Induced Neuroprotection Independently From PrP^{Sc} Accumulation in a Mouse Model for Prion Disease Treated With Simvastatin

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Background: The misfolding and aggregation of specific proteins has emerged as a key feature of several neurodegenerative diseases. In prion diseases, progressive disease and neuronal loss are associated with the accumulation of PrP^{Sc}, the misfolded isoform of PrP^{C}. Previous in vitro studies suggest that cholesterol-lowering drugs inhibit the conversion of PrP^{C} to PrP^{Sc} and the accumulation of the latter, possibly through the disturbance of cholesterol-rich membrane domains (lipid rafts).

Objective: To examine the effect of simvastatin, a cholesterol-lowering drug, on prion disease progression and survival.

Design: Controlled animal study.

Setting: University medical center research laboratory.

Subjects: Female mice from the FVB/N strain.

Interventions: Peripheral and central nervous system inoculations with scrapie Rocky Mountain Laboratory inoculum.

Main Outcome Measures: Clinical, immunological, pathological, and molecular assays were performed.

Results: Simvastatin delayed disease progression, leading to increased survival in peripheral as well as central nervous system inoculations. Simvastatin's beneficial effect is mediated through the L-mevalonate pathway; however, it is independent of brain cholesterol levels. Interestingly, simvastatin treatment induced PrP^{Sc} accumulation in parallel with an induced neuroprotective effect. In accordance, we found that simvastatin induced immunomodulatory mechanisms in the brains of infected mice, affecting expression levels of specific microglial chemokines and cytokines.

Conclusions: Simvastatin delays prion disease progression and increases survival in vivo, independently of the pathogenic conversion of PrP^{C} to PrP^{Sc}. We show that simvastatin's effects on neuroprotection are correlated with downregulation of Cox2 levels and induction of microglial activation in prion-infected mouse brains.

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P RION DISEASES ARE A GROUP OF fatal neurodegenerative disorders characterized by the accumulation of PrP^{Sc}. PrP^{Sc} is a posttranslational refolded protein generated from the normal cellular prion protein PrP^{C}. PrP^{C} is attached to membranes through its glycosylphosphatidylinositol (GPI) anchor, which localizes PrP^{C} to specific membrane microdomains enriched with cholesterol and sphingomyelin, known as lipid rafts.[1-6]

It has been suggested that the interaction of PrP^{C} with membranes may play a role in the pathogenic conversion of PrP^{C} to PrP^{Sc}. Several lines of evidence implicate lipid rafts in the conversion of PrP^{C} to PrP^{Sc}.[5] Specifically, PrP^{C} and PrP^{Sc} colocalize to rafts,[2,7] and cholesterol depletion from rafts decreases PrP^{Sc} formation in prion-infected cell lines.[8-10] The localization into lipid rafts is important for PrP^{C} conversion. It was shown that replacing the GPI anchor with a transmembrane domain inhibited both PrP^{C} localization to rafts and conversion to PrP^{Sc} in cells infected with prions.[8,11,12] These in vitro results suggested that cholesterol in rafts might play a role in disease pathogenesis.

In prion diseases, the interval between inoculation and appearance of a clinical disease is termed the *incubation period*, during which neurological and biochemical changes as well as accumulation of PrP^{Sc} in the brain can be detected. This stage can be designated a preclinical stage, which is characterized by the accumulation of the neurotoxic agent(s) to subpathological levels. Accordingly, a preclinical stage occurs in other neurodegenerative diseases such as

Parkinson disease and Alzheimer disease. In Parkinson disease, for example, it has been shown that symptoms appear only at a late stage, when about 70% of the dopaminergic neurons are degenerated.[13] Therefore, delayed disease progression may serve as a potential therapeutic approach in neurodegeneration.

The 3-hydroxy-3-methylglutaryl–coenzyme A reductase inhibitors (statins) inhibit the synthesis of 3-hydroxy-3-methylglutaryl–coenzyme A reductase and its conversion into L-mevalonate, thereby inhibiting the synthesis of all mevalonate pathway products that are mediators in cholesterol biosynthesis. Recent data suggested that statins have pleiotropic effects including neuroprotection in neurodegenerative diseases such as Alzheimer disease, multiple sclerosis, stroke,[14] and prion diseases.[15,16] Although statins have shown neuroprotective benefits in neurological injury and diseases,[17,18] there is only limited information to date regarding the effect of statins on the progression of neurodegenerative diseases or their incubation time.

Here we used prion disease as a model for progressive neurodegeneration and tested the effect of simvastatin on prion disease progression, PrP^{Sc} levels, and neurotoxic effects in mice infected with the scrapie Rocky Mountain Laboratory (RML) strain. We show that simvastatin significantly delayed time of death in prion-infected mice (either intracerebrally or intraperitoneally) by virtue of its effect on prolonged incubation time. Surprisingly, the delayed onset is associated with higher levels of the pathogenic PrP^{Sc} form. No effect on brain cholesterol content was detected, but we show that simvastatin acted exclusively through the L-mevalonate pathway. Examinations of the treated and untreated scrapie-infected brains revealed that the beneficial effects of simvastatin involved reduced neuronal loss in specific brain regions. We show that simvastatin acted to modulate inflammatory mechanisms and downregulate Cox2 levels.

METHODS

ANIMALS

Animals were housed at a specific-pathogen-free, temperature-controlled room (22°C ± 1°C) with a cycle of light for 12 hours and dark for 12 hours (lights on at 06:00 hours) and free access to food and water. All of the animal procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the institute research committee.

PREPARATION OF THE RML STANDARD INOCULUM

Mouse-adapted scrapie prion inoculum was prepared from brains of scrapie-sick FVB/N mice (mean [SD] incubation time, 140 [15] days) as described previously.[19] Brains were homogenized (1:10 wt/vol) by 10 strokes of a dounce homogenizer in buffer containing 10mM TRIS hydrochloride at pH 7.4; 300mM sucrose; 5mM EDTA; 1mM magnesium chloride; and protease inhibitor cocktail (Sigma, Rehovot, Israel). The homogenate was spun at 1000g for 5 minutes at 4°C, and the supernatant was divided in aliquots and kept at –70°C. This 10% brain homogenate was then diluted by 10-folds in bovine serum albumin, 1%, in phosphate-buffered saline. The FVB/N female mice (Harlan, Rehovot) were inoculated intracerebrally with the scrapie RML inoculum at 4 weeks of age (30 μL of the inoculum was injected at the left hemisphere) or intraperitoneally at 7 weeks of age (with 200 μL of the inoculum). Different RML inoculum preparations were used for each experiment; therefore, some variations in incubation times are expected as a result of the disease severity during brain extraction and variability in preparations.

SIMVASTATIN ADMINISTRATION

A reported quality of simvastatin is that it efficiently penetrates the central nervous system.[20] For a dosage of 20 mg/kg/d, a 10-mg capsule of simvastatin (Teva, Petah-Tikva, Israel) was ground and solubilized in 75 mL of acidic water at pH 5.5 (the pH of the drinking water in the specific-pathogen-free animal facility). We calculated the final drug concentration in the drinking water according to the average amount of water consumed by each mouse (approximately 5 mL/d) and the average body weight of the mature, healthy animals (approximately 33 g). Accordingly, one 10-mg capsule was solubilized in 150 mL and 750 mL to obtain concentrations of 10 and 2 mg/kg/d, respectively. A fresh simvastatin solution was added to the drinking water daily.

EXPERIMENTAL DESIGN

For intracerebrally inoculated mice, each independent experiment (total of 4) included experimental groups consisting of scrapie-infected mice and healthy controls with and without simvastatin treatment (n=15-20) (experiment 4 also included prion-infected mice and healthy controls treated with 20 mg/kg/d of L-mevalonate in parallel with the simvastatin treatment [n=4-6]). Mice were euthanized at representative times, ie, appearance of symptoms (experiment 1, 115 days post infection [dpi]; experiment 2, 105 dpi; experiment 3, 104 dpi; and experiment 4, 118 dpi [n=4-5])[21] or progressive disease (experiment 1, 144 dpi; experiment 2, 130 dpi; experiment 3, 136 dpi; and experiment 4, 148 dpi [n=4-5]) or when the mice were terminally ill (ie, when they were unable to reach food and water, referred to as time of death) (n=5-7). For the L-mevalonate–treated mice (experiment 4), we only determined time of death. For intraperitoneally inoculated mice (experiment 5), healthy controls and prion-infected mice were treated with either a constant low simvastatin dosage (2 mg/kg/d) or a constant high dosage (20 mg/kg/d) immediately after inoculation (n=15-17). Mice were euthanized for spleen analyses at 30 and 45 dpi. The rest of the mice were kept alive to determine disease progression and time of death. In experiments 1 through 4, mice that died during the course of the experiment due to reasons unrelated to the disease were omitted from the final analyses. Mice were observed daily for typical clinical signs of scrapie, including altered gait, kyphosis, ataxia, disorientation, somnolence, and wasting.[22] We designated the time of scrapie symptom appearance as when at least 2 of the indicated symptoms were observed in an individual mouse for at least 4 consecutive days. In experiments 1, 3, and 4, mice were appointed to simvastatin treatment at 72 dpi. We used a protocol of increasing dosages starting at 2 mg/kg/d for 2 weeks followed by 20 mg/kg/d and continuing until termination. In experiment 2, mice were appointed to simvastatin treatment at 41 dpi. One group was treated at a low constant dosage of 2 mg/kg/d, and an additional group was treated with increasing dosages of 2, 10, and 20 mg/kg/d in 3-week intervals and then received 20 mg/kg/d until termination. Mouse brains were divided into hemispheres. One hemisphere was processed for biochemical analyses[23] and the second was frozen immediately for cryosections.[21]

