fig. 1C) compared with controls at 56 dpi (P < 0.005, Bonferroni t test) (Fig. 1D) and to the same prion-infected mice at 48 dpi (P < 0.001, Bonferroni t test) (Fig. 1B). No difference in BL was found (P > 0.5, Bonferroni t test) between prion-infected mice at 48 dpi (Fig. 1B) and control mice at 56 dpi (Fig. 1D). Based on these results, we chose BL values ≥2 × 10⁶ photons/s as indicative of luc expression induced by prion replication. In prion-infected mice, the median time for an increase in Gfap-luc transgene expression was 55 ± 2.8 dpi (Fig. 1E). Signs of neurologic dysfunction in the same mice were apparent ∼62 d later, yielding a median incubation time of 117 ± 1.1 dpi (1E). Control mice neither showed increased Gfap-luc expression nor developed neurologic dysfunction throughout the course of the experiment.

We also inoculated groups of 12 Tg(Gfap-luc) mice with RML prions either i.p. or by oral gavage. Mice inoculated i.p. showed increased CNS BL after a median incubation time of 129 ± 12.4 dpi and died after a median incubation time of 167 ± 22.3 dpi. Mice inoculated by oral gavage exhibited elevated CNS BL after a median incubation time of 154 ± 9.4 dpi and died after a median incubation time of 216.5 ± 5.7 dpi (Fig. S1). For these routes of infection, increased BL preceded clinical signs by ∼40–60 d, despite longer incubation periods compared with i.c. inoculation.

To determine the kinetics of prion accumulation, reactive astrocytic gliosis, and BL in prion-inoculated Tg(Gfap-luc) mice, we measured PrPSc, GFAP mRNA, and BL between 21 and 61 dpi. PrP 27-30, the protease K-resistant fragment of PrPSc, was detected in the brains of infected mice beginning at 42 dpi and progressively increased thereafter (Fig. 2A). Immunohistochemistry for PrP also showed PrPSc deposits in brain tissue taken at 42 dpi (Fig. 3). GFAP mRNA levels as measured by qRT-PCR were elevated at 56 dpi, at which time the BL had also increased.

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Fig. 1. BL in Tg(Gfap-luc) mice i.c. inoculated with RML prions (n = 12) indicates reactive astrocytic gliosis. (A) BL measured from the brains of prion-inoculated mice (black circles) begins to increase at 55 dpi. BL in control Tg(Gfap-luc) mice inoculated with 1% normal brain homogenate (NBH) (n = 4, gray squares) remains low throughout the incubation period. (B–D) Photos of representative Tg(Gfap-luc) mice, with overlays of the circular area above the brain from which BL was quantified. The BL measured, ×10^6 photons/s, from each mouse brain is shown below each image. The BL measured from the brains of prion-infected mice significantly increased (**, P < 0.001, Bonferroni t test) from 48 dpi (B) to 55 dpi (C). Similarly, BL measured from infected mice at 55 dpi (C) was also significantly (*, P < 0.005) greater than in control mice inoculated with NBH and imaged at 56 dpi (D). No significant difference (N.S., P > 0.5) was measured between RML-inoculated mice at 48 dpi (B) and control mice at 56 dpi (D). Based on this result, we determined that astrocytic gliosis was detectable at BL values ≥2.0 × 10^6 photons/s. (E) Kaplan–Meier plots for incubation times based on the appearance of neurological signs (gray squares) or on the onset of reactive astrocytic gliosis (black circles). The median onset time for clinical signs was 117 ± 1.1 dpi (95% confidence interval), whereas that for gliosis was 55 ± 2.8 dpi.

Fig. 2. During prion infection, increases in PrPSc preceded increases in BL (A) and GFAP mRNA (B). (A) Protease-resistant PrPSc (circles, right ordinate) in the brains of RML-infected Tg(Gfap-luc) mice began to accumulate at 42 dpi and increased steadily. PrPSc levels were quantified at each time point by densitometry of Western blottings. BL (triangles, left ordinate) in the brains of infected Tg(Gfap-luc) mice increased beginning at 56 dpi, −14 d after PrPSc accumulation. BL measured from the brains of control mice inoculated with 1% NBH did not increase (squares). (B) Correlating with an increase of BL, GFAP mRNA also increased at −56 dpi. Error bars indicate the SE, based on at least 9 mice for BL measurements and 3 mice for all other measurements.

(Fig. 2B). Immunohistochemistry for GFAP showed reactive astrocytic gliosis in the brain beginning at 49 dpi (Fig. 3). Control Tg(Gfap-luc) mice did not show PrPSc in their brains as detected by immunohistochemistry (Fig. 3) or increased GFAP levels as monitored by qRT-PCR, immunohistochemistry, and BL.

