Titanium dioxide photocatalytic inactivation of prions

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INTRODUCTION

Prions are postulated to be the infectious agents of a family of transmissible, fatal, neurodegenerative disorders affecting both humans and animals. The possibility of prion transmission constitutes a public-health risk that confronts regulatory authorities everywhere. The main problem in handling prions is the fact that they are extremely resistant to standard decontamination methods. Thus, the use of harsh and expensive practices to destroy prions is inevitable. The development of applicable and efficient prion-inactivation practices is still highly important for the prevention of accidental transmission. In the search for effective and environmentally friendly methods to eliminate organic compounds and bacteria, much attention has been focused on the so-called advanced oxidation processes. These are based on the formation of hydroxyl radicals, which are known to possess a high reductive potential. This study tested the potential of titanium dioxide, an inexpensive and completely inert reagent, to inactivate prions in a heterogeneous photocatalytic process. Initial in vitro experiments were followed by a bioassay with the scrapie strain 263K in Syrian hamsters. The results obtained from this study indicate that titanium dioxide photocatalytic treatment of scrapie-infected brain homogenates reduces infectivity titres significantly.

INTRODUCTION

Prions are proteinaceous infectious particles postulated to be the causative agents of fatal neurodegenerative diseases known as transmissible spongiform encephalopathies (TSEs), including Creutzfeldt-Jakob disease (CJD) in humans, bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep and goats and chronic wasting disease in wild ruminants (Prusiner, 1998). They are composed mainly, if not solely, of an abnormal isoform, designated PrPSc, of the normal host-encoded cellular PrP protein (PrPC).

Prion transmission constitutes a public-health risk, especially for surgical patients, health-care workers and hospital laboratory personnel. In the acquired prion diseases, the infectious agent enters the body either through ingestion of contaminated food or during medical treatments with contaminated biological materials or surgical tools. It is noteworthy that all forms of the human TSEs are transmissible, including the sporadic and inherited ones (Dormont, 2002). Infectivity can also be harbouredin tissues of humans or animals that are either subclinically infected or in the preclinical stage of the disease. Such individuals, although carriers of the infectious agent (Hill & Collinge, 2003), display no symptoms.

Prions are unusually resistant to the conventional physical and chemical methods of decontamination commonly used to inactivate other infectious agents. Standard methods for the inactivation of TSE infectious agents used in everyday practice include treatment with bleach containing 20 g active chlorine l⁻¹, exposure to formic acid, and boiling with 1% SDS or 1 M sodium hydroxide (Taylor, 1999). Unfortunately, in many cases these methods have proven to be inefficient (Rutala & Weber, 2001).

Advanced oxidation processes (AOPs) comprise a group of alternative chemical treatments used for the decontamination of water and air polluted with organic compounds. The different AOPs [e.g. titanium dioxide (TiO₂)/UVA photocatalysis, Fe²⁺/H₂O₂ (the Fenton system), O₃/UVB] share the same chemical feature, which is the generation of free hydroxyl radicals, 'OH (Agustina et al., 2006). 'OH radicals
are strong oxidizing agents that attack organic compounds non-selectively, leading ultimately to their mineralization. TiO$_2$ is a non-toxic compound used widely as a pigment in food and cosmetics. It is inexpensive, non-corrosive and displays catalytic activity upon exposure to natural or artificial light (UVA). When illuminated, the TiO$_2$ particles act as semiconductors, generating OH radicals and other reactive oxygen species by electrochemical reactions conducted at the surface of the photocatalyst (Mills & Le Hunte, 1997; Agustina et al., 2006).

Here, we report results concerning the heterogeneous photocatalytic degradation of PrP and PrP$^{\text{Sc}}$, as well as the inactivation of an experimental scrapie infectious agent under artificial UVA illumination using TiO$_2$ as a catalyst. One of the potential objectives of the study was the development of a scaled-up method for the elimination of TSE pathogens before their environmental release from facilities that produce large amounts of possibly contaminated waste, such as hospitals, laboratories, abattoirs or even farms. The method outlined could also be adapted for decontamination of surgical instruments and smaller medical devices. The present work does not describe an inactivation protocol for any specific application, but should be seen as a first approach, demonstrating the effectiveness of TiO$_2$-based photocatalytic treatment in the field of prion inactivation.