IMMUNOHISTOCHEMICAL ANALYSIS AND HEMATOXYLIN-EOSIN STAINING

Horizontal sections (8 µm thick) of frozen brain were taken between 1.5 and 2.5 mm deep (dorsal) and fixed. For bromodeoxyuridine (BrdU) staining, the sections were fixed in acetic acid, 5%, and ethanol, 95%, for 10 minutes at –20°C, followed by hydrogen peroxide, 0.3%, in methanol for 15 minutes at room temperature (mouse anti-BrdU, 1:20; Dako, Glostrup, Denmark; and neuronal nuclei antibody, 1:50; Chemicon International, Inc, Temecula, California). For ionized calcium binding adaptor molecule (IBA-1), glial fibrillary acidic protein (GFAP), and major histocompatibility complex (MHC) class II, the sections were fixed in formaldehyde, 4% (IBA-1, 1:200; Wako Chemicals, Osaka, Japan; GFAP, 1:200; Dako; and MHC class II clone IBL 5/22, 1:50; Chemicon International, Inc). For intercellular adhesion molecule 1 (ICAM-1), sections were fixed in fresh cold acetone (1:200; Dako) and in paraformaldehyde, 4% (calbindin, 1:200; Biotest Ltd, Kfar Saba, Israel). Following incubation with the primary antibody (according to the manufacturers' recommendations), sections were incubated with a goat-antimouse IgG secondary antibody conjugated to Alexa Fluor 488 (dilution, 1:100; Molecular Probes, Inc, Eugene, Oregon) for 50 minutes at room temperature or with antirabbit cy5 (dilution, 1:100; Molecular Probes, Inc). Counterstaining was performed with 4′,6-diamidino-2-phenylindole (DAPI). For the expression of MHC class II and calbindin, to measure the thickness of the CA1 neuronal layer, and to determine BrdU-immunoreactive neuronal cells, images of a total of 16 microscopic fields (4 fields for each mouse brain, at magnification ×100) containing cerebellum or hippocampus were obtained under identical conditions from each group and stored using a video camera. The positively stained cells (BrdU, MHC II) or Purkinje cells (calbindin) and the positively stained blood vessels (ICAM-1) were counted and the thickness of the CA1 layer was measured. For hematoxylin-eosin staining, sections were incubated in hematoxylin for 20 minutes at room temperature and then in eosin, 2%, for 2 minutes at room temperature (Sigma).

BrdU INJECTIONS

Mice were intraperitoneally injected with 50 µg of BrdU per gram of body weight (RPN201; Amersham Biosciences, Buckinghamshire, England) at 12-hour intervals (3 injections starting at 104 dpi [experiment 4] or 5 injections starting at 118 dpi [experiment 5]). Mice were euthanized 12 hours after the last BrdU injection. Brains were removed and processed for immunohistochemical analysis.

FLOTATION ASSAY (RAFT ISOLATION)

Flotation of detergent-insoluble complexes was performed as described previously[24] with some modifications.[25] In brief, mouse brains were homogenized 1:10 wt/vol in an ice-cold buffer containing 150mM sodium chloride, 25mM TRIS hydrochloride (pH 7.5), 5mM EDTA, and Triton X-100 (Sigma), 1%. Insoluble particles were spun down and the lysate was loaded at the bottom of ultracentrifuge tubes (TLS-55; Beckman Instruments, Inc, Fullerton, California). An equal volume of ice-cold Nycodenz (Biological Industries, Beit Haemek, Israel), 70%, in TNE (25mM TRIS hydrochloride [pH 7.5], 150mM sodium chloride, and 5mM EDTA) was added and mixed with the lysate. An 8% to 35% Nycodenz linear step gradient in TNE was then overlaid above the lysate (200 mL each of 35%, 25%, 22.5%, 20%, 18%, 15%, 12%, and 8% Nycodenz). The tubes were spun at 55 000 rpm for 4 hours at 4°C in a TLS-55 rotor (200 000g). Fractions were collected from the top to the bottom of the tube. Each fraction was applied to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotted with anti-PrP monoclonal antibody 6H4 (Prionics AG, Schlieren, Switzerland).

WESTERN BLOTTING

For the detection of PrP^{Sc} protein levels, 15-µg samples of protein measured with the bicinchoninic acid kit (Pierce Biotechnology, Rockford, Illinois) were treated with proteinase K (Sigma). Digestion was performed with 0.04-mg/mL proteinase K for 30 minutes at 37°C. PrP^{Sc} was detected with anti-PrP monoclonal antibody 6H4 (Prionics AG). For the detection of PrP^{Sc} in spleen, spleen homogenates (100 µg of protein) were treated with 0.1-mg/mL proteinase K for 1 hour at 37°C. Insoluble material was collected after ultracentrifugation at 45 000 rpm for 1 hour at 4°C and analyzed. PrP^{Sc} was detected with anti-PrP monoclonal antibody IPC1 (Sigma).

CHOLESTEROL MEASUREMENTS

Brain homogenates were prepared as described in the "Preparation of the RML Standard Inoculum" section. Five micrograms of cholesteryl acetate (internal standard) was added to samples containing 0.05 mg of protein of total brain homogenate. Samples were then extracted in 3 volumes of chloroform and methanol (2:1) for 1 hour in cold. Phases were separated by centrifugation at 2000g for 5 minutes. The lower phase was collected with careful omission of the upper phase. After evaporation of the chloroform under argon, the sample was resuspended in 0.15 mL of isopropanol. From the sample, 0.02 mL was analyzed by high-performance liquid chromatography using a combined set of 2 columns (attached in line) (Chromolith 100/4.6 mm; RP 18e; Merck and Co, Inc, Whitehouse Station, New Jersey). Samples were separated with isopropanol and acetonitrile (1:1) at 1 mL/min with a controlled temperature set at 12°C.

STATISTICAL ANALYSES

To compare survival between prion-infected groups with and without simvastatin treatment, we used the Breslow test (SPSS version 14 statistical software; SPSS Inc, Chicago, Illinois). The different experiments and protocols did not vary in time of death; therefore, data were combined according to the experimental groups. Survival curves were determined according to Kaplan-Meier curves.

LOW-DENSITY-ARRAY REAL-TIME TAQMAN POLYMERASE CHAIN REACTION ANALYSIS

Tissue samples were snap-frozen within 5 minutes following brain removal and stored in liquid nitrogen. Total RNA was isolated by homogenization with RNeasy isolation kit (Qiagen, Valencia, California). The integrity and quantity of the RNA were evaluated using real-time polymerase chain reaction (PCR) for the housekeeping genes: 18S, glyceraldehyde-3-phosphate dehydrogenase real-time PCR experiments were carried out according to the manufacturer's instructions (Applied Biosystems, Foster City, California). Quantitative real-time Mouse Immune Panel TaqMan PCR was performed using low-density arrays and the 7900 robotic real-time PCR system (Applied Biosystems) (see the list of 96 genes at http://docs.appliedbiosystems.com/pebiodocs/00115187.pdf). Data were collected using instrument spectral compensations by the SDS 2.2 software (Applied Biosystems) and analyzed using the threshold-cycle relative quantification method.

Table 1. Survival Increase With Simvastatin Treatment in Scrapie-Infected Mice

Group and Inoculation	Time of Death, Mean (SE), dpi	Experiment No.	Time of Death, dpi
Scrapie infected, without simvastatin treatment			
Intracerebral	148.4 (3.9) (n=24)	1[a]	120, 150, 152, 154, 178
		2[b]	112, 120, 123, 147, 163
		3[a]	104, 156, 163, 166, 166, 167, 167
		4[a]	143, 148, 150, 150, 153, 155, 155
Intraperitoneal	226.4 (34.3) (n=10)	5[a]	129, 229, 234, 234, 237, 237, 241, 241, 241, 241
Scrapie infected, with increasing simvastatin dosage of 2-20 mg/kg/d[c]			
Intracerebral	166.7 (2.2) (n=20)	1[a]	164, 170, 180, 184, 192
		2[b]	157, 164, 165, 165
		3[a]	142, 156, 163, 176, 176, 176, 177
		4[a]	169, 169, 169, 176
Intraperitoneal	243.0 (11.2) (n=10)	5[d]	234, 234, 234, 236, 237, 237, 249, 250, 251, 268
Scrapie infected, with constant simvastatin dosage of 2 mg/kg/d			
Intracerebral	161.7 (3.5) (n=4)	3[b]	151, 165, 165, 166
Intraperitoneal	239.1 (2.5) (n=9)	5[d]	229, 234, 234, 234, 237, 237, 244, 251, 252

Abbreviation: dpi, days post infection.
[a] Start of simvastatin treatment at 72 dpi.
[b] Start of simvastatin treatment at 41 dpi.
[c] Statistically significant according to Breslow tests, $P<.001$.
[d] Start of simvastatin treatment at 7 weeks.