Convinced that BLI could be used as a surrogate for prion infection, we asked whether the interval from inoculation to the point of infection in BL could be used to measure the prion titer in the inoculum. To investigate this possibility, we inoculated nine additional groups of 12 mice with serial 10-fold dilutions, ranging from 10^{-2} to 10^{-10}, of a 10% (wt/vol) Rocky Mountain Laboratory (RML) brain homogenate. All mice were imaged weekly until they developed clinical signs or the experiment was terminated (Fig. 4A; Fig. S2). We observed an inverse correlation between the inoculated dose and the interval from inoculation to the BL point of infection, corresponding to ≥2 × 10^6 photons/s. Using the method of Spearman–Kärber (26), we calculated a prion titer of 8.3 ± 0.3 log ID_{50} units/mL based on the appearance of neurological signs, and a titer 9.3 ± 0.3 log ID_{50} units/mL as determined by BLI. The difference in these calculated titers is due to fewer censored animals and a greater number of preclinical cases in the BLI dataset (Table S1). In some prion-infected mice that died without developing overt neurological deficits, reactive astrocytic gliosis was detected by BLI. In other mice inoculated with high dilutions, some were positive by BLI, but failed to develop clinical signs of neurological dysfunction throughout the course of the experiment. Based on the calculated titer, we plotted the dose of prions in the inoculum as a function of the median incubation times for appearance of clinical signs (gray curve in Fig. 4B) or for astrocytic gliosis based on BLI (black curve in Fig. 4B). Also, we found a linear correlation between the median incubation times.
as determined by the onset of neurological signs and by the point of inflection in BL (Fig. 4C).

Discussion

The studies reported here indicate that the point of inflection in BL can serve as a surrogate marker for prion replication. The inverse relationship between prion dose in the inoculum and the BL point of inflection was maintained over the entire range of dilutions investigated (Fig. 4B). The accumulation of PrPSc in prion-infected Tg(Gfap-luc) mice, which we detected as early as 42 dpi, preceded increases in GFAP mRNA and BL that were detected at 55 dpi (Figs. 2 and 3). Our findings are consistent with the temporal progression of prion infection reported previously (22).

It will be important to cross the Tg(Gfap-luc) mice with various mice, constructed by us and others, that express WT, chimeric, and mutant PrP transgenes. In a preliminary study, we found that bigenic (Gfap-luc:MoPrP-A) mice, which overexpress WT mouse PrP-A at ∼8-fold levels of FVB mice, exhibited a BL point of inflection at ∼45 dpi. Based on signs of neurological dysfunction, these Tg(Gfap-luc:MoPrP-A) mice had a median incubation time of 52 dpi. Whether higher levels of luc expression will be able to detect gliosis earlier in such mice remains to be determined.

Determining the earliest sites of Gfap expression in prion-infected mice will be important in attempting to optimize the bioassay of prions using BLI. In a preliminary study, the trauma of i.c. inoculation was found to incite transient astrocytic gliosis. It may be possible to reduce this gliotic response by using a smaller bore needle and possibly by administering some antiinflammatory drug at the time of or before inoculation. Intraperitoneal inoculation is unfortunately not a useful approach, because it greatly prolongs the incubation time as measured either by the onset of neurological dysfunction or by BLI (Fig. S1) (27, 28).

Defining the pathway from prion multiplication to Gfap up-regulation will be paramount to optimizing BLI. GFAP is an intermediate filament protein and, as such, is a member of a large multigene family of highly regulated, abundant cytoskeleton proteins. Astrocyte precursors in the CNS usually express vimentin as the major intermediate filament protein, but during maturation, vimentin expression is down-regulated and GFAP expression increases (29, 30). GFAP is generally recognized as an astrocyte maturation marker. In the mature CNS, Gfap expression is regulated dynamically by signals originating from astrocyte-neuronal interactions, as well as neuroendocrine and inflammatory modulators including steroids, cytokines, and growth factors (31, 32).

Changes in gene expression induced by prions were followed throughout the incubation time by measuring mRNA transcripts in murine brains (23). Analysis of a large dataset (≈20 million data points) revealed ∼500 differentially expressed genes (DEGs). Among the highest DEGs during scrapie infection was Gfap, as well as some microglial specific genes. Of particular interest, the expression levels for IFN-γ, leukemia inhibitory factor (LIF), IL-2, IL-6, TNF-α, basic fibroblast growth factor (bFGF), IL-1α, and IL-1β, all of which are known to up-regulate Gfap expression, did not change throughout the course of the incubation time (23). In RML-infected FVB mice, induction of GFAP was observed between 56–70 dpi, and was closely followed by up-regulation of ciliary neurotrophic factor (CNTF), colony stimulating factor (CSF)-1, and TGF-β1. An earlier study showed up-regulation of the cytokine TGF-β1 after infection, but not of IL-1β, TNF-α, IL-6, or IL-10 (33). In contrast to these foregoing results, several studies reported up-regulation of IL-1β, TNF-α, or IL-6 in the late stage of experimental prion disease (34–38).

