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Scratch-wounding renders cultivated cells less permissive to prion infection ☆

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Abstract

Using permissive cell lines of epithelial or neuroglial origin, we found that scratch-wounding a small proportion of the recipient cells prior to prion exposure strongly reduced the cell culture's susceptibility to infection. We provide evidence suggesting that wound-triggered inhibition of prion infection was mediated by the release of nucleotides in the extracellular medium of injured cultures. While cell wounding or ATP treatment of unwounded target cells inhibited de novo infection, we found that they had no effect on steady-state infected cultures, indicating that these treatments affect the early stages of infection. These findings support the view that cells have the capacity to modulate their permissiveness to prion infection in response to external stimuli, such as a signalling molecule.

Keywords: Prion; Extracellular ATP; Scrapie; PrP; Cell model; Cell wounding

Prion diseases, or transmissible spongiform encephalopathies, include scrapie in sheep and goats, bovine spongiform encephalopathy in cattle, and Creutzfeldt–Jakob disease in humans. This group of fatal neurodegenerative disorders is characterized by spongiform change, neuronal loss, and accumulation in the infected tissues of a detergent-insoluble and protease-resistant isoform of the cellular PrP protein [1,2]. The causative infectious agent, or prion, is thought to be an abnormal form of PrP, or a precursor of it [3]. Prions multiply by triggering the conversion of the normal cellular form of PrP into the abnormal conformer. While prion multiplication is absolutely dependent on the presence of the PrP protein [4,5], several convincing lines of evidence indicate that expression of PrP is not sufficient to confer permissiveness to any given cell type. Indeed, mouse tissues expressing high levels of PrP, either naturally or after genetic engineering, do not necessarily accumulate prion infectivity [6,7]. Strong cellular selectivity unrelated to PrP expression is also observed in cellular models of prion infection as very few PrP-expressing cell lines are able to replicate prions [8,9]. The current view is that prion multiplication requires a number of cellular and/or molecular factors, that may be restricted to a few cell types. However, these prion multiplication-controlling factors have yet to be identified.

In this study, we found that cellular mechanical injury affects the susceptibility of cultivated permissive cells to prion infection as scratch-wounding inhibits de novo infection of permissive target cells. We provide evidence that this inhibitory effect may be mediated by the release of nucleotides in the extracellular medium. These findings introduce the notion that an external biological

☆ *Abbreviations:* PrP, cellular prion protein; PrPres, abnormal prion protein resistant to proteinase K digestion; PK, proteinase K; PBS, phosphate-buffered saline; mAb, monoclonal antibody, dox, doxycycline; PIPLC, phosphatidylinositol-specific phospholipase C.

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stimulus, e.g., an injury or a signalling molecule, can modulate cell permissiveness to prion infection.

Materials and methods

Chemicals. ATP, UTP, and GTP were from Pharmacia, while ADP was from Roche. EGTA and apyrase with a low ATPase/ADPase ratio (grade VII) were from Sigma–Aldrich.

Cells. Rov cells [10] were maintained in α-minimal essential medium (α-MEM) containing 10% fetal calf serum, 100 U/ml penicillin, and 10 μg/ml streptomycin, and were split at a 1:4 dilution weekly. To induce the expression of the transfected ovine PrP protein, the cultures were treated with 1 μg/ml doxycycline (dox). MovS cells (the MovS6 clone) are mouse Schwann-like cells established from mice transgenic for the ovine PrP which constitutively express this protein [11]. These cells were cultured in a mixture of Dulbecco's modified Eagle's medium and F12 medium (3/4, 1/4, respectively) containing 10% foetal calf serum, 100 U/ml penicillin, and 10 μg/ml streptomycin, and were split at a 1:10 dilution weekly.