RESULTS

SIMVASTATIN TREATMENT INCREASED SURVIVAL IN PRION-INFECTED MICE

Healthy mice were maintained and treated in parallel with mice infected intracerebrally with RML scrapie inoculum. To study the effect of simvastatin on disease progression, the drug was administered to the mice via the drinking water. We used either a constant simvastatin dosage of 2 mg/kg/d throughout the treatment or an increasing dosage of 2 to 20 mg/kg/d. The rationale of using an increasing dosage was to overcome potential desensitization of the mice to the drug.[26] Animals of each group were euthanized at times representing disease progression or kept alive to determine time of death and then euthanized at severe illness. Overall, simvastatin delayed the appearance of early prion disease symptoms by a mean (SE) of approximately 22 (5) days in mice receiving an increasing dosage of 2 to 20 mg/kg/d (n=15) and approximately 19 (4) days in mice receiving a constant dosage of 2 mg/kg/d (n=8). We refer to this delay in symptom appearance as prolonged disease incubation time. Experiments 1 through 4 did not differ statistically in survival; therefore, we combined the data from all of the 4 independent experiments. In accordance, simvastatin dosages of 2 to 20 mg/kg/d significantly increased survival of scrapie-infected mice by approximately 21 days ($P<.001$; n=20-24) (**Table 1** and **Figure 1**). The effect of the low simvastatin dosage on survival was less pronounced with approximately 14 days (n=4). To increase the certainty of the results, we used a different RML inoculum in each of the 4 experiments.

To verify the relevance of simvastatin in delayed disease progression, we also infected healthy mice intra-
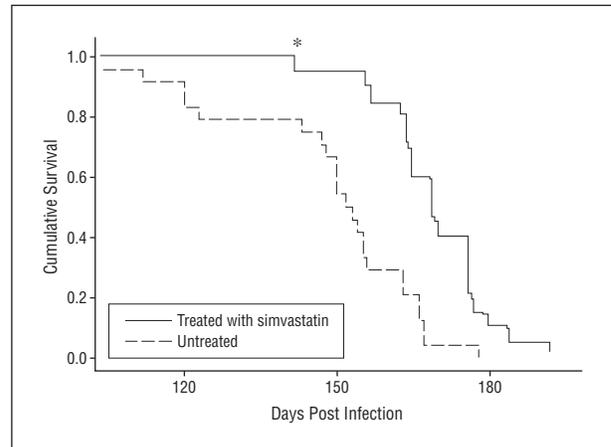

Figure 1. Simvastatin increased survival in prion-infected mice. Kaplan-Meier survival curve for scrapie-infected mice inoculated intracerebrally with the Rocky Mountain Laboratory inoculum, in a summary of 4 independent experiments. Simvastatin therapy at a dosage of 2 to 20 mg/kg/d was started at 72 days post infection (experiments 1, 3, and 4) or 41 days post infection (experiment 2) (n=24 and 20, respectively) (see Table 1 for experiments). *$P<.001$ by the Breslow test.

peritoneally with RML scrapie inoculum. Mice were infected at 7 weeks of age and subjected to simvastatin treatment immediately afterward (simvastatin at constant dosages of 2 mg/kg/d or 20 mg/kg/d). Overall, both simvastatin treatments increased survival of intraperitoneally infected mice by a mean (SE) of 17 (3.5) days (n=9-10) (Table 1). In this peripheral inoculation, the increased survival can result from prolonged disease incubation time or delayed peripheral accumulation of PrP[Sc] and delayed neuroinvasion. To this aim, we analyzed PrP[Sc] levels in the spleens of scrapie-infected mice by immunoblots. At 30 dpi, we found reduced levels of
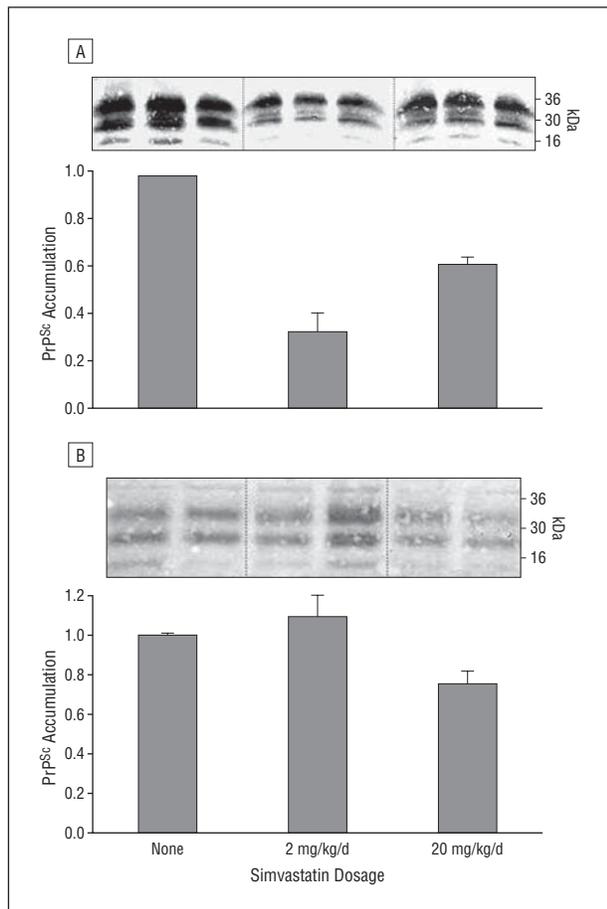
Figure 2. Simvastatin-inhibited accumulation of PrP^{Sc} in spleens of scrapie-infected mice inoculated intraperitoneally. The y-axes show relative PrP^{Sc} levels normalized to those in scrapie-infected mice without simvastatin treatment. Samples of spleen homogenates (100 mg) of scrapie-infected mice untreated or treated with simvastatin were digested with 0.1-mg/mL proteinase K and subjected to immunoblotting using anti-PrP monoclonal antibody IPC1 (Sigma, Rehovot, Israel). A, Spleens at 30 days post infection. B, Spleens at 45 days post infection.

PrP^{Sc} in spleens of mice treated with both simvastatin dosages, ie, 2 or 20 mg/kg/d (n=3) (**Figure 2**A); at 45 dpi, reduced PrP^{Sc} levels were detected in animals treated with the high simvastatin dosage but not with the low simvastatin dosage (n=2) (Figure 2B). This result suggests that simvastatin increased survival in intraperitoneally infected mice through delayed peripheral prion spread.

SIMVASTATIN DID NOT AFFECT BRAIN CHOLESTEROL LEVELS

We next determined brain cholesterol levels in total brain homogenates of simvastatin-treated and untreated mice. No significant alterations in cholesterol levels attributed to simvastatin treatment were detected (**Table 2**). Therefore, simvastatin inhibition of disease progression is independent of brain cholesterol levels.

We next analyzed the effect of simvastatin on the localization of PrP^{Sc} in brain membranes and specifically in lipid rafts. Membranal rafts containing both PrP^C and PrP^{Sc} have been shown to float on Nycodenz gradients as a function of their lipid composition. Lowering cholesterol levels in vitro resulted in heavier rafts that were found in lower frac-

Table 2. Cholesterol Levels in Brain Homogenates of Simvastatin-Treated and Untreated Mice

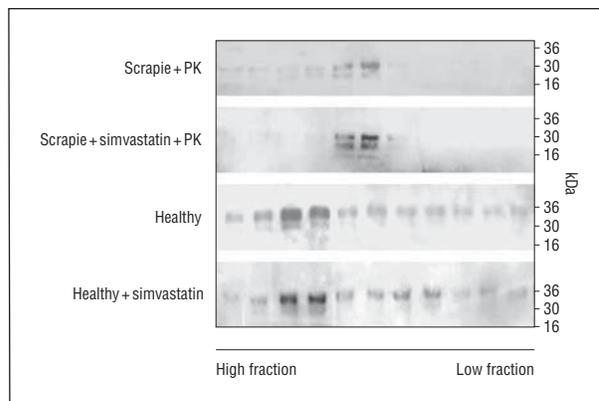| Group	Cholesterol, Mean (SE)[a]
Healthy, untreated (n=5)	35.9 (0.7)
Healthy, treated with constant simvastatin dosage of 2 mg/kg/d (n=5)	33.3 (2.1)
Healthy, treated with increasing simvastatin dosage of 2-20 mg/kg/d (n=5)	35.4 (1.5)

[a] Units are micrograms per milligram of protein.