BLI has been used to study a mutant SOD Tg model of amyotrophic lateral sclerosis (25). BL increased in the lumbar spinal cord and peripheral nerves of Tg(SOD1,G93A) mice at 4–5 weeks of age when the mice were still asymptomatic. The onset of hind-limb paralysis at 16 weeks of age was also associated with an additional increase in BL, but this increase was
confined to the lumbar region of the spinal cord. BLI has also been used in Tg(Gfap-luc) mice to study kainic acid lesions, autoimmune encephalomyelitis, ischemic injury, and pneumococcal meningitis (39–42).

Because Gfap expression is so responsive to perturbations in the CNS, BLI of mice expressing the Gfap-luc transgene is likely to find wide application in the study of mouse models exhibiting features of neurodegenerative diseases including Parkinson’s diseases (PD). Reactive astrocytic gliosis is a prominent attribute of PD (43). Because mouse models of PD lack well defined endpoints and rely on complex behavioral assessments of memory or motor function deficits, mice that express the Gfap-luc transgene might offer more readily measurable endpoints of such disease models. If consistent, readily detectable changes can be quantified by BLI, then such an approach may find wide application in studies of the pathogenesis of these and other neurodegenerative diseases.

BLI offers the ability to interrogate repeatedly cellular functions within the brain throughout the prion incubation time, during which much of the mice remain asymptomatic. Our results demonstrated that up-regulation of the Gfap gene occurred long before neurological dysfunction developed in Tg-(Gfap-luc) mice inoculated with prions. Interestingly, the expression of luc remained elevated up to the time of death. BLI of neurodegeneration-induced gliosis not only provides a surrogate marker that is detectable in asymptomatic mice propagating prions, but it also provides an objective measure of prion infection. Most important, BLI permits the quantitative measurement of prion infectivity, because the time interval from inoculation to the BLI point of infection is inversely proportional to the dose of prions inoculated i.c. Because BLI is more rapid, sensitive, and objective than measuring the onset of neurologic dysfunction, it may become the method of choice for studies in which prion infectivity is measured.

Methods

Source of Mice and Husbandry. Tg FVB/N-Tg(Gfap-luc)-Xen mice, or Tg(Gfap-luc) mice for simplicity, are hemizygous and were provided by Caliper Life Sciences and have been described previously (42). Tg(Gfap-luc) mice were maintained by breeding with FVB/N mice. The presence of the Gfap-luc transgene in offspring was determined by PCR. The 1-kb PCR product from the sequence internal to the luc gene was amplified using the sense and antisense primer pair 5'-TGATTTCATACCGGAGG-3' and 5'-CCAAAACAAACACCGGCCG-3', both at 0.4 μM in the reaction mix. PCR conditions were 97 °C for 3 min, followed by 35 cycles of 94.5 °C for 30 s, 58 °C for 90 s, and 72 °C for 90 s, followed by a final cycle of 72 °C for 10 min.

Prion Isolates and Transmission. CD1 mouse-adapted RML prions were used as inocula; the RML prion strain was originally derived from sheep with scrapie and has been serially passed in mice for many generations (44). Brain homogenates (10% wt/vol) in PBS (pH 7.4) were obtained by three 30 s strokes of a PowerGen homogenizer (Fisher Scientific). Serial log dilutions ranging from −1 to −10 for inoculation were obtained by further diluting brain homogenates in 5% (wt/vol) bovine albumin fraction V (ICN) and PBS. For i.c. inoculation, weaning mice were injected into the right parietal lobe with 30 μL of inoculum using a 27-gauge, disposable hypodermic syringe. For oral inoculation, weaning mice were inoculated by oral gavage using 200 μL of a 1% (wt/vol) brain homogenate. For i.p. inoculations, mice were injected with 200 μL of a 1% (wt/vol) brain homogenate. Inoculated animals were examined daily for clinical signs and were killed when they showed clinical signs of neurological dysfunction, and scored for prion disease based on standard diagnostic criteria (45, 46). All mouse studies were reviewed and approved by the University of California, San Francisco (UCSF) Institutional Animal Care and Use Committee.

BLI. Mice were imaged weekly using an IVIS Spectrum imaging system (Caliper). In brief, mice were injected i.p. with a solution of D-luciferin potassium salt (Caliper) in PBS, pH 7.4 (Invitrogen), receiving 150 mg D-luciferin per kilogram body weight. The mice were then anesthetized using an isoflurane-oxygen gas mix and imaged after 10 min for 30–60 s under anesthesia. BLI was quantified from images using Living Image 3.0 software (Caliper).