Experimental infection of wounded cell cultures by ovine prions. Confluent Rov or MovS cultures, grown in 12-well tissue culture plates, were wounded according to a geometrical pattern (30 horizontal and 30 vertical scratches) with a 19 G gauge needle. The protein content of wounded Rov cultures was assessed after immediate solubilization of wounded cultures in Triton/DOC lysis buffer. In some experiments, cultures were pretreated with 5 mM EGTA for 45 min in complete culture medium to disrupt cellular junctions. A few minutes after wounding, the cell culture medium was removed and replaced by fresh culture medium containing 0.25% of sonicated infectious brain homogenate (from tg338 transgenic mice terminally affected with sheep prions [12]). Two days later, the infectious medium was removed, the monolayers were rinsed once in phosphate-buffered saline (PBS), and the cells were left for 2 more days in regular culture medium before being passaged in one T-25 cm^2 flask. One week later, the cultures were rinsed with cold PBS, lysed at 4 °C in 1.5 ml Triton/DOC lysis buffer (50 mM Tris–HCl, pH 7.4; 0.5% Triton X-100, and 0.5% Na-Deoxycholate), clarified (2000 rpm, 1 min), and stored at −20 °C.

Detection of the normal and abnormal forms of PrP in cell lysates. To detect PrP or PrPres, proteins from cell lysates were methanol-precipitated or digested with proteinase K (PK), respectively, as described [10]. Aggregated PK-resistant PrP was collected by centrifugation at 13,000 rpm for 20 min at room temperature. Pellets from methanol precipitation or PK digestion were boiled in Laemmli buffer, subjected to SDS/PAGE electrophoresis, and transferred to nitrocellulose membranes. Blots were stained with anti-PrP ICSM18 mAbs [13] and revealed with an enhanced chemiluminescence detection system (ECL, Amersham Pharmacia). Signals were quantified with NIH image software.

Immunoprecipitation of cell surface PrP. Dox-induced Rov cells grown to confluence in 12-well tissue culture plates were scratch-wounded. Eighteen hours later, the cultures were placed on ice and anti-PrP monoclonal antibody 4F2 (ascites, 1/50) [14] was added for 1 h in regular culture medium. The monolayers were then extensively washed in PBS buffer, lysed with lysis buffer, and the cellular extracts were clarified (2000 rpm, 1 min). The antibody–PrP complexes were adsorbed with 30 μl protein A–Sepharose beads (Invitrogen), incubated on a wheel at 4 °C for 1 h, and washed three times with lysis buffer. The immunoprecipitated polypeptides were analysed by Western blot.

Nucleotides and apyrase treatments. Confluent Rov cells were wounded in the presence of apyrase (3 U/ml in complete culture medium) or were treated with either ATP or UTP (100 μM). Nucleotides- and wounded, apyrase-treated Rov cultures were then challenged with sheep prions by adding the infectious, sonicated, brain homogenate to the culture medium.

Wounding of persistently infected cultures. Chronically infected Rov cells were grown to confluence in 12-well tissue culture plates and were scratch-wounded as indicated before. Twenty-four hours later, each well was amplified in one T-25 cm^2 flask. One week later, the cultures were rinsed with cold PBS and lysed at 4 °C in 1.5 ml lysis buffer.

Dye transfer. Intercellular coupling was determined using the microinjection dye transfer method as described [15]. Rov cells were plated in 35-mm Petri dishes and microinjections were performed the day after seeding. Single cells were microinjected for 1 s with 5%(w/v) lucifer yellow CH (diluted in 0.33 M lithium chloride) using capillaries driven by a micromanipulator (IMT2-SYF, Tokyo, Japan) coupled to a pressure control unit (Eppendorf model 5242, Hamburg, Germany). All manipulations were performed under a microscope equipped with epifluorescence (Olympus, Rungis, France). The glass capillaries (Clark Electromedical Instruments) were prepared by an automatic horizontal puller (Narishige, Tokyo, Japan). Ten minutes after microinjection, cells were fixed with 4% formaldehyde in PBS and the numbers of dye-coupled cells were determined.