Figure 3. Simvastatin did not affect the distribution of PrP^{Sc} or PrP^C on flotation gradients. Samples of brain homogenates of scrapie-infected mice (130 days post infection, experiment 2) untreated or treated with simvastatin were digested with proteinase K (PK) (0.04 mg/mL at 37°C for 30 minutes) and subjected to flotation gradients (see "Methods" section). Fractions were collected from top to bottom of the gradient, corresponding to their density, ie, low and high fractions, respectively. For the detection of PrP^{Sc}, all of the fractions were immunoblotted with anti-PrP monoclonal antibody 6H4. Healthy brains were analyzed by the flotation gradient in parallel, with the exclusion of PK digestion.

tions of the gradient and affected PrP distribution to the rafts.[8-10] It is therefore possible that alterations in the positioning of raft proteins in a Nycodenz gradient may represent changes in cholesterol distribution or levels in the cellular membranes. To this effect, we compared the position of PrP^{Sc} in flotation gradients in simvastatin-treated and untreated mice. PrP^{Sc} and PrP^C distributions on the Nycodenz gradient were not affected by simvastatin treatment (**Figure 3**). Therefore, simvastatin affects disease progression through mechanisms unrelated to properties of membrane rafts.

REDUCED NEURONAL LOSS IN BRAINS OF SIMVASTATIN-TREATED, SCRAPIE-INFECTED MICE

Functional benefits derived from the pleiotropic effects of statins suggest neuroprotective effects.[17] Using hematoxylin-eosin staining of frozen brain sections (105 dpi, experiment 2), we found that a considerable number of Purkinje neurons were lost in the prion-infected mice at this time (**Figure 4**A). This loss of Purkinje neurons is in agreement with a previous report.[27] However, hematoxylin-eosin staining of simvastatin-treated brains indicated the presence of Purkinje neurons to the levels in
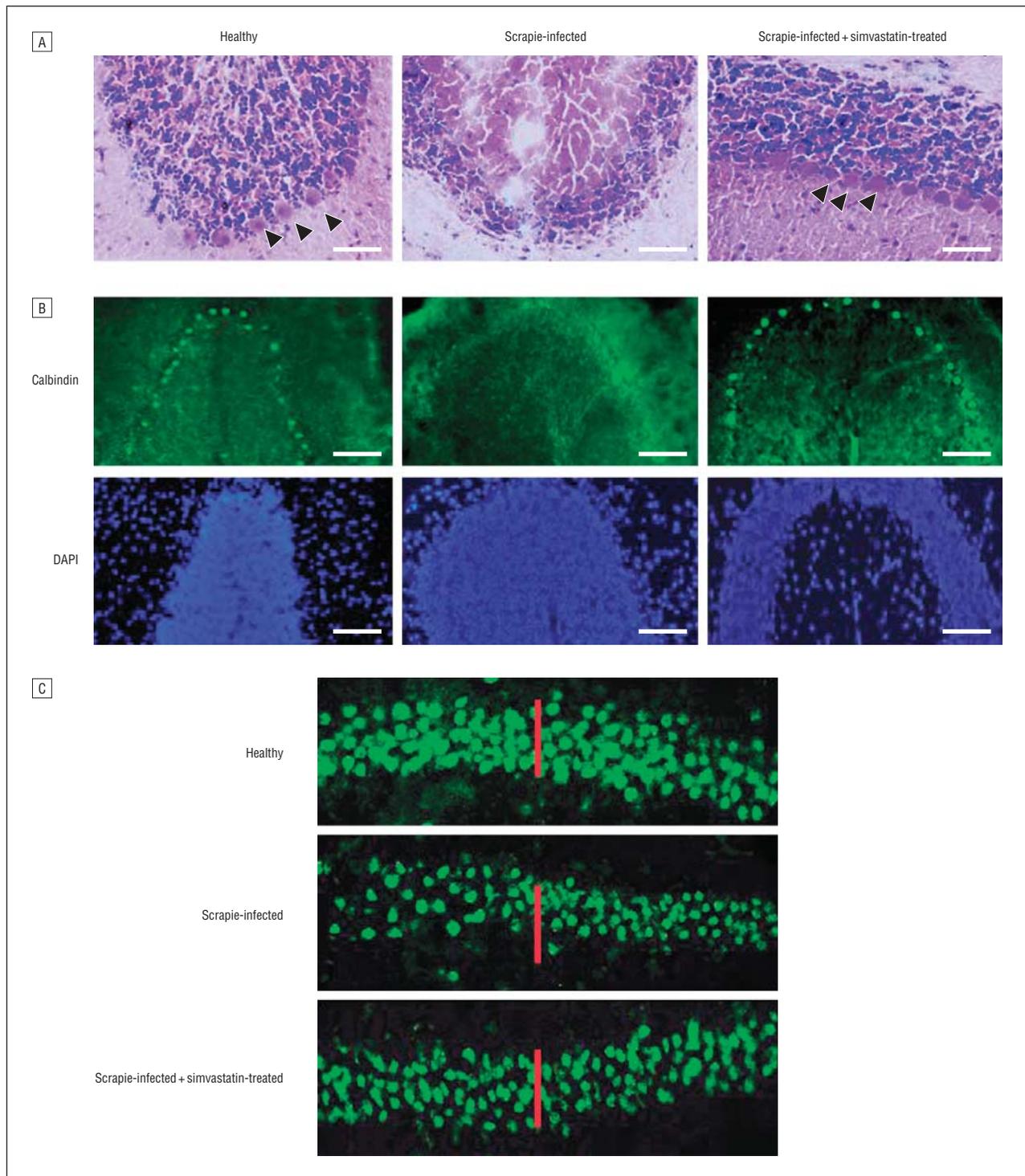
Figure 4. Neuroprotection in scrapie-infected mice treated with simvastatin. Horizontal cryosections of healthy, scrapie-infected, and scrapie-infected, simvastatin-treated (at dosages of 2-20 mg/kg/d) mouse brains (105 days post infection, experiment 2). A, Hematoxylin-eosin staining. Arrowheads indicate Purkinje cells; bars, 50 μm. B, Immunohistochemical analysis with anticalbindin antibody as a marker for Purkinje cells and counterstaining with 4′,6-diamidino-2-phenylindole (DAPI). Bars indicate 50 μm. C, Calbindin neuronal staining of the CA1 neuronal layer. Bars indicate 30 μm.

healthy brains (Figure 4A), suggesting a neuroprotective effect of simvastatin in prion-infected brains. Importantly, no pathological effect of simvastatin treatment was observed in the healthy mice.

To confirm the effect of simvastatin treatment on Purkinje neurons in the scrapie-infected mouse brains, we stained horizontal sections containing the cerebellum for calbindin, a 28-kDa calcium-binding protein that is abundant in Purkinje neurons. As shown in Figure 4B and consistent with the hematoxylin-eosin staining results (Figure 4A), a 75% reduction in the number of Purkinje neurons was detected in the prion-infected brains. Simvastatin had a protective effect on the actual number of Purkinje neurons positively stained with calbindin in the prion-

Table 3. Simvastatin Effect in Scrapie-Infected and Healthy Brains[a]

Group	Mean (SD)					
	Calbindin[b]	CA1[c]	BrdU[d]	IBA-1[e]	MHC II[f]	ICAM-1[g]
Scrapie infected, untreated	7.6 (1.4)	0.70 (0.10)	1.8 (0.2)	2.4 (0.6)	2.6 (0.5)	7.9 (1.2)
Scrapie infected, with constant simvastatin dosage of 2 mg/kg/d	11.3 (1.8)	ND	ND	ND	ND	ND
Scrapie infected, with increasing simvastatin dosage of 2-20 mg/kg/d	21.5 (1.6)	0.96 (0.07)	2.4 (0.5)	3.3 (0.8)	4.8 (0.7)	5.4 (0.9)
Healthy, untreated	29.8 (2.3)	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	3.3 (0.6)
Healthy, with increasing simvastatin dosage of 2-20 mg/kg/d	28.7 (0.3)	1.02 (0.08)	1.3 (0.2)	1.6 (0.4)	1.7 (0.3)	3.4 (0.7)

Abbreviations: BrdU, bromodeoxyuridine; IBA-1, ionized calcium binding adaptor molecule; ICAM-1, intercellular adhesion molecule 1; MHC II, major histocompatibility complex class II; ND, not determined.

[a] All of the sections were taken from animals euthanized at 105 days post infection (experiment 2; 3-4 brains for each treatment, 16-20 microscopic fields) except for MHC II (118 days post infection, experiment 4).
[b] Average number of cells staining positively for calbindin per field.
[c] Relative thickness of CA1 neuronal layer presented as the ratio to that in healthy mouse brain.
[d] Average number of cells staining positively for BrdU per field, presented as the ratio to those in healthy mouse brain.
[e] Arbitrary units measured as pixels per area in semiquantitative Western blot.
[f] Average number of cells staining positively for MHC II per field, presented as the ratio to those in healthy mouse brain.
[g] Average number of blood vessels stained with ICAM-1 per field.

infected groups (**Table 3**). This protective effect of simvastatin was dose dependent, with an increase of 50% and 70% in the number of Purkinje neurons at the low dosage of 2 mg/kg/d and the high dosage of 2 to 20 mg/kg/d, respectively. This neuroprotective effect of simvastatin in prion-infected mice was consistent in all of the animals tested (each group, n=4). No effect of simvastatin on Purkinje cells was observed in the healthy mice (Table 3).