Western Blotting. For Western immunoblotting analysis, 10% (wt/vol) brain homogenates were prepared in PBS containing 2% (wt/vol) N-lauroylsarcosine sodium salt (Sigma-Aldrich) by three runs of 15 s in a Precellys 24 homogenizer (Bertin Technologies). Samples of 5% brain homogenates were incubated with 20 μg/mL of proteasome K (PK, New England Biolabs) for 1 h at 37 °C. PK digestion was stopped with 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich), and samples were centrifuged at 100,000 × g for 1 h at 4 °C. Pellets were resuspended in 90 μL of PBS containing 2% (wt/vol) N-lauroylsarcosine sodium salt before 30 μL of 4× NuPage LDS sample buffer (Invitrogen) was added and the samples were boiled for 5 min. For electrophoresis, 35 μL of undigested and PK-digested samples were loaded onto the gels. SDS gel electrophoresis and Western blotting were performed using NuPage Novex 4–12% Bis-Tris gels and the iBlot dry blotting system (Invitrogen). PrP was detected with the human-mouse (HuM) recombinant antibody fragment.

Fig. 4. The inoculated prion dose is related to BL in Tg(Gfap-luc) mice. (A) Median BL measured from the brains of Tg(Gfap-luc) mice inoculated with 10% RML brain homog¬enate at the following serial log dilutions: −1 (red), −2 (turquoise), −3 (blue), −4 (violet), −5 (brown), −6 (green), and −7 (pink). BL from Tg(Gfap-luc) mice inoculated with 1% NBH is shown as a control (black). (B) The number of days from the point of inoculation to the BLI threshold (black curve) as a function of the serial dilution factor (gray curve). BLI detects prion infection from −1 log dilution higher than the incubation-time assay. (C) For each dilution, median incubation times for clinical signs plotted against the median incubation times for astrocytic gliosis show a linear relationship. Error bars indicate the SE.
(recFab) P covalently bound to HRP and developed with the enhanced chemiluminescent detection system (Amersham Biosciences) (47).

Quantitative RT-PCR. Brains from euthanized mice were extracted and stored in RNAlater solution (Ambion) at 4 °C overnight and then transferred to –80 °C for long-term storage. In short, brains were taken up in 10 volumes of TRI reagent and homogenized using three turns of 1 s in a Precellys 24 homogenizer (MO Bio Laboratories). Total RNA was extracted using the RiboPrep kit (Ambion). Residual DNA was eliminated by DNase treatment using the Turbo DNA-free kit (Ambion). RNA yield was measured using a NanoDrop 1000A spectrophotometer (Thermo Scientific). RNA quality was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies) at the UCSF Helen Diller Family Comprehensive Cancer Center Genome Analysis Core facility. Complementary DNA was made using the SuperScript III Platinum Two-Step qRT-PCR kit with SYBR green (Invitrogen). Real-time PCRs were performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems) at the UCSF Genomics Core Facility using the following sense and antisense primer sets: 


5. Changes in gene expression levels were determined relative to 18S-rRNA, a reference that did not change significantly in our samples.

Histopathology. Tissue samples were either immediately frozen after removal or immersion-fixed in 10% buffered formalin and embedded in paraffin. To evaluate vacuolation, 8-µm-thick brain sections were stained with hematoxylin and eosin (H&E). Reactive astrocytic gliosis was evaluated by immunostaining for GFAP using polyclonal rabbit antiserum 20334 (Dako). Detection of PrPSc from formalin-fixed tissue sections was performed with the HuM-1 mouse PrP-specific monoclonal antibody after hydrolytic autoclaving, as previously described (48).

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Fig. S1. Transgenic (Tg)(Gfap-luc) mice were inoculated i.p. \((n = 12)\) or by oral gavage \((n = 12)\) with a 1\% RML brain homogenate. Mice inoculated i.p. developed reactive astrocytic gliosis after a median incubation time of \(129 \pm 12.4\) days postinoculation (dpi) (gray curve) and died after a median incubation time of \(167 \pm 22.3\) dpi, whereas mice inoculated by oral gavage developed reactive astrocytic gliosis after a median incubation time of \(-154 \pm 9.4\) dpi (black curve) and died after a median incubation time of \(-216.5 \pm 5.7\) dpi. Error bars indicate the SE.
Fig. S2. Ten groups of 12 Tg(Gfap-luc) mice were inoculated with serial log dilutions, ranging from $-1$ to $-10$, of a 10% RML prion brain homogenate. Dilution-dependent imaging periods after inoculation are shown. For higher dilutions, imaging periods started and ended later.
Table S1. Differences in scoring for clinical signs and reactive astrocytic gliosis in Tg(Gfap-luc) mice inoculated with prions

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Numbers in parentheses indicate the number of mice that scored positive for reactive astrocytic gliosis, but not clinical signs.