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# A Hypothalamic Neuronal Cell Line Persistently Infected with Scrapie Prions Exhibits Apoptosis

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**Neuronal death and vacuolation are characteristics of the CNS degeneration found in prion diseases. Relatively few cultured cell lines have been identified that can be persistently infected with scrapie prions, and none of these cells show cytopathologic changes reminiscent of prion neuropathology. The differentiated neuronal cell line GT1, established from gonadotropin hormone releasing-hormone neurons immortalized by genetically targeted tumorigenesis in transgenic mice (P. L. Mellon, J.J. Windle, P. C. Goldsmith, C. A. Padula, J. L. Roberts, and R. I. Weiner, Neuron 5:1–10, 1990), was examined for its ability to support prion formation. We found that GT1 cells could be persistently infected with mouse RML prions and that conditioned medium from infected cells could transfer prions to uninfected cells. In many but not all experiments, a subpopulation of cells showed reduced viability, morphological signs of neurodegeneration and vacuolation, and features of apoptosis. Subclones of GT1 cells that were stably transfected with the *trkA* gene encoding the high-affinity nerve growth factor (NGF) receptor (GT1-trk) could also be persistently infected. NGF increased the viability of the scrapie-infected GT1-trk cells and reduced the morphological and biochemical signs of vacuolation and apoptosis. GT1 cells represent a novel system for studying the molecular mechanisms underlying prion infectivity and subsequent neurodegenerative changes.**

The prion diseases are a group of neurodegenerative disorders of humans and animals (17, 38). These illnesses are caused by proteinaceous infectious pathogens called prions that are devoid of nucleic acid (37). Prions are composed largely, if not entirely, of the scrapie isoform of the prion protein ($PrP^{Sc}$) (38). $PrP^{Sc}$ is derived from the cellular PrP isoform ($PrP^{C}$) by a posttranslational process (3) which involves a profound conformational change (34). The PrP gene resides on the short arm of chromosome 20 in humans and on chromosome 2 in mice (51). After synthesis in the endoplasmic reticulum, $PrP^{C}$ is transported to the cell surface where it is bound by a glycosylphosphatidyl inositol anchor (52).

Although studies of PrP metabolism in cultured cells have advanced our understanding of $PrP^{Sc}$ formation, the small number of cell lines that support prion infection has limited these investigations. Though mouse neuroblastoma cells infected with mouse prions have been used most widely (3, 7, 9, 40), some investigations have employed rat pheochromocytoma cells infected with mouse prions (42–44). The results with rat pheochromocytoma cells are puzzling since there is a significant species barrier between rats and mice (35). A brain cell line was isolated from the brain of a scrapie-infected Syrian hamster which was reinfected after passage of the cells in culture. These cells were particularly useful in studies of the subcellular localization of $PrP^{Sc}$ (54, 55). While no obvious cytopathology has been described in these cultured cell systems, profound changes in stress proteins have been recorded in scrapie-infected neuroblastoma cells (57) as well as marked attenuation of receptor-mediated intracellular 1,4,5-inositol triphosphate levels (62) and $Ca^{2+}$ release (24).

Recently, well-differentiated neuronal cell lines (GT1) originating from the central nervous system (CNS) were established from gonadotropin-releasing hormone (GnRH) neurons immortalized by genetically targeted tumorigenesis in transgenic mice (31). The GT1 cell lines are highly differentiated GnRH neurons that express, process, and regulate GnRH physiologically (30, 60). To render the GT1 cells responsive to nerve growth factor (NGF), they were transfected with the *trkA* receptor (GT1-trk) (64). Treatment of the GT1-trk cells with NGF stimulated neurite outgrowth and increased cell survival in serum-free medium. These characteristics of the GT1 cells suggested that they might be used for studies of prion biology.

We report that both GT1 and GT1-trk cells can be persistently infected with scrapie prions and both accumulate $PrP^{Sc}$. In contrast to scrapie-infected neuroblastoma cells, the GT1 cells showed pronounced neurodegenerative changes reminiscent of those observed in the CNS of humans and animals dying of prion disease. This cell culture system may prove useful for dissecting the molecular events that feature in the pathogenesis of prion diseases.

## MATERIALS AND METHODS

**Reagents.** Proteinase K was obtained from Beckman Instruments. The polyclonal antibodies RO73 (PrP) and LR1 (GnRH) have been described previously (references 48 and 47, respectively) (LR1 was a gift of P. Goldsmith). Monoclonal antibodies to simian virus 40 large T antigen (SV40 TAg) and *bcl2* were obtained from Pharmingen, San Diego, Calif., a monoclonal antibody to p53 was from Oncogene Science, Cambridge, Mass., and a polyclonal antibody to neuron-specific enolase was from Chemicon, Temecula, Calif. Immunoblotting was done

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by using the enhanced chemiluminescence blotting technique of Amersham Corporation. Secondary antibodies for immunofluorescence were fluorescein isothiocyanate-conjugated immunoglobulins obtained from Boehringer-Mannheim. Cell culture media and solutions were obtained from the UCSF Cell Culture Facility. β-NGF (highly purified; mouse) and Pefabloc inhibitor were purchased from Boehringer-Mannheim. Poly-L-lysine for coating of slides was from Sigma. DNA probes for Northern blot analysis were used as purified fragments as described previously (20, 46, 64). [$^{32}$P]dCTP (5,000 Ci/mmol) and [$^{35}$S]Met (1,000 Ci/mmol) were obtained from Amersham and New England Nuclear, Boston, Mass., respectively. G418 was from Gibco, Grand Island, N.Y., and protein A-Sepharose was from Pierce. All other chemicals were purchased from Sigma.