Results

Cell wounding inhibits prion transmission to exposed cells

Epithelial Rov cells expressing a stably transfected ovine PrP gene are one of the few cell systems permissive to sheep prion multiplication [10]. Upon exposure to infectious inoculum, Rov cells become infected and accumulate abnormal PrP at high levels. Effective infection of Rov cells can routinely be assessed by immunodetection of de novo produced abnormal PrP as early as one passage post-exposure. While studying the effect of various cellular stresses on cell permissiveness to prion infection, we noticed that wounding Rov cultures could result in significant impairment of prion transmission. To investigate further this intriguing observation, PrP-expressing Rov cells grown as confluent monolayers were scratch-wounded with a sterile needle according to a simple geometrical pattern. The total cell loss represented about 10% (see Materials and methods) so that a large majority of the cells remained physically unaffected by the mechanical injury. Shortly after wounding (i.e., within a few min), cultures were subjected to prion infection by exposure to fresh medium containing the infectious brain homogenate. The inoculum was removed, infected cells were passaged and grown for 1 week before measuring abnormal PrP content. PrPres levels were found to be consistently lower in wounded cultures, as shown by a representative experiment in Fig. 1A. Quantification showed that wounding resulted in a 3-fold inhibition of PrPres levels ($64 \pm 3\%$ inhibition, $n = 12$, see Fig. 4). As the levels of Rov-derived PrPres reflect the level of prion infection [16], these data indicated that wounding had a strong inhibitory effect on prion transmission to Rov cells. To determine whether or not this observation was a unique feature of rabbit epithelial Rov cells, we used mouse glial, Schwann-like MovS cells, another cell system permissive for sheep prions [11]. Wounding MovS cultures prior to exposure to

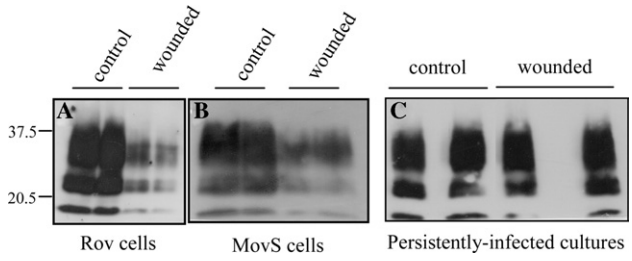

Fig. 1. Wounding permissive cells inhibits transmission of prion infection but not prion multiplication. Duplicate confluent monolayers of epithelial Rov cells (A) or neuroglial MovS cells (B) were wounded or left undisturbed. Fresh culture medium containing sheep prions (0.25% of brain homogenate) was then added to wounded and control cultures for 2 days. The infectious inoculum was removed, exposed cells were split and grown for 1 week. Cell cultures were solubilized with detergent, proteins from cell lysates were digested with PK and aggregated, PK-resistant PrP (PrPres) was spun down and analysed by immunoblotting. PrPres from the inoculum was not detectable under these experimental conditions (data not shown) and therefore, as also shown previously [10], the signals observed one week post-exposition correspond to abnormal PrP generated in the inoculated cultures. The position of molecular mass marker proteins is indicated (in kDa). (C) Confluent monolayers of infected Rov cultures, multiplying steady-state levels of prions, were either wounded or left undisturbed. One day later, the cultures were split, grown for one week before being analysed for PrPres content by immunoblotting.

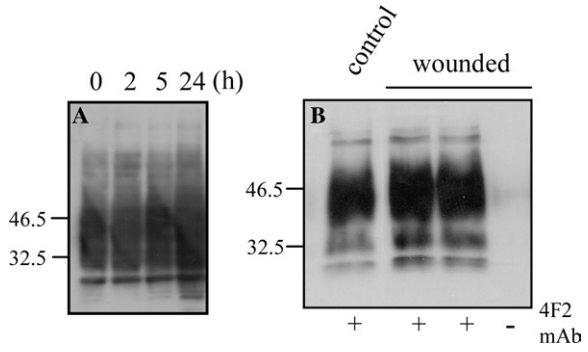

Fig. 2. Wounding Rov cultures does not affect cellular PrP expression. (A) Monolayers of confluent Rov cells were lysed before or 2, 5 or 24 h after wounding. The equivalent of 50 μg of proteins was methanol-precipitated and levels of cellular PrP were determined by immunoblotting. (B) Immunoprecipitation of cell surface PrP. Control or wounded Rov cultures were incubated at 4 °C with (+) or without (−) 4F2 anti-PrP mAb. Antibody-bound PrP was then immunoprecipitated and analysed by Western blot with ICSM18 mAb. The position of molecular mass marker proteins is indicated (in kDa).