A similar neuroprotective effect was detected in the CA1 neuronal layer of the hippocampus. These neurons are degenerated and lost in prion disease.[28] Using calbindin staining on frozen brain sections (105 dpi, experiment 2; each group, n=4), we found smaller neurons at the CA1 neuronal layer with a diffused staining and an approximately 25% reduction in the thickness of the neuronal layer in the prion-infected mouse brains (Table 3 and Figure 4C). However, the size of neurons and the thickness of the CA1 neuronal layer appeared normal in the prion-infected mice treated with simvastatin at dosages of 2 to 20 mg/kg/d. No effect of simvastatin was detected in the healthy brains (Table 3). Therefore, simvastatin exerted a general neuroprotective (or neurorestorative) effect in scrapie-infected mice.

CELL PROLIFERATION IN THE SUBVENTRICULAR ZONE AND DENTATE GYRUS

The number of cells positively stained for BrdU was significantly higher in the subventricular zone and dentate gyrus of prion-infected, simvastatin-treated brains than in healthy brains. Specifically, we documented increases of approximately 134%, 182%, and 240% in BrdU staining in healthy mice treated with simvastatin, scrapie-infected mice, and scrapie-infected, simvastatin-treated mice, respectively (Table 3 and **Figure 5**). This result indicates that at the neurodegenerative stage, there is a certain amount of cell proliferation; simvastatin treatment further induced this proliferation in healthy brains and, to a further extent, in scrapie-infected brains. Therefore, the neuroprotective effects of simvastatin include cell proliferation.

INCREASED PrP[Sc] LEVELS IN SIMVASTATIN-TREATED SCRAPIE-INFECTED BRAINS

Simvastatin's effect on PrP^C and PrP^{Sc} protein levels in brain extracts at early symptom appearance and at progressive disease was determined by immunoblotting using anti-PrP monoclonal antibody 6H4. Higher levels of PrP^{Sc} were consistently detected in the simvastatin-treated groups at both times as compared with the scrapie control group (without simvastatin treatment). A representative immunoblot showing 2 brains of each treated group at early symptom appearance (105 dpi, experiment 2) and progressive disease (130 dpi, experiment 2) is shown in **Figure 6**A and C. Simvastatin had no effect on PrP^C levels in healthy mice (Figure 6B). The increased PrP^{Sc} level in the simvastatin-treated, scrapie-infected brains may result from the neuroprotective effect of simvastatin, ie, the surviving neurons continuously generate PrP^{Sc}. However, it may also suggest that simvastatin acts to increase survival in a mechanism independent of PrP^{Sc} accumulation.

SIMVASTATIN EFFECTS ARE MEDIATED THROUGH THE L-MEVALONATE PATHWAY

The absence of any effect on cholesterol levels or PrP^{Sc} distribution on rafts raised the question of whether simvastatin acts through the L-mevalonate pathway to delay disease progression. We found that an L-mevalonate dosage of 20 mg/kg/d significantly reversed the effect of simvastatin (**Table 4**) and that administration of L-mevalonate to scrapie-infected mice appeared toxic, with reduced survival compared with scrapie-infected, untreated mice. Therefore, we conclude that simvastatin acts through the L-mevalonate pathway in its beneficial effects.
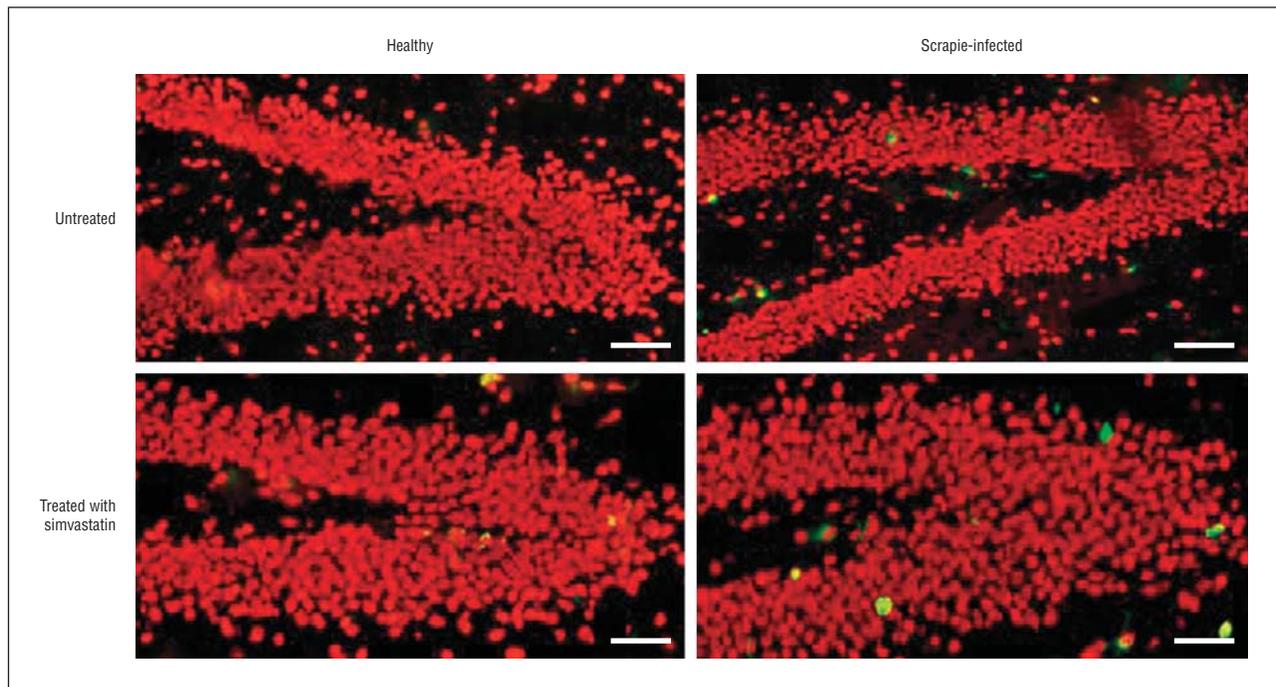
Figure 5. Simvastatin-induced cell proliferation in prion-infected mice. Bromodeoxyuridine immunostaining at the dentate gyrus of healthy and scrapie-infected mice untreated or treated with simvastatin (at a dosage of 20 mg/kg/d) at early disease symptom appearance (118 days post infection, experiment 4). Bars indicate 50 µm.

Inhibiting the L-mevalonate pathway will result in a further downstream inhibition of L-mevalonate intermediates such as geranylgeranyl pyrophosphate and farnesyl pyrophosphate, which represents major intermediates for the posttranslational modification of proteins. One potent candidate protein for this posttranslational modification is Ras protein. On its farnesylation, Ras is activated and transported to the membranes. However, we did not detect any effect of simvastatin on Ras localization in the treated and untreated brains. We conclude that the pleiotropic effects of simvastatin are mediated through prenylation of proteins other than Ras in our prion model.

INDUCED GLIAL ACTIVATION IN BRAINS OF SIMVASTATIN-TREATED, SCRAPIE-INFECTED MICE

It is well established that the immune system has a critical role in the progression of prion pathogenesis. A typical immune response or perivascular leukocyte accumulation is absent in prions; however, glial cells were shown to be activated in Creutzfeldt-Jakob disease and a rodent model of prions.[29] On the other hand, it was reported that simvastatin acts to inhibit neuroinflammation in neurodegeneration[30] and specifically in prion disease.[16]

To study the effect of simvastatin treatment on glial activation in our scrapie-infected mice, we stained frozen brain sections (105 dpi, experiment 2) of treated and untreated prion-infected mice as well as of healthy mice with anti–GFAP and IBA-1 antibodies as markers for astrocytic and microglial activation, respectively. As expected, activation of both astrocytosis and microglia was observed in the prion-infected animals throughout the brain compared with the healthy brains (**Figure 7**A and B). However, further ac-

tivation of astrocytosis and microglia was observed in scrapie-infected, simvastatin-treated brains (Figure 7A and B), with a more pronounced induced astrocytosis than microglial activation. Therefore, the delayed disease progression and increased survival are correlated with induced astrocytosis and microglial activation. Interestingly, astrocytosis in prion-infected, simvastatin-treated mice was dose dependent, with induced activation at the constant low dosage of 2 mg/kg/d and further induction with the increasing simvastatin dosage of 2 to 20 mg/kg/d. The images in Figure 7 are taken from the dentate gyrus, a representative area indicating activation throughout the brain.