**Cell culture and mode of infection.** GT1-trk cells represent a subclone of the murine neuronal hypothalamic cell line GT1-1 (31) which was stably transfected with the *trkA* gene and previously labeled as GT1-1 *trk9* (64). The nontransfected subclones GT1-1, GT1-3, and GT1-7 and the mouse neuroblastoma cell lines N2a and ScN2a have been described previously (3, 7, 31). Cells were grown in Dulbecco's modified Eagle's (DME-H21) medium containing 5% fetal calf serum (FCS)–5% horse serum supplemented with 300 μg of G418 per ml if necessary (GT1-trk and ScGT1-trk) or DME-H16 medium containing 10% FCS (ScN2a and N2a). NGF (100 ng/ml) was applied for the indicated times and freshly added with each medium change. Infection was done in one well of a 12-well plate (Nunc) with purified mouse Rocky Mountain Laboratory strain of mouse prions derived from the Chandler isolate (RML prions) at a theoretical multiplicity of infection of 50 to 100 50% infectious dose ($ID_{50}$) units per cell. Prion rods were purified from brains of CD-1 Swiss mice that showed clinically via a discontinuous sucrose gradient (39), dilution in phosphate-buffered saline (PBS), and ultracentrifugation for 24 h at 24,000 rpm. The pellet was redissolved in DME medium containing 5% FCS and 5% horse serum, transferred to sterile tubes, and precipitated in ethanol. After centrifugation at 60,000 × *g* for 1 h, the final pellet was washed in 70% ethanol and redissolved in 500 μl of normal cell medium. Recipient cells were plated at a cell density of 5,000 to 10,000 cells per well (12-well plate) 2 days prior to infection (with and without NGF). One hour before infection, the cells were washed twice with cold PBS and starved by the addition of Earle's balanced salt solution (BSS) containing 5% dialyzed FCS. The inoculum was added to this solution overnight; cells were then washed with PBS and normally propagated. Mock infection of controls was performed in parallel. For the infections with conditioned medium, the supernatants were either sterile filtered (25 μm; Nunc) or centrifuged twice, first at 463 × *g* for 15 min at 4°C and then at 16,000 × *g* for 15 min at room temperature. Briefly, 10 ml of freshly harvested cell-free supernatant (24 h old) was added to subconfluent cultures in 10-cm-diameter plates and incubated overnight. After 24 h, cells were washed twice with PBS and normally passaged for 6 to 8 weeks.

**Cell viability, proliferation rates, and cell survival assays.** Cell viability was measured by Trypan blue exclusion dye. Briefly, confluent cell cultures were washed twice in cold PBS, trypsinized, and suspended in DME medium containing 5% horse serum and 5% FCS (to inactivate the trypsin). After centrifugation at 800 rpm for 5 min, cells were resuspended in PBS and an aliquot was incubated in 0.4% Trypan blue (Sigma) according to the recommendations of the manufacturer and counted in a hemocytometer. To assess cell growth, cells were plated with $2 \times 10^6$ cells per dish (10 cm diameter). At intervals after plating, cells were washed and trypsinized and cells excluding Trypan blue were counted in a hemocytometer. All counts were made in duplicate. For experiments in serum-free medium, cells were washed three times with serum-free DME H21 medium and cultured in the same. Cell survival was quantified after trypsinization and Trypan blue staining in a hemocytometer.