sheep prions also resulted in reduced levels of PrPres (Fig. 1B). We then examined if inhibition by wounding would also occur when infection was already established in permissive cells. Persistently infected Rov cultures, replicating steady-state levels of prions, were wounded and levels of abnormal PrP were determined one week later. PrPres levels were similar in control or wounded infected cultures (Fig. 1C), indicating that wounding does not inhibit the process of prion multiplication once initiated.

To determine if wounding had any effect on the level of PrP expression, confluent PrP-expressing Rov cultures were lysed and analysed for PrP expression levels, up to 24 h after wounding. Immunoblotting analysis showed that PrP levels in the wounded Rov culture remained unchanged (Fig. 2A). We also verified whether wounding would alter the presence of PrP at the cell surface. Anti-PrP monoclonal antibodies were incubated with wounded or control Rov cultures, in order to immunoprecipitate plasma membrane-bound PrP. Fig. 2B shows that wounding did not appreciably affect the presence of PrP at the cell surface.

ATP treatment inhibits prion infection

The magnitude of the inhibition of prion transmission was far too large to be merely the consequence of cell removal resulting from wounding. This suggested that injured cells produce an inhibitory signal that could then act on the cells of the culture. Small molecules can be transferred between adjacent cells through gap junctions, hydrophilic pores across the plasma membrane [17]. To test for the presence of functional gap junctions, individual Rov cells from a confluent culture were microinjected with yellow Lucifer fluorescent dye. In sharp contrast to what was observed with primary cultures of epithelial cells from rat liver (Fig. 3A), microscopic examination revealed little, if any, dye transfer between injected and adjacent Rov cells (Fig. 3B). To further address this point, cellular junctions between Rov cells were disrupted by transiently treating confluent cultures with EGTA (Fig. 3C). We first checked that transmission of prions to a physically individualized cell population was as efficient as transmission to an equivalent number of confluent cells (data not shown). Then, EGTA-treated Rov cultures were wounded and infected. Wounding EGTA-treated Rov cultures resulted in $58 \pm 4\%$ ($n = 4$) inhibition of prion transmission (Fig. 3D), indicating that EGTA treatment had no effect on wound-mediated inhibition of prion infection. Thus, cell contacts between Rov cells were not required for the putative wound-induced, inhibitory signals to diffuse within the culture.

We then asked if such inhibitory activity was present in the growth medium of wounded cultures as the result of a release by the cells responding to the mechanical injury. Media from Rov cultures were collected 5 min, 15 min, 2 h, 8 h or 24 h after wounding and tested for a possible inhibitory effect on prion transmission. After addition of infectious brain homogenate, these conditioned media were used to infect recipient Rov cultures. Similar levels of Rov-derived abnormal PrP were found in the exposed cultures (data not shown) showing that no inhibitory activity was detectable in the tested conditioned media. A possible explanation for this negative result could be that the postulated released inhibitory signal might act in a paracrine fashion and/or be