SIMVASTATIN-INDUCED MHC CLASS II ACTIVATION IN PRION-INFECTED BRAINS

We next stained healthy and scrapie-infected brains that were untreated and treated with simvastatin at early symptom appearance (118 dpi, experiment 4) for MHC class II and found that MHC class II activation correlated with glial activation. Specifically, we detected increased MHC class II activation in prion-infected brains and a further increase in prion-infected, simvastatin-treated brains (Figure 7C). Therefore, in contrast to a previous study,[16] simvastatin treatment did not inhibit MHC class II activation; on the contrary, there is further activation of MHC class II in the treated brains. No effect on MHC class II staining was detected in healthy brains treated with simvastatin.

SIMVASTATIN INHIBITED ICAM-1 EXPRESSION IN PRION-INFECTED BRAINS

The expression of ICAM-1, a protein constitutively expressed in healthy brains, is dramatically induced by in-
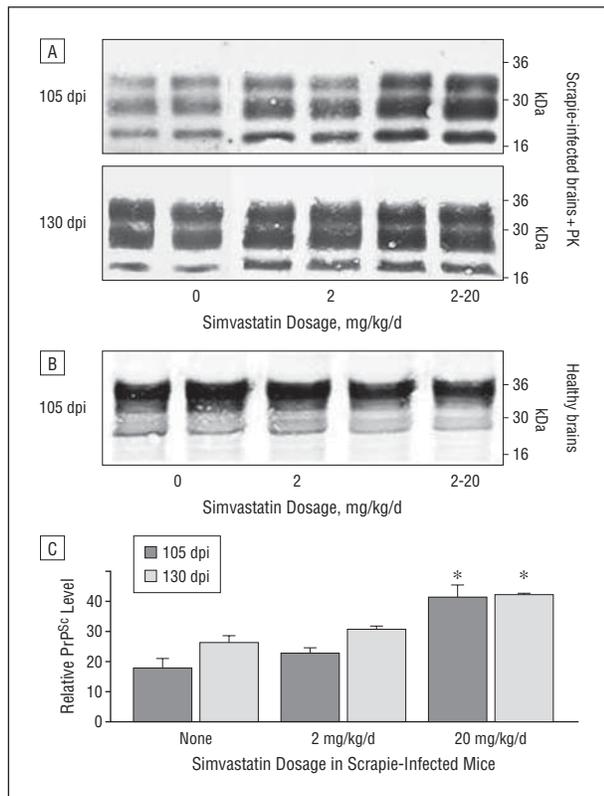
Figure 6. Treatment with simvastatin increased the accumulation of PrP^Sc but not PrP^C. A, Mice intracerebrally infected with scrapie Rocky Mountain Laboratory inoculum and subsequently either left untreated or treated with 2 different dosages of simvastatin (constant low dosage of 2 mg/kg/d or increasing dosage of 2-20 mg/kg/d). Mice from each group (n=5) were euthanized at 105 days post infection (dpi) and 130 dpi (experiment 2). For the detection of PrP^Sc, samples of brain homogenates were treated with 0.04-mg/mL proteinase K (PK) for 30 minutes at 37°C; detection of PrP used immunoblotting with anti-PrP monoclonal antibody 6H4. A representative blot from 2 mice in each treatment group is shown. B, Uninfected mice were untreated or treated with simvastatin, then euthanized in parallel with the scrapie-infected mice. Digestion with PK was not performed. C, Densimetric quantification of PrP^Sc levels in Western blots using Un-Scan-It software (Silk Scientific, Inc, Orem, Utah) (n=5 brains in each group). Data are presented as mean±SD. Significantly higher PrP^Sc levels are detected in drug-treated, scrapie-infected mice. *P=.04 (2-tailed t test).

flammatory conditions including proinflammatory cytokines secreted by microglia. In the inflamed brain, ICAM-1 is a marker for leukocyte infiltration, which was shown to occur in patients with Creutzfeldt-Jakob disease and in murine models of prion disease.[31] Importantly, its expression was shown to be downregulated by simvastatin[32] and by specific cytokines.[33] We stained frozen brain sections of treated and untreated mice (105 dpi, experiment 2) with an anti–ICAM-1 antibody. A higher number of brain blood vessels that positively stained for ICAM-1 was detected in scrapie-infected brains than in healthy brains (Table 3). Importantly, ICAM-1 immunoreactivity was considerably reduced (60%) in the brains of scrapie-infected mice treated with simvastatin at dosages of 2 to 20 mg/kg/d (Table 3). Simvastatin treatment of healthy mice did not change the immunostaining of these brains with the anti–ICAM-1 antibody. Our results therefore suggest that simvastatin treatment inhibited the inflammatory response leading to ICAM-1 expression in the brains of scrapie-infected mice.

Table 4. Simvastatin Effect on Disease Progression Is Mediated Through L-Mevalonate Pathway

Group	Time of Death, Mean (SE), dpi	Time of Death in Experiment 5, dpi
Control	148.4 (3.9) (n=7)	143, 148, 150, 150, 153, 155, 155
Simvastatin dosage of 20 mg/kg/d	166.7 (2.2) (n=4)	169, 169, 169, 176
L-Mevalonate^a	127.0 (13.1) (n=6)	86, 86, 137, 150, 150, 153
Simvastatin dosage of 20 mg/kg/d + L-mevalonate^b	150.4 (4.1) (n=5)	141, 141, 153, 155, 162

Abbreviation: dpi, days post infection.
^a Significantly different from scrapie-infected control group; Breslow test, P<.05.
^b Significantly different from scrapie-infected mice treated with a simvastatin dosage of 20 mg/kg/d; Breslow test, P<.05.

EFFECTS OF SIMVASTATIN ON THE IMMUNE PROFILE OF PRION-INFECTED MICE

To further characterize the effects of simvastatin on the brain immune profile, we performed TaqMan low-density-array analyses (Applied Biosystems). For this, we generated complementary DNA from total brain RNA (mice euthanized at early symptom appearance; 118 dpi, experiment 4; n=4 for each group), and the immune profiles of healthy and prion-infected brains with and without simvastatin treatment were quantitatively analyzed. Expression values for target genes were normalized to the concentration of angiotensin II receptor type 2 RNA, which showed the least variation among reference genes. Among the 96 genes that were analyzed, we identified two with an expression pattern correlating with PrP^Sc levels, namely *Cxcl10* and *Ccl3*, ie, the levels of these genes were upregulated in prion-infected mice and further upregulated in prion-infected, simvastatin-treated mice (data not shown). Importantly, these genes were previously shown to be upregulated in prion-infected mice.[34] Further, we identified 6 genes that were substantially affected by simvastatin treatment in the prion-infected brains. These are *Ccl2*, *Ccl5*, *Cxcr3*, *Cxcl 11*, *IL1*β, and *Cox2* (**Figure 8**). The effects on expression of these genes cannot be explained by the increased PrP^Sc protein levels. The expression levels of *Ccl2*, *Ccl5*, *Cxcr3*, and *IL1*β are slightly elevated in prion-infected mouse brains and substantially activated in prion-infected, simvastatin-treated mouse brains. These upregulated genes may indicate T-cell activation and proinflammatory response. Interestingly, simvastatin treatment reduced *Cxcl11* expression levels in the absence of a detectable effect on interferon γ levels, a result that may suggest inhibition of T-cell activation. Together, the results indicate a multifaceted effect of simvastatin, acting in parallel to induce and inhibit T-cell activation in the prion-infected brain.

Importantly, simvastatin treatment inhibited *Cox2* expression in the scrapie-infected brains (Figure 8F). *Cox2* is constitutively expressed in the brains and is induced on inflammation. Its expression in the brain has been associated with proinflammatory activities and involved in neurodegeneration.[30] Therefore, its inhibition by simva-