**Immunoblot analysis.** Confluent cell cultures were washed twice in cold PBS and lysed in 1 ml of cold lysis buffer (10 mM Tris-HCl [pH 7.5], 100 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, 0.5% deoxycholate) for 10 min. After removal of insoluble debris by centrifugation at 16,000 × *g* for 40 s, samples were split between those with and those without proteinase K (PK) digestion. Samples without PK digestion were supplemented with inhibitors (5 mM phenylmethylsulfonyl fluoride [PMSF] and 0.5 mM Pefabloc) and 3 M guanidine (Gdn)-HCl and directly precipitated in methanol. The remaining samples were incubated with 40 μg of PK per ml for 1 h at 37°C; digestion was stopped with PMSF (to 5 mM) and Pefabloc (to 0.5 mM), and the samples were denatured with 3 M GdnHCl for 10 min and precipitated in methanol (10 volumes). After centrifugation at 3,300 × *g* for 30 min at 4°C, the pellets were redissolved in 100 μl (PK) or 200 μl (without PK) of TNE buffer, 50 to 100 μl of gel loading buffer (7% sodium dodecyl sulfate [SDS]; 30% glycerin, 20% mercaptoethanol, and 0.01% bromphenol blue in 90 mM Tris-HCl [pH 6.8]) was added, and the samples were transferred to Eppendorf tubes. After boiling for 10 min, an aliquot (30 to 50 μl) was analyzed on a 12.5% polyacrylamide gel (25). For greater separation, Tricine peptide gels (16.5%) were used according to the method of Schägger and von Jaggow (45) (1 to 10 μl of loading buffer). For Western blot analysis, the proteins were electrotransferred to nitrocellulose membranes (Hoefer Scientific, San Francisco, Calif.). The membrane was blocked with 5% nonfat dry milk in TBST (0.05% Tween 20, 100 mM NaCl, 10 mM Tris-HCl [pH 7.8]), incubated overnight with the primary antibody (RO73; 1:5,000) at 4°C, and stained using the enhanced chemiluminescence blotting kit from Amersham Corporation.

**Metabolic radiolabeling and immunoprecipitation assay.** Confluent cells were washed twice with ice-cold PBS and incubated 1 h in Earle's BSS containing 5% dialyzed FCS. The medium was replaced with fresh Earle's BSS (10% FCS) supplemented with 400 μCi of L-[$^{35}$S]methionine (NEN) and incubated for 4 h. Cells were rinsed twice in PBS and chased in normal medium containing FCS for 16 h. After different chase periods, cells were washed twice in ice-cold PBS and lysed in cold lysis buffer (100 mM NaCl, 10 mM Tris-HCl [pH 7.8], 10 mM EDTA, 0.5% Triton X-100, 0.5% deoxycholate); insoluble debris was removed by centrifugation at 3,300 × *g* for 40 s at room temperature. Prior to immunopurification of $PrP^{Sc}$, the lysates were digested with PK (40 μg/ml; 1 h at 37°C). Digestion was stopped with 5 mM PMSF and 0.5 mM Pefabloc, and the samples were denatured with 3 M Gdn thiocyanate (SCN) and precipitated with methanol. The final pellets were resuspended in 1 ml TNS (100 mM NaCl, 10 mM Tris-HCl [pH 7.8], 1% Sarkosyl), and the antibody (RO73; 1:1,000) was incubated overnight at 4°C. Protein A-Sepharose beads (immobilized protein A on Trisacryl; Pierce, Radkon, Ill.) were then added for 1 h at room temperature. The immunoadsorbed proteins were washed in TNS buffer and analyzed on SDS–12.5% polyacrylamide gels (25) followed by autoradiography on X-Omat AR films (Kodak).

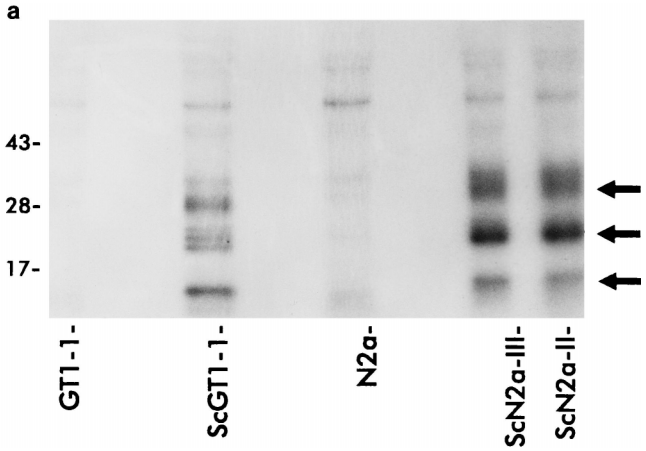


FIG. 1. Production of PK-resistant PrP in infected cells. (a) Immunoprecipitation assay of de novo-synthesized PrP 27-30. The polyclonal antiserum RO73 was used. Only PK-digested samples are shown. Molecular size markers are indicated on the left. Arrows indicate the three PK-resistant PrP bands. Notably, there are differences in molecular size between ScGT1-trk and ScN2a cells. (b) Intracellular accumulation of $PrP^{Sc}$ in scrapie-infected cells, as shown by immunocytochemistry. Indirect immunofluorescence was done under conditions that resulted in a typical dot-like cytosolic staining in scrapie-infected cells; RO73 was used as antibody. Panels A and B show N2a and ScN2a-III cells as controls. Panels C and E show the parental GT1-trk cells, and panels D and F show the infected ScGT1-trk cells. $PrP^{Sc}$ accumulation is also detectable in neurites. Cells in panels E and F were treated with NGF (100 ng/ml) 24 h prior to fixation. Notably, NGF treatment had profound effects on the morphology of ScGT1-trk cells.

**Immunocytochemistry.** Cells were plated on poly-L-lysine-coated plastic 8-chamber slides (Permanox, Nunc, Inc., Naperville, Ill.) at low density 1 to 3 days prior to staining. Cells were washed twice in ice-cold PBS and fixed in 10% formalin in PBS for 30 min. After sequentially treating with 1% $NH_4Cl$ in PBS, 0.5% Triton X-100 in PBS, and 3 M GdnHCl (only for $PrP^{Sc}$ immunostaining) for 5 min each, slides were washed four times with PBS and blocked by incubation in 5% milk solution in PBS for 30 min. The first antiserum was added at 1:1,000 dilution in PBS containing 5% milk and incubated overnight at 4°C. After five washes in PBS, fluorescein isothiocyanate-conjugated secondary antisera (1:50 dilution in 5% milk in PBS) were used and immunostaining was accomplished according to standard procedures. Slides were mounted in antifading solution (5% propylgallate in 70% glycerol, 100 mM Tris-HCl [pH 9.0]) and kept dry at −20°C.

**Electron microscopy.** Cells were harvested by trypsinization or mechanical scraping, washed with PBS, and fixed in 0.1 mM sodium cacodylate-buffered fixative containing 3.0% (wt/vol) paraformaldehyde, 0.2% (vol/vol) glutaraldehyde, 1.0% (wt/vol) sucrose, and 3 mM $CaCl_2$ at 4°C for 2 h (26). Fixed samples were rinsed with sodium cacodylate buffer overnight, postfixed with 1.0% (wt/vol) $OsO_4$, en bloc stained with 2.5% (wt/vol) uranyl acetate, and then dehydrated with graded ethanol and embedded in Polybed 813 epoxy resin. Cells for immunogold electron microscopy were submitted to the same procedure except for the omission of osmication. Immunogold labeling was performed on 80- to 90-nm ultrathin sections collected on uncoated nickel grids with a three-step biotin antibiotin-gold conjugate method described previously (26). For ultrastructural localization of PrP, the rabbit antiserum RO73 (diluted 1:100) was used. Sections were subjected to denaturation of PrP with 3 M GdnSCN in 50 mM Tris-HCl, pH 7.4, for 10 min at room temperature and then rinsed with PBS

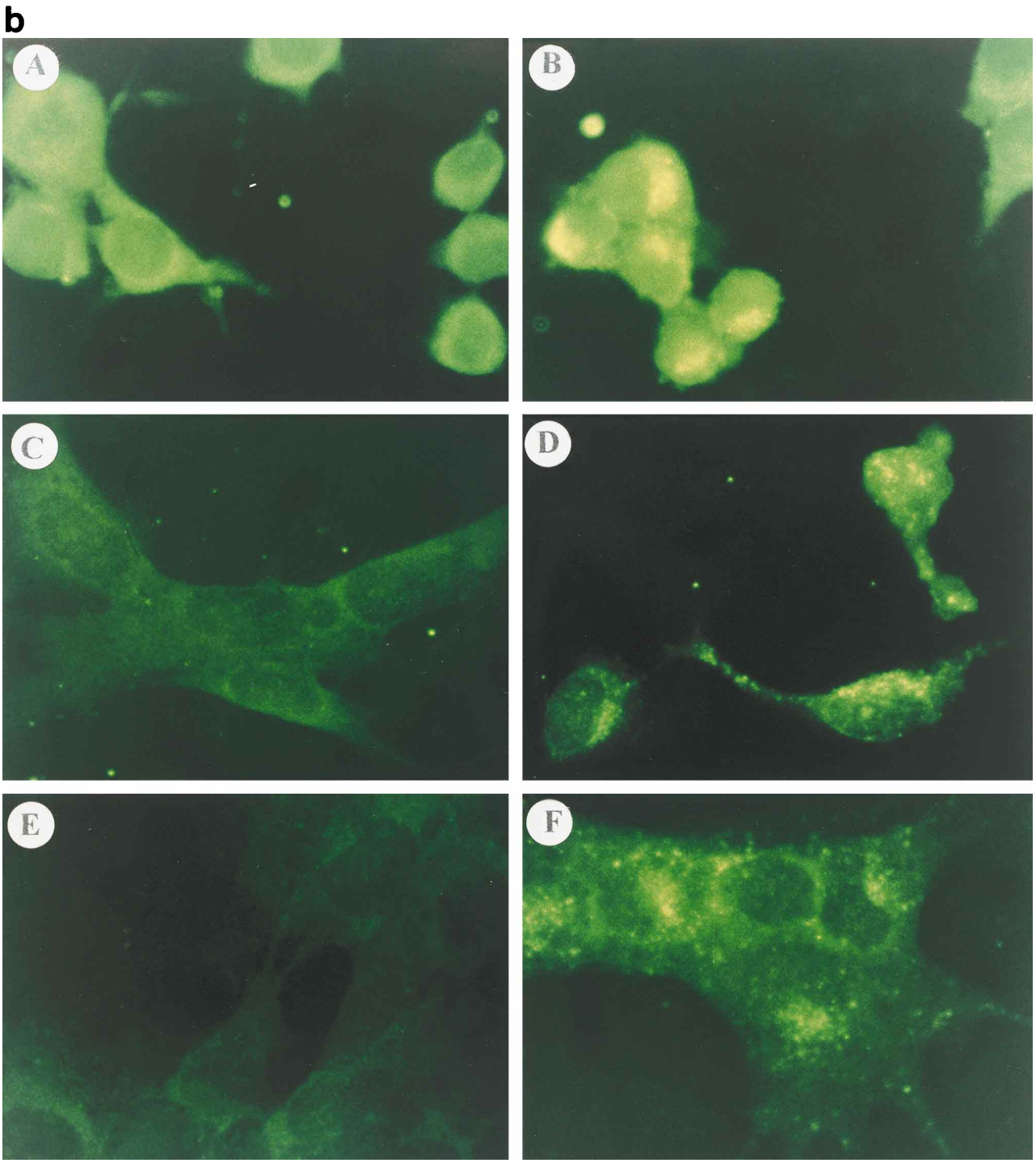


FIG. 1—*Continued.*

before antibody treatment. Control sections were treated identically except for the omission of primary antibody by substitution with nonimmune serum. Normal and immunolabeled sections were counterstained with uranyl acetate and lead citrate prior to ultrastructural analysis with a JEOL 100-CX electron microscope.

**RNA analysis.** Total cytosolic RNA was isolated by cell lysis in Nonidet P-40 (NP-40) buffer (10 mM Tris-HCl [pH 7.4], 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40) and nucleus removal. The RNA was analyzed on Northern blots exactly as described previously (45). All probes and hybridization conditions have been described previously (20, 64). Washes were done under high-stringency conditions in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 65°C. Blots were exposed to Kodak X-Omat AR films for 1 to 7 days.

**DNA fragmentation assay.** Low-molecular-weight DNA soluble in Triton X-100 was obtained according to the method of Newell et al. (33). Briefly, $10^7$ cells were washed twice with cold PBS and lysed in 1 ml of hypotonic lysis buffer for 5 min (5 mM Tris-HCl [pH 7.4], 5 mM EDTA, 0.5% Triton X-100). The lysates were centrifuged at 14,000 × *g* for 20 min. The supernatants were incubated with RNase (DNase free; Boehringer-Mannheim) at 50 μg/ml for 2 h at 37°C, deproteinized by extraction once in phenol-chloroform and once in chloroform-isoamylalcohol (24:1), and precipitated in 2.5 volumes of ethanol (96%; with 1/10

TABLE 1. ScGT1-trk cell equivalents bioassayed in CD-1 mice[a]

| No. of cells per inoculum | Expt A | | | Expt B | | | Expt C | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean incubation time to illness (days) | Mice | Log $ID_{50}/10^7$ cells | Mean incubation time to illness (days) | Mice | Log $ID_{50}/10^7$ cells | Mean incubation time to illness (days) | Mice | Log $ID_{50}/10^7$ cells |
| GT1-trk | | | | | | | | | |
| $10^6$ | | 0/5 | | | 0/5 | | | 0/5 | |
| ScGT1-trk | | | | | | | | | |
| $10^6$ | 140 | 5/5 | 5.1 | 142 | 5/5 | 4.9 | | NT[b] | |
| $10^5$ | 152 | 5/5 | 5.1 | 141 | 5/5 | 6.0 | 134 | 5/5 | 6.6 |
| $10^4$ | 166 | 5/5 | 5.0 | 145 | 5/5 | 6.7 | 144 | 5/5 | 6.8 |
| $10^3$ | 181 | 5/5 | 4.8 | 170 | 4/4 | 5.7 | 160 | 5/5 | 6.5 |
| $10^2$ | 184 | 4/5 | 5.4 | 173 | 4/4 | 6.5 | 177 | 5/5 | 6.2 |
| 10 | | NT | | | NT | | 188 | 4/5 | 6.3 |
| Mean titer[c] | | | 5.1 | | | 6.0 | | | 6.5 |

[a] Results of three independent experiments that were terminated after 300 days.
[b] NT, not tested.
[c] Mean titer of results of experiments A, B, and C was 5.8 log $ID_{50}/10^7$ cells.

volume 3 M sodium acetate). After centrifugation at 3,300 × *g* for 1 h, the pellets were washed in 70% ethanol and resuspended in 50 μl Tris-EDTA buffer (pH 7.5) supplemented with gel-loading buffer. An aliquot of 10 μl was subjected to electrophoresis on a 1.9% agarose gel and stained with ethidium bromide.

**Bioassays.** GT1-trk and ScGT1-trk cells were taken at various passages after primary infection (the earliest at 7 months after primary infection). Inocula from confluent cell cultures were washed twice in PBS, pelleted after trypsinization, and adjusted to $2 \times 10^8$ cells of inoculation buffer (5% bovine serum albumin in PBS) per ml. Cells were inactivated by five rounds of freeze-thawing ($N_2$ and 56°C water bath) under sterile conditions. Aliquots (100 μl) of this homogenate were diluted in 1 ml of inoculation buffer and then serially diluted 10-fold over a range of $2 \times 10^7$ to $2 \times 10^1$ cell equivalents per ml of inoculation buffer. For each sample, 30 μl was injected intracerebrally into five CD-1 Swiss mice (Charles River Laboratories). Positive control experiments done in parallel consisted of scrapie-infected mouse brain homogenate. Mice were diagnosed as having scrapie by clinical examination (8), and diagnosis was confirmed by histopathological examination including histoblots (53).

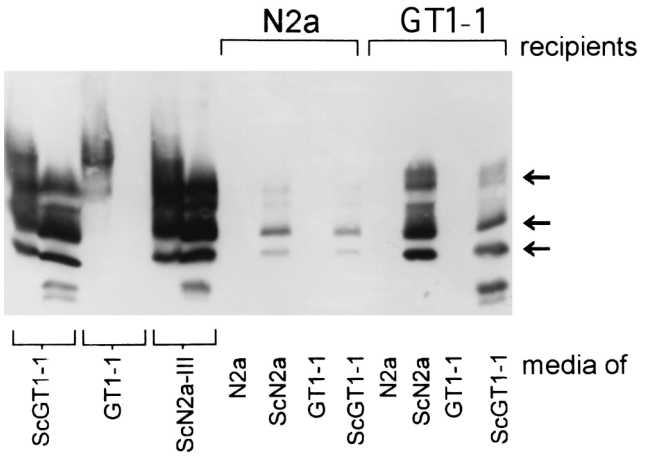


FIG. 2. Conditioned medium of prion-propagating cells can be used for infection of cultured cells. Immunoblot analysis of cells infected with cell-free medium. RO73 was used as antiserum, and Tricine peptide gels (16.5%) were used for higher separation. For this experiment supernatants were filtered through 25-μm-pore-size filters. Lanes 1 to 6 show donor cells without (lanes 1, 3, and 5) and with (lanes 2, 4, and 6) PK treatment (40 μg/ml for 1 h). Lanes 7 to 14 represent PK-treated cell lysates of recipient cells 6 weeks after infection. Lanes 7 to 10 were N2a cells as recipients, lanes 11 to 14 were GT1-trk cells. Donor cells for the conditioned media are indicated at the bottom. Conditioned medium from both infected cells was infective, but GT1-trk cells were better recipients. Note the differences in molecular size of PrP in GT1-trk cells infected with ScN2a- or ScGT1-trk-derived prions (especially lanes 12 and 14).

## RESULTS

**ScGT1-trk cells produce $PrP^{Sc}$.** To establish murine cell lines which persistently propagate prions, subclones of the hypothalamic neuronal cell line GT1 were inoculated with purified mouse RML prions (GT1-1, GT1-3, GT1-7). The *trkA*-transfected GT1-trk cells were studied most extensively

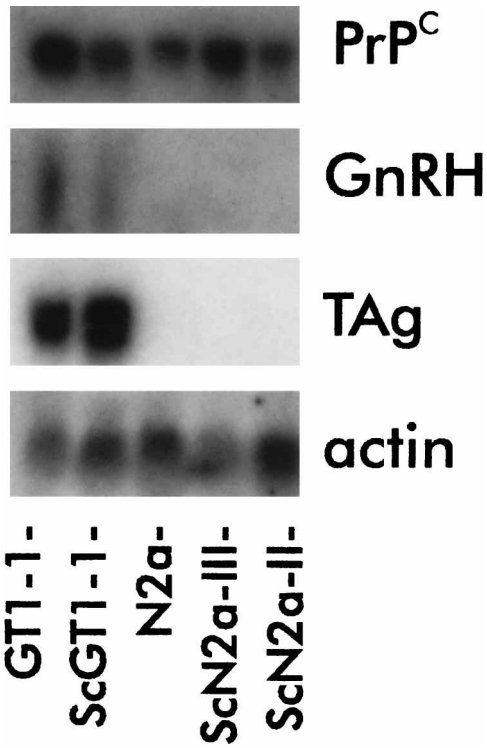


FIG. 3. Expression of selected genes in infected and parental cells. Northern blot analysis for PrP, GnRH, SV40 TAg, and actin. Equal amounts of total cytosolic RNA were loaded, separated, and transferred to nylon membranes. Lanes 1 and 2 contain GT1-trk and ScGT1-trk RNAs, respectively. Lanes 3, 4, and 5 contain RNAs from N2a, ScN2a-III, and ScN2a-II cells, which do not express GnRH or SV40 TAg. Actin expression was used as an internal control for loading. There were no significant differences between infected and uninfected cells.

TABLE 2. Viabilities of GT1-trk and ScGT1-trk cells and effect of NGF

| Cell line | % Viable cells by Trypan blue exclusion test | | | *P* value (*t* test) |
|---|---|---|---|---|
| | No NGF | NGF | NGF discontinuation | |
| Short-term treatment[a] | | | | |
| ScGT1-trk | | | | |
| Day 5 | 78 (75–81) | 82 (80–84) | | <0.01 |
| Day 10 | 76 (74–79) | 83 (81–86) | | <0.001 |
| After discontinuation | | | 75 (74–78) | <0.001 |
| GT-trk | | | | |
| Day 5 | 86 (85–89) | 87 (86–89) | | >0.05 |
| Day 10 | 89 (87–91) | 90 (88–91) | | >0.05 |
| After discontinuation | | | 87 (85–90) | >0.05 |
| Long-term treatment[b] | | | | |
| ScGT1-trk | 78 (74–81) | 85 (83–87) | | <0.05 |
| After discontinuation | | | 76 (73–78) | <0.01 |
| GT1-trk | 89 (86–91) | 90 (88–92) | | >0.05 |
| After discontinuation | | | 87 (85–88) | >0.05 |

[a] Results of three independent experiments ($n = 6$); 100 ng of NGF per ml for 5 or 10 days; discontinuation of NGF for 10 days after 10 days of treatment.
[b] Results of three independent experiments ($n = 3$); 20 to 100 ng of NGF per ml for over 3 months; discontinuation of NGF for 4 weeks after 3 months of treatment.

(previously labeled GT1-1-trk9 [64]). Three scrapie-infected cell lines named ScGT1-trk were derived by treating GT1-trk cells with 100 ng of NGF/ml for 4 days prior to infection. Another scrapie-infected cell line (ScGT1-trk) was derived in the absence of NGF. The ScGT1-trk cells exhibited diminished viability and altered morphology. All four ScGT1-trk cell lines expressed $PrP^{Sc}$ in similar amounts and showed similar changes in morphology and viability; these alterations seemed to be constant over a period of 18 months. Scrapie-infected GT1-trk cells exhibited three bands on immunoblots which were resistant to PK digestion that were also found in ScN2a cells (data not shown). These PrP 27-30 molecules exist as diglycosylated, monoglycosylated, and unglycosylated polypeptides. ScGT1-trk cells processed several months after the cells used for the initial experiments showed similar amounts of $PrP^{Sc}$ (data not shown). Subcloning of the ScGT1-1 cells did not produce any cell lines with increased levels of $PrP^{Sc}$.

To discriminate between de novo-generated $PrP^{Sc}$ and traces of $PrP^{Sc}$ in the inoculum, metabolic labeling was performed, followed by immunoprecipitation (Fig. 1a). The lysates were incubated with PK prior to immunoprecipitation. The ScGT1-trk cells as well as the ScN2a-II and ScN2a-III cells showed radiolabeling patterns typical of $PrP^{Sc}$ (Fig. 1a, three bands marked by arrows).

Indirect immunofluorescence analysis combined with GdnSCN denaturation demonstrated the intracellular accumulation of $PrP^{Sc}$ as evidenced by the intense fluorescence signals in the cytoplasm of ScN2a (Fig. 1b, panel B) (56) and ScGT1-trk (panels D and F) cells. Uninfected controls showed no evidence of $PrP^{Sc}$ (panels A, C, and E). The immunofluorescence staining in ScGT1-trk cells was more punctate than that found in ScN2a cells. Treatment of ScGT1-trk cells with NGF seemed to diminish the $PrP^{Sc}$ signal in many of the cells (panel F).

**Bioassays of ScGT1-trk cells in mice.** GT1-trk and ScGT1-trk cells were collected in three independent experiments after many passages and bioassayed for scrapie prions in CD-1 Swiss mice (Table 1). Similar prion titers were obtained whether the inoculum contained $10^6$ or $10^2$ ScGT1-trk cells. None of the mice inoculated with GT1 cells developed scrapie. ScGT1-trk cells passaged ~25 times had a titer of $10^{5.1}$ $ID_{50}$ units/$10^7$ cells, those passaged ~40 times had a titer of $10^{6.0}$ $ID_{50}$ units/$10^7$ cells, and those passaged ~55 times had a titer of $10^{6.5}$ $ID_{50}$ units/$10^7$ cells. These findings indicate that prion infectivity after numerous cell passages is produced de novo and is not the result of the inoculum.

**Conditioned medium from cultured ScGT1-trk cells is infectious.** To test whether cell-free supernatants of infected cultures contain prions, we harvested conditioned medium from confluent cell cultures after 24 h. After separation of the ScGT1-trk cells from the medium by centrifugation, the supernatant fractions were applied overnight to uninfected GT1-trk or N2a cells (see Materials and Methods). After 6 to 8 weeks of passaging, the cells were analyzed for $PrP^{Sc}$ (Fig. 2). GT1-trk cells exposed to conditioned medium harvested from ScGT1-trk cells produced $PrP^{Sc}$ as demonstrated by the presence of PK-resistant PrP in cell extracts. Eight of 10 attempts to transfer prion infectivity with cultured medium were positive as assayed by immunoblotting after 8 weeks. Our findings indicate that prions can spread from cell to cell and may provide new insights into the spread of prions in the CNS of humans and animals.

**Gene expression in prion-infected cells.** Given the possibility that, during or after infection, cells were selected that genetically differed from control cells, we compared levels of expression of genes known to be expressed in the parent cells. The levels of mRNAs encoding PrP, GnRH, SV40 TAg, and actin were similar for GT1-trk and ScGT1-trk cells (Fig. 3). In addition, the levels of mRNAs encoding the *trkA* receptor and superoxide dismutase were similar in GT1-trk and ScGT1-trk cells (data not shown). Similar levels of $PrP^{C}$, SV40 TAg, and neuron-specific enolase were found by immunoblotting, and distribution of GnRH peptides in GT1-trk and ScGT1-trk cells were similar as judged by immunofluorescence assays (data not shown). These data demonstrate that infected and parental GT1-trk cells did not differ in the expression of a variety of genes, which argues that a major selection event did not result from prion infection.

**Cell death due to prions could be prevented by NGF.** The viability of persistently infected ScGT1-trk cells was assessed by Trypan blue exclusion over a period of 12 months. Approximately 85% of uninfected control GT1-trk cells were viable compared to ~75% of infected ScGT1-trk cells ($P < 0.001$) (Table 2). Scrapie infection not only reduced the viability of

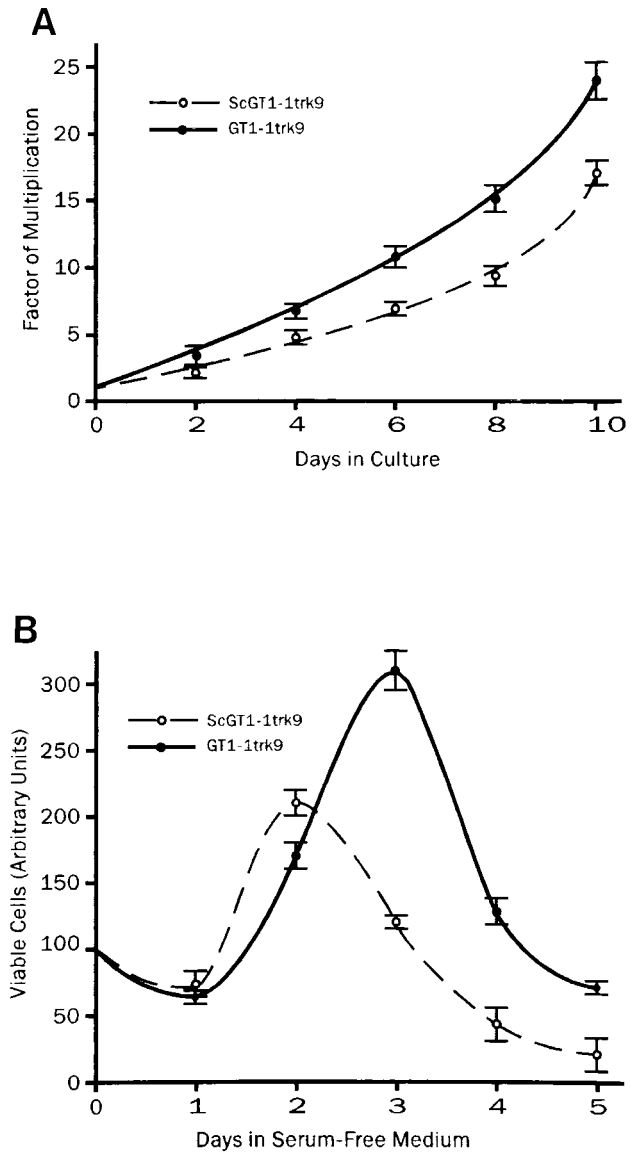


FIG. 4. Effect of scrapie infection on proliferation of cells in serum-containing medium and on survival in serum-free medium. (A) GT1-trk (closed circles) and ScGT1-trk (open circles) cells were grown for the indicated periods in serum-containing medium. Number of living cells was determined and is indicated as multiplication factor ($n = 2$). (B) Cells were maintained in serum-containing medium until day zero. Cultures were then washed three times with serum-free medium and maintained in the same. The number of viable cells was determined at each time point (arbitrary units; $n = 2$).

these cells but also diminished their proliferation. The doubling time for GT1-trk cells in normal medium was between 1.5 to 2 days and that for ScGT1-trk cells was between 2 to 2.5 days (Fig. 4A). We also compared the survival of the scrapie-infected and control cells in serum-free medium. About 70% of GT1-trk cells were still alive and viable after 5 days, whereas only 20% of the prion-infected cells were alive after this period of time ($P < 0.01$) (Fig. 4B). In the experiment shown, it appears that remaining traces of FCS allowed cell proliferation for a few days after the medium was changed