**METHODS**

**Substrates for TiO$_2$ digestion treatments.** BSA was obtained commercially (A-3059; Sigma). Recombinant proteins MBP–rhuPrP (maltose-binding protein fused to human PrP aa 23–230), rhuPrP (human PrP aa 23–230) (Sachsamangolou et al., 2004) and bovine and ovine PrP were expressed in *Escherichia coli* cells. All of the recombinant proteins were used at a final concentration of 0.15 g l$^{-1}$ in the photocatalytic treatments. PrP$^{\text{Sc}}$-infected brain homogenates (CJD, BSE, sheep scrapie, hamster scrapie, mouse scrapie and mouse BSE) were also treated photocatalytically with TiO$_2$. Infectious material was used either as crude brain homogenate or after PrP$^{\text{Sc}}$ biochemical enrichment, as described below.

**Photocatalytic experiments.** TiO$_2$ P-25 (cat. no. 23.8595.0000.26; Degussa) was used for all photocatalytic experiments. In the P-25 formulation, the TiO$_2$ particles are non-porous; their anatase: rutile ratio is 3:6:1 and their mean surface area is 50 m$^2$ g$^{-1}$. Experiments were performed in disposable 1.5 ml plastic tubes. The reaction mixture in each tube was maintained in suspension by stirring. Irradiation was carried out by using two parallel 8 W black light/blue fluorescent tubes, mounted in standard 8 W fluorescent tube holders (TLD 8W/08; Philips). Light intensity in the region of 340–400 nm was measured by using a photometer/radiometer (PMA 2100; Solar Light). The initial light intensity used in the photocatalytic experiments was evaluated to be 6·1 mW cm$^{-2}$.

In all photocatalytic treatments except those with BSA and recombinant proteins, a protease inhibitor cocktail (P2714; Sigma) was included to ensure that no protein degradation by endogenous proteases occurred. The final volume of each reaction was 50 μl, containing TiO$_2$ diluted from an 8 g l$^{-1}$ stock solution in distilled water, the indicated concentrations of H$_2$O$_2$ from a freshly prepared 3% solution and the appropriate amounts of the organic load and distilled water. Samples were exposed to UVA light for 2 h (recombinant proteins, BSA) or 12 h (brain homogenates and enriched PrP$^{\text{Sc}}$ preparations).

BSA and the recombinant prion proteins were illuminated in the presence of TiO$_2$ (2 g l$^{-1}$) and H$_2$O$_2$ (0.5 g l$^{-1}$). After exposure to UVA light, aliquots of each sample containing the equivalent of 3 μg of each protein were electrophoresed through polyacrylamide gels and stained with Coomassie brilliant blue.

Brain homogenates (10%, w/v) were diluted 10-fold in 50 μl of a suspension containing 4 g TiO$_2$ 1$^{-1}$ and 4 g H$_2$O$_2$ 1$^{-1}$. Control homogenates without the reactants were run in parallel. After UVA exposure, the remaining proteins, if any, were precipitated with 10 vols methanol overnight at −80°C. Precipitated proteins from each 0.5 mg brain equivalent starting sample were separated on 12% polyacrylamide gel and stained with silver as described previously (Polymenidou et al., 2002).

Enriched PrP$^{\text{Sc}}$ preparations were treated similarly with 4 g TiO$_2$ 1$^{-1}$ and 4 g H$_2$O$_2$ 1$^{-1}$ for 12 h and proteins were precipitated with 10 vols methanol overnight at −80°C. Pellets were resuspended in 100 μl 2.5% O’Farrell sample buffer, containing 125 mM Tris/HCl (pH 6.8), 5% (w/v) SDS, 25% (v/v) glycerol and 7.5% (v/v) 2-mercaptoethanol (O’Farrell, 1975). Aliquots of 30 μl of the suspension, corresponding to 3 μg brain equivalent, were used for PrP$^{\text{Sc}}$ detection on Western blots.

**PrP$^{\text{Sc}}$ enrichment.** PrP$^{\text{Sc}}$ was isolated quantitatively as described previously (Polymenidou et al., 2002). Sheep, hamster and mouse scrapie samples, as well as BSE samples, were treated with proteinase K (P6556; Sigma) at a concentration of 30 μg ml$^{-1}$, whilst the sporadic CJD samples were treated with 50 μg ml$^{-1}$. Proteinase K digestion was performed for 1 h at 37°C in a theromixmer with shaking at 500 r.p.m. The PrP$^{\text{Sc}}$-enriched material was treated photocatalytically as described above and afterwards diluted to 100 μl with 5 × O’Farrell sample buffer. The equivalent of 3 μg brain was used for PrP$^{\text{Sc}}$ detection on immunoblots.

**Electrophoresis and immunoblotting.** SDS-PAGE and immunodetection of PrP$^{\text{Sc}}$ were performed as described previously (Polymenidou et al., 2002; Sachsamangolou et al., 2004). Blots were probed with the monoclonal anti-PrP antibody 6H4 (a generous gift from Prionics). Proteins were visualized using a CDP-Photostar chemiluminescence kit (New England Biolabs) following the manufacturer’s instructions.

**Bioassays.** Brain from hamsters in the final state of disease after inoculation with scrapie strain 263K (Kimberlin & Walker, 1977) and normal hamster brain were kindly provided by Dr Ruth Gabizon (Hadassah University, Jerusalem, Israel). Homogenates (10%, w/v) were prepared from both scrapie and control hamster brain in cold PBS containing 10 mM Tris/HCl (pH 7.4) and 300 mM sucrose using a Polytron apparatus (Kinematica). After centrifugation at 1000 g for 5 min at 4°C, the supernatant was aliquotted and stored at −80°C for future use.

For preparation of treated inocula, brain homogenate was diluted fourfold prior to photocatalysis. After the photocatalytic treatment, the mixture was diluted a further 2.5 times with sterile PBS containing 1% BSA. Aliquots of 50 μl of this material were used for each inoculation.

Forty male golden Syrian hamsters, 30–40 days old, were obtained from Charles River Laboratories. They were handled according to the regulations of the local ethics committee (reference number 13/985) in a Biosafety Level 3 containment facility. Fifteen animals were each injected intracerebrally with 50 μl treated material, which initially contained the infectivity of 1% (w/v) brain homogenate.
Ten matched control hamsters were also injected intracerebrally with 50 μl scrapie-infected hamster brain homogenate [1 % (w/v) homogenate prepared from a 10-fold dilution of a 10 % (w/v) homogenate with PBS plus 1 % (w/v) BSA]. Fifteen negative-control animals were injected with 1 % (w/v) normal hamster brain homogenate.

**Clinical evaluation.** Two observers, blind to the identity of the inoculated homogenate, performed the clinical evaluation. After day 50 post-inoculation, hamsters were examined daily for clinical symptoms characteristic of scrapie, as described previously (Prusiner et al., 1984). The onset date of the disease was considered to be the day when at least two typical symptoms were observed. Animals were sacrificed when they were terminally ill or as otherwise stated.

Statistical analysis was carried out with a χ2 test using PRISM 4.0 software (GraphPad).

**RESULTS AND DISCUSSION**

**In vitro experiments**

Initially, we tested the effect of TiO2 photocatalysis on purified homogeneous proteins. This was monitored by using Coomassie brilliant blue staining of photocatalysis products following SDS-PAGE. Treatment with TiO2/H2O2 resulted in the complete degradation of all of the proteins that were used as test substrates, i.e. BSA and recombinant PrPs from different species (Fig. 1a).

The necessity for illumination was seen in control experiments, where reaction mixtures containing the test substrates were treated in the dark in the presence of TiO2 and H2O2. Without illumination, BSA and purified prion proteins remained unaffected in the presence of both TiO2 and H2O2 (Fig. 1b, lanes 1–5).

The addition of a powerful oxidizing reagent such as H2O2 or potassium peroxydisulfate (K2S2O8) to TiO2 suspensions is a well-known procedure and in many cases leads to an increased rate of photooxidation (Poullos et al., 2003; Agustina et al., 2006), especially when the initial organic load is high. H2O2 acts as an electron scavenger, which reacts with electrons at the conduction band of the TiO2 molecule to generate additional OH radicals (Parra et al., 2001), thus potentiating the effect of TiO2. However, protein oxidation promoted solely by H2O2, at the concentrations used, could not account for the observed levels of protein oxidation. Reaction mixtures containing purified recombinant proteins and H2O2 were illuminated as above. As shown in Fig. 1(b, lanes 6–10) proteins remained unaffected by this treatment.

Non-specific protein oxidation mediated by TiO2 in the presence of UVA light was confirmed in experiments in which the complex protein content of infected brain homogenates from various species was present in the photocatalytic reaction. After treatment with TiO2/H2O2, complete degradation of the proteins was achieved (Fig. 2a), as assessed by silver staining. In control reactions, the proteins appeared not to be degraded after similar exposure to UVA in the absence of the photocatalyst and H2O2. These results suggested that photocatalytic treatment can be used not only for elimination of the prion protein, but for the entire protein load.

Quantitatively purified PrPSc preparations from clinical TSE cases and experimental animal TSE models were treated photocatalytically with TiO2 in the presence of H2O2. This treatment resulted in the apparent degradation of the pathological prion protein. After 12 h illumination, PrPSc was no longer detectable on Western blots (Fig. 2b) developed with high-sensitivity enhanced chemiluminescence reagent.

**Bioassay**

The effectiveness of the photocatalytic treatment was evaluated further by an in vivo study. TiO2-treated and untreated brain homogenates from hamsters terminally ill with scrapie strain 263K were analysed on a Western blot before the initiation of the bioassays. As expected, PrPSc
could only be detected in the untreated sample. In the TiO$_2$/H$_2$O$_2$-treated inoculum, no PrP$^{Sc}$ could be detected, even after extended film-exposure times (Fig. 3a).

Whilst improvements in the sensitivity of PrP Western blot analysis make it possible to detect quantities of the protein at the nanogram level, the technique is still not adequate for infectivity determination. Based on our 10-fold serial-dilution experiments (Fig. 3b), the PrP signal arising in the untreated inoculum (500 µg brain equivalent) disappeared completely after a 1000-fold dilution containing 5 µg brain tissue.

Inoculated hamsters were examined daily for symptoms of the disease after day 50 post-inoculation. The scrapie-inoculated animals entered the clinical disease phase 69.4 ± 4.85 days post-inoculation and the terminally ill animals were sacrificed. Twelve out of 15 hamsters inoculated with TiO$_2$/H$_2$O$_2$-treated material presented some clinical symptoms about 50 days later than the positive controls (mean incubation period, 119.6 ± 9.73 days). Interestingly, the clinical phase in those animals was significantly extended, with disease symptoms lasting for over 2 months compared with a 2 week disease course seen with the positive controls. Three hamsters injected with the TiO$_2$/H$_2$O$_2$-treated inoculum were sacrificed asymptotically at 380 days post-inoculation. Fig. 4 shows the survival curves of all three hamster groups tested. Variations were statistically significant, as assessed by a $\chi^2$ test ($P<0.0001$).

All scrapie control animals developed the disease in the shortest time frame that has been reported previously for this particular model. Therefore, it was not feasible to determine precise measurements of the infectivity titre. However, the effectiveness of TiO$_2$ treatment in more concentrated samples was prominent and in favour of our proposition.

Brain tissues from all animals were examined for the presence of proteinase K-resistant PrP on Western blots in order to confirm the presence of the disease. Traces of PrP$^{Sc}$ were detectable in the brains of the three asymptomatic animals, suggesting either a preclinical state of the disease or a subclinical infection due to the low infectivity titre of the inoculum (Hill & Collinge, 2003). Hamsters belonging to the negative-control group remained asymptomatic for the time span of the experiment.

**Conclusions**

TiO$_2$ photocatalytic treatment has been shown to be effective in vitro for the destruction of nucleic acid molecules (Ashikaga et al., 2000) as well as proteins. Experiments performed in our laboratory showed degradation of nucleic acids (data not shown) and purified recombinant PrP$_S$ and PrP$^{Sc}$. Although there is no direct evidence for nucleic acid involvement in the pathogenesis of TSEs, there are indications of interactions between prion protein and nucleic acids (Murdoch et al., 1990; Akowitz et al., 1994; Cordeiro et al., 2001; Adler et al., 2003). The fact that TiO$_2$, in the presence of UVA light, non-specifically oxidizes and destroys organic substances including proteins and nucleic acids suggests that it would cause the indistinguishable inactivation of any kind of organic component that might also be involved in TSE pathogenesis. Additionally, TiO$_2$ has been found to be effective against both bacteria (Bekbolet & Araz, 1996; Maness et al., 1999) and viruses (Sjogren & Sierka, 1994; Watts et al., 1995). This property can be seen as a very important secondary benefit in parallel with its use in the field of prion inactivation.
Taking into account our experimental results for the photocatalytic inactivation of the TSE pathogen, it is clear that the photocatalytic treatment of liquid waste should be evaluated further as a powerful tool for disinfection of the TSE infectious agent. The method described could also be adapted for decontamination of surgical instruments. The non-specific activity of TiO$_2$ does not exclude inactivation of unknown organic substances that may be involved in TSE pathogenesis. Of course, protocols for specific applications will have to be optimized and validated. However, the use of a low-cost and biologically inactive catalyst and the possibility of activating it with solar light, combined with the simple equipment required for this method, can offer economically reasonable, user- and environmentally friendly solutions to the processing of prion-contaminated liquid waste.

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