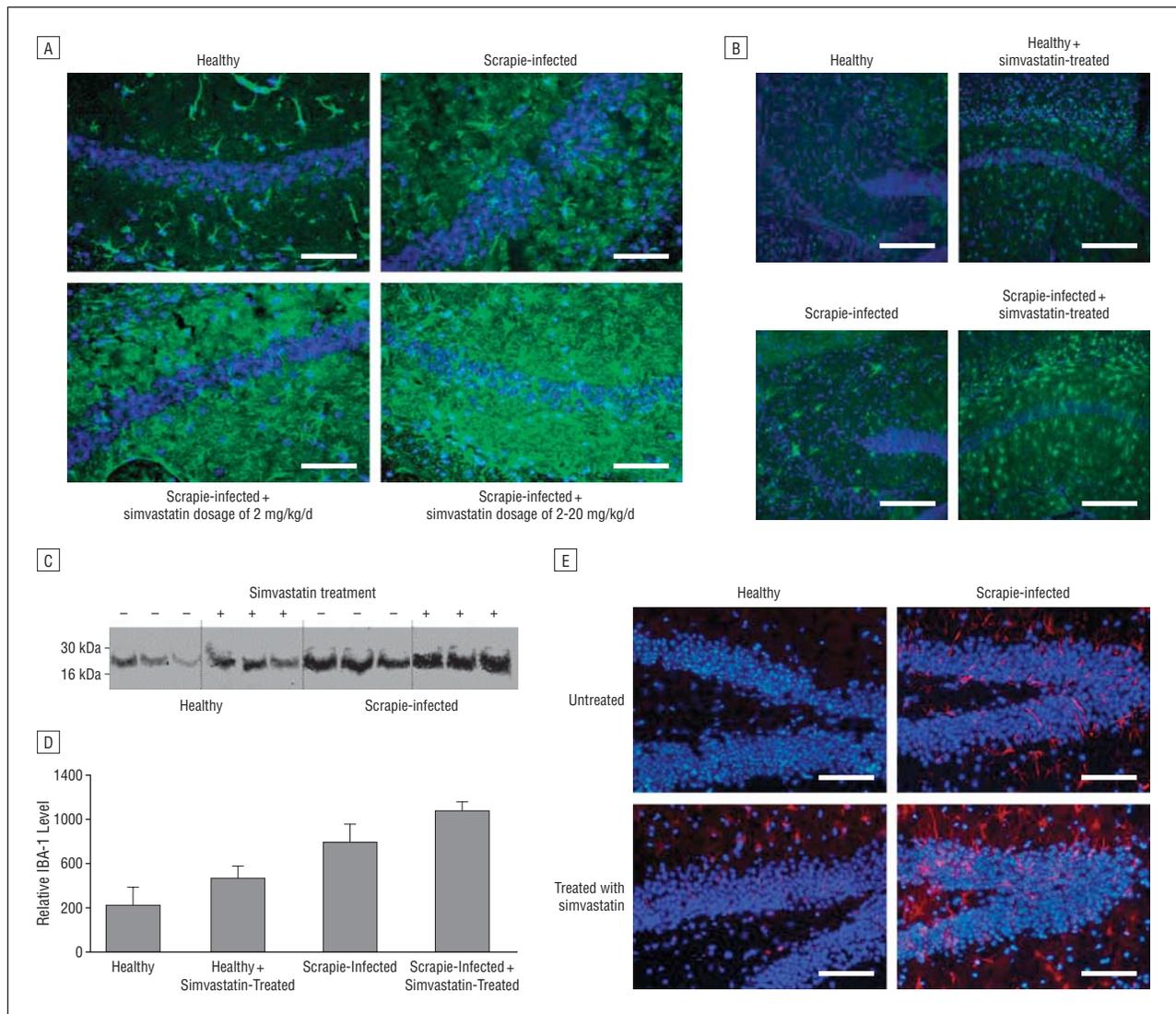**Figure 7.** Glial activation in scrapie-infected mice with and without simvastatin treatment. A, Astrocytosis. Immunohistochemical analysis of glial fibrillary acidic protein expression as a marker for astrocytes. Frozen sections were from healthy mice, scrapie-infected, untreated mice, or scrapie-infected mice treated with simvastatin (constant low dosage of 2 mg/kd/d or increasing dosage of 2-20 mg/kg/d) at 105 days post infection (experiment 2), with 4′,6-diamidino-2-phenylindole (DAPI) counterstaining. Bars indicate 50 μm. B, Microglial activation. Immunohistochemical analysis was performed as in A, with anti–ionized calcium binding adaptor molecule (IBA-1) antibody. Bars indicate 50 μm. C, Immunoblot of brain homogenates (50 μg) of healthy and scrapie-infected mice untreated or treated with simvastatin at 105 days post infection (experiment 2), probed with anti–IBA-1 antibody. D, Quantitation of immunoblot in C, presented as mean±standard deviation (n=3). E, Major histocompatibility complex class II activation. Immunohistochemical analyses with anti–major histocompatibility complex class II antibody. Frozen sections were from healthy and scrapie-infected mice untreated or treated with simvastatin at a dosage of 20 mg/kg/d (118 days post infection, experiment 4). Bars indicate 50 μm.

statin may partly explain the neuroprotective effects observed.

COMMENT

We show that administration of simvastatin, a cholesterol-lowering drug, to prion-infected mice significantly delayed disease progression, leading to increased survival. Simvastatin affected disease progression in both peripheral (intraperitoneal) and central nervous system (intracerebral) inoculations. Interestingly, the increased incubation time occurred despite a marked increase in PrP^{Sc} levels, no detectable effect on the properties of detergent-insoluble membrane domains (ie, cholesterol-rich rafts), and no detectable effect on brain cholesterol levels. The increased survival of the prion-infected mice treated with simvastatin is associated with a striking neuroprotective effect: glial activation and cell proliferation at the hippocampus. We show that while simvastatin treatment did not affect cholesterol levels or membrane properties, its effects are mediated through the L-mevalonate pathway. Analysis of the immune profile of prion-infected, simvastatin-treated mice suggests an induced activation of astrocytes and microglia with inhibition of proinflammatory activities mediated by *Cox2*.

The accumulation of PrP^{Sc} is a hallmark of prion diseases, and its concentration was shown to correlate with disease severity.[35] Nevertheless, prion disease pathogenesis and transmission were previously shown to occur in the absence of PrP^{Sc}.[36,37] We show, for the first time to
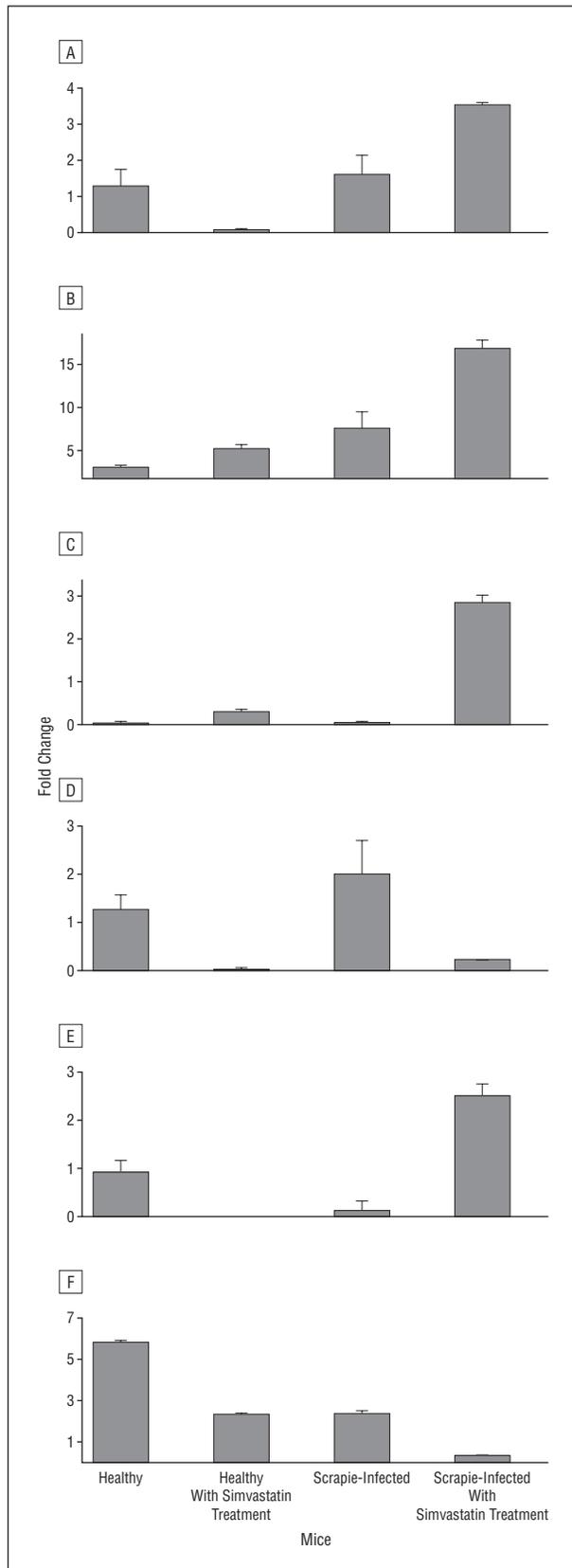
Figure 8. Low-density microarray analysis of mouse immune panel. Fold changes of altered genes with a threshold expression level higher than 2 in healthy mice, healthy mice treated with simvastatin, prion-infected mice, and prion-infected mice treated with simvastatin (simvastatin dosage was 20 mg/kg/d) (n=4 brains for each group). The genes were *Ccl2* (A), *Ccl5* (B), *Cxcr3* (C), *Cxcl11* (D), *IL1*β (E), and *Cox2* (F). Error bars indicate SD.

our knowledge, evidence for the existence of an inverse correlation, ie, increased levels of the pathogenic prion protein, PrP[Sc], despite inhibition in disease progression. We hypothesize that this unique effect is related to a larger portion of surviving scrapie-affected neurons present in the simvastatin-treated animals. These neurons may continue to generate PrP[Sc] in the affected cells, up to the point where the prion pathogenic mechanisms overcome the neuroprotective effect conferred by the drug. These results also suggest that the neuronal toxic effects of PrP[Sc] are dependent on additional factors, for example, factors involved in inflammation that act to induce neuronal death in prion disease.

Our in vivo results presented herein do not coincide with the in vitro results observed in cell-cultured experiments where cholesterol-lowering drugs actually affected cholesterol levels and PrP[Sc] accumulation, resulting in lower PrP[Sc] levels. The differences may be explained by variations in drug concentrations, efficiency of absorption, and drug clearance in the 2 experimental models.

Based on our results, we suggest that the mechanism by which simvastatin delays prion disease in the mouse model involves its pleiotropic effects.[38] Importantly, we show that the beneficial effects of simvastatin are mediated through the L-mevalonate pathway. We suggest that specific protein prenylation, but not Ras farnesylation, is involved in these beneficial effects of simvastatin. In addition to their designed effect on cholesterol levels, statins induce anti-inflammatory effects[39] as well as antioxidative effects.[40] Together, these effects may contribute to the overall beneficial effect of simvastatin in prion diseases.

The presence of chronic inflammation in prion diseases, occurring in the absence of overt leukocyte infiltration, has been demonstrated.[41] This inflammation occurs as a local response and is driven by glial cells, which have been shown to be activated in prion diseases.[42] However, the primary activator of glial cells in prion diseases has not been identified and there is no direct evidence that PrP[Sc] itself is proinflammatory.[43] We report a general correlation between increased PrP[Sc] protein levels, induced glial activation, and neuroprotection. However, in neurodegenerative diseases such as Alzheimer disease, Parkinson disease, and prion diseases and more so in multiple sclerosis, microglial activation is correlated with neuronal degeneration.[44-46] On the other hand, increasing evidence suggests that differentially activated microglial cells may have a general role in neuronal survival and neurogenesis.[47-49] In this regard, we suggest that simvastatin treatment affects the secretion of chemokines and cytokines from activated microglia and astrocytes.[50]

Our immune profile analyses have pointed out several gene candidates for mediating a neuroprotective response. Specifically, we found increased levels of expression for *Ccl2*, *Ccl5*, *Cxcr3*, and *IL1*β and reduced levels for *Cxcl 11* and *Cox2*. The immune response involves a broad variety of factors; however, it is suggested that the importance of an individual factor changes during the course of a disease[51] or as a result of changes in patterns of expression in other immune factors.[51] Although Ccl2 and Ccl5 chemokines and Cxcr3 were shown to have a specific role as mediators of inflammation, it is sug-

gested that specific chemokines, including Ccl2 and Ccl5, have an additional role as neuroprotective agents and that their protective action is dependent on the specific chemokine concentrations, cellular microenvironment, and stage of development of the target neuron.[52,53] Interestingly, Cxcl11 binds with high affinity to the Cxcr3 receptor and recruits activated helper T cells, type 1, to sites of inflammation. In this regard, the finding that Cxcl11 levels are reduced while Cxcr3 levels are upregulated in simvastatin-treated, prion-infected brains may explain the neuroprotective effect of simvastatin herein. Further, it implicates more complex interactions among pivotal factors of the inflammatory response. An example of the complex effect of a specific factor is the role of interleukin 1β (IL-1β) activity in the brain. The effects of IL-1β range between promoting neuronal survival[54] and exacerbating neuronal damage.[55] Finally, reduced *Cox2* expression supports an anti-inflammatory response in the simvastatin-treated, prion-infected mice. Based on these results, we suggest that simvastatin promotes an overall balance of microglial-induced chemokines and cytokines shifting toward neuroprotection.

Experimental manipulations of the immune system were previously shown to significantly increase survival time and delay prion disease onset. The rationale was that increased survival time serves as an indicator of slowing disease progression. However, this experimental approach was limited to the peripheral propagation of PrP[Sc] protein and its neuroinvasion. We show that simvastatin acts to delay both the events involved in peripheral propagation as well as central nervous system–specific neurotoxic effects.

Recently, Mok et al[16] reported that simvastatin increased survival in a murine prion model (inoculated intracerebrally). Importantly, the results from our study and that by Mok et al complement each other for prion strain (139A in the study by Mok et al and RML in our study) and mouse strain (C57/B6 in the study by Mok et al and FVB/N in our study). However, the two studies differ in the protocol of drug administration: with a high simvastatin dosage of 100 mg/kg/d starting at 100 dpi and given through the mouse chow pellets (in the study by Mok et al) vs low simvastatin dosages of 2 and 2 to 20 mg/kg/d starting at 41 and 72 dpi and given through the drinking water (in our study). Importantly, despite the differences in the protocols for drug administration, the effect on survival is similar, with 16 to 20 days of increased survival in the study by Mok et al and approximately 21 days of increased survival in our study. In line with our findings, no effects on brain cholesterol levels were detected and increased survival is attributed to simvastatin's pleiotropic effects.

However, the two studies differ significantly in the mechanisms leading to increased survival. Mok et al did not report an effect on PrP[Sc] levels, neurosurvival, or cell proliferation, whereas we report increased PrP[Sc] levels with simvastatin treatment and impressive effects of the drug on neuroprotection and cell proliferation in the infected brain.

Furthermore, the studies differ significantly in the results of the immune profile analyses. Whereas Mok et al reported that simvastatin acted to inhibit microglial ac-

tivation (through the inhibition of MHC class II expression) in prion-infected mice, we report a more complex response to the treatment, specifically, elevation of certain factors that may indicate induced proinflammatory response (ie, GFAP, IBA-1, and MHC II expression, and upregulation of chemokines and cytokines Ccl2, Ccl5, Cxcr3, and IL-1β). In parallel, we detected inhibition of other factors that may act to inhibit the proinflammatory response, ie, downregulation of Cxcl 11 and Cox2, or correlate with an inhibited inflammatory response, ie, reduced levels of ICAM-1. Collectively, our results indicate a multifaceted effect of simvastatin, inducing and inhibiting pivotal players in inflammation that together act to inhibit disease progression.

Recent studies have shown that statins act as direct inhibitors of induction of MHC class II expression by interferon γ and thus as repressors of MHC class II–mediated T-cell activation. This effect of statins is mediated by inhibition of the inducible promoter IV of the class II transactivator.[56] The specific disagreement in MHC class II results (in our study and the study by Mok et al) is of specific interest. Because no effect on classical proinflammatory cytokines such as interferon γ or tumor necrosis factor α was detected, simvastatin's effects on MHC class II activation (herein) or inhibition (in the study by Mok et al) cannot be simply explained by its action to inhibit inflammatory response overall.[16] Rather, it is more likely that a complex response with broad cellular implications is activated. The differences in observations between the two studies may result from the different drug dosages (100 mg/kg/d in the study by Mok et al vs 2-20 mg/kg/d in our study) and drug administration (chow pellets in the study by Mok et al vs drinking water in our study). In this regard, it is also important to note the effect of duration of drug administration, starting at 100 dpi in the study by Mok et al and 41 or 72 dpi in our study and present until termination. In conclusion, the two studies show results indicating that simvastatin prolongs disease incubation time and increases survival independently from its designed action to inhibit cholesterol biosynthesis. However, further study is needed to understand the alternative protective mechanisms activated by simvastatin.

To date, there is neither a cure nor a symptomatic therapy for prion diseases. Most of the experimental efforts are focusing on finding reagents that will inhibit the conversion of PrP[C] to PrP[Sc] and, thereafter, the accumulation of the abnormal prion protein isoform in cells. We show here, for the first time to our knowledge, that reagents with a mechanism of action independent from PrP[Sc] formation and accumulation can delay the onset of prion disease. Simvastatin treatment significantly delays prion disease onset in mice infected with prions. The simvastatin treatment was administered before symptom appearance; therefore, at this point, we suggest considering administering this treatment to individuals at risk such as healthy carriers of pathogenic mutations in the prion protein. While most cases of human prion diseases are sporadic, about 10% of the cases are associated with pathogenic mutations at the PrP gene.[57] The combination of a codon 200 mutation (E200K) and the polymorphism at codon 129M is the most common form of familial

Creutzfeldt-Jakob disease. It is particularly prevalent in Jewish persons of Libyan and Tunisian origin, who have an incidence of Creutzfeldt-Jakob disease about 100 times higher than the worldwide average.[58-60]

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Announcement

Trial Registration Required. In concert with the International Committee of Medical Journal Editors (ICMJE), *Archives of Neurology* will require, as a condition of consideration for publication, registration of all trials in a public trials registry (such as http://ClinicalTrials.gov). Trials must be registered at or before the onset of patient enrollment. This policy applies to any clinical trial starting enrollment after July 1, 2005. For trials that began enrollment before this date, registration will be required by September 13, 2005, before considering the trial for publication. The trial registration number should be supplied at the time of submission.

For details about this new policy, and for information on how the ICMJE defines a clinical trial, see the editorial by DeAngelis et al in the January issue of *Archives of Dermatology* (2005;141:76-77). Also see the Instructions to Authors on our Web site: www.archneurol.com.