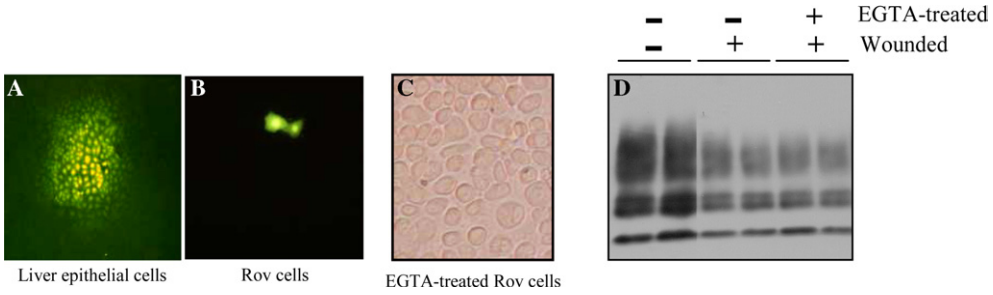

Fig. 3. Rov cells do not express functional gap junctions and wound-mediated inhibition of prion infection does not require cellular contacts. Individual liver epithelial (A) or Rov (B) cells were microinjected with Lucifer yellow. Epifluorescence images show the lack of dye intercellular transfer between neighbouring Rov cells. Magnifications, 100× (A) and 400× (B). Duplicate confluent cultures of Rov cells were treated for 45 min with 5 mM EGTA to disrupt cellular junctions. The resulting individualized cell populations, shown by phase contrast microscopy (C, magnification, 400×), and control confluent Rov cultures were then wounded and exposed to prions. The cultures were then subcultivated and cell-derived PrPres levels of a representative experiment were determined 1 week later (D).

mediated by a short-lived molecule. This prompted us to examine the possible involvement of triphosphate nucleotides. Nucleotides are important extracellular signalling molecules whose release has been demonstrated within milliseconds after mechanical stimulation of various cell types, including epithelial cell lines [18–21]. To test if such molecules could mediate the observed inhibition of prion transmission, Rov cells were infected in the presence of various concentrations (1–100 μM) of ATP. Quantification of PrPres accumulation showed that 100 μM ATP (Figs. 4A and B) or 100 μM UTP (data not shown) treatments reproducibly inhibits prion infection of Rov cells, pointing to nucleotide triphosphate as possible candidates for the wound-dependent inhibition. In contrast, infection in the presence of ADP or GTP did not inhibit prion infection (Fig. 4A). Next, before being exposed to prions, Rov cells were wounded in the presence or in the absence of apyrase, an enzyme that hydrolyses 5′ nucleotide triphosphates. PrPres levels in the exposed cultures show that apyrase largely restored efficient infection of wounded Rov cultures (Fig. 4B). These data are consistent with the involvement of extracellular nucleotides in the wound-mediated inhibition of prion transmission. Treatment of persistently infected Rov cultures for up to 1 week with 100 μM ATP had no effect on the accumulation of abnormal PrP (data not shown).

Discussion

While addressing the effect of various cellular stresses on cellular susceptibility to prion infection, we found that scratch-wounding affects transmission of prion infection to permissive cells. This intriguing observation was investigated further by showing that transmission of prion infection to two different permissive recipient cell lines was impaired by prior wounding. The magnitude of the observed inhibitory effect suggested that cells responding to mechanical injury produced an inhibitory molecule able to diffuse and to act on surrounding cells. The absence of functional gap junctions between the epithelial Rov cells along with the fact that cell–cell contacts were not required for wound-mediated inhibition of prion transmission did not support the possibility that the signalling molecule was transferred to the other cells in the culture through cellular junctions, but rather that a putative inhibitory signal was secreted in the

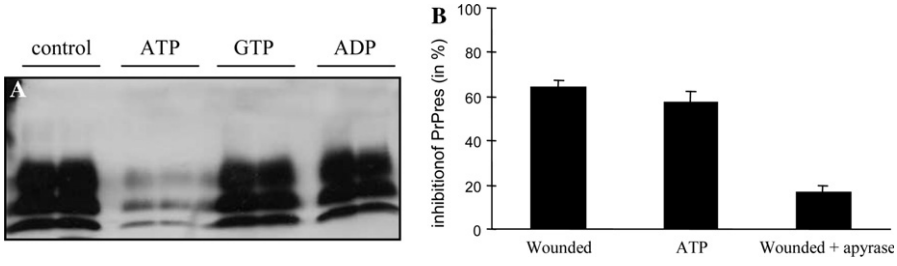

Fig. 4. Nucleotides are involved in wound-mediated inhibition of prion transmission. (A) Duplicate confluent Rov cultures were incubated for 5 min in the presence or in the absence (control) of 100 μM of the indicated nucleotides before adding the infectious brain homogenate. Processing of the infected cultures and PrPres detection were as in Fig. 1. (B) Data are expressed as means ± SEM of inhibition of PrPres levels compared to levels in untreated Rov cultures (12, 7, and 4 determinations for wounded, ATP, wounded + apyrase, respectively). Confluent Rov cultures were exposed to infectious inoculum after one of the following treatments: incubation with 100 μM ATP, wounding in the presence or in the absence of apyrase (3 U/ml), an enzyme that hydrolyses nucleotides. Processing of the infected cultures and PrPres detection were as in Fig. 1. Treatment with apyrase did not affect the infection of unwounded Rov cells (not shown).

extracellular medium. Nucleotides are released by a variety of cells, including epithelial, after cellular damage or in response to mechanical stimulation [18–21] and several lines of evidence indeed suggest that release of such molecules could mediate inhibition of prion infection in response to scratch-wounding: (i) as observed for wounding, ATP treatment did not interfere with prion multiplication in already infected cultures but strongly inhibited the transmission of prion infection to uninfected permissive cells, (ii) consistent with an effect triggered by the release of nucleotides, resistance to prion infection was induced rapidly following wounding and appeared to be a long-lasting effect, (iii) the efficiency of prion transmission was restored when permissive cells were wounded in the presence of apyrase, an enzyme that cleaves 5′ purine and pyrimidine nucleotides into monophosphate nucleotides.

The origin of nucleotides released by Rov cultures in response to scratch-wounding is presently unclear. As the intracellular concentration of ATP ranges from 3 to 5 mM [22], cellular damage of cells removed by the injury could result in the release of large amounts of ATP into the extracellular environment. Alternatively, surviving cells along the wound site could respond to the mechanical stimulation by releasing nucleotides, high local concentrations of which could lower the susceptibility of neighbouring cells to prion infection. An effect restricted to neighbouring cells due to high local concentration of extracellular nucleotides could explain why, in our experiments, media conditioned by wounded cultures failed to inhibit prion transmission. In this study, transmission of prion infection was performed shortly after wounding but preliminary experiments indicate that resistance to infection triggered by cell wounding is a rather stable phenotype as it can still be evidenced 24 h after wounding.

ATP release is far from being restricted to response to mechanical stimulation. It occurs in a wide range of tissues and promote numerous diverse cellular effects [23]. Extracellular nucleotides act on responsive cells through a large family of purinergic receptors. P2X are ion-gated channels which lead to calcium influx while P2Y receptors are linked to G-coupled proteins responsible for intracellular calcium mobilization [24]. Although the presence of purinergic receptors on Rov cells was not examined in the present study, these receptors have been detected in astrocytes and neurons [25], and in many other cell types including a wide spectrum of epithelial cells [24]. However, the mechanisms by which nucleotides and wounding inhibit prion infection have still to be established. The presence of PrP at the cell surface is critical for successful de novo infection as incubation of permissive cells with anti-PrP mAb or with PIPLC has been shown to prevent prion transmission [26]. However, we did not find any evidence that wound-mediated inhibition of prion infection could be due to alteration in expression and trafficking of cellular PrP. Cell cultures neither responded to scratch-wounding by down-regulating PrP expression nor by preventing its normal trafficking to the cell surface. We did not observe any inhibitory effect of scratch-wounding or of nucleotide treatment when prions were already multiplying in infected cultures. This indicated that these treatments did not interfere with prion multiplication per se but rather inhibited the process of prion transmission to recipient permissive cells. Thus, nucleotide treatment or wounding may provide new tools to investigate the early phases of prion transmission to cultivated permissive cells. More generally, our findings show that an external biological stimulus can modulate cell permissiveness to prion infection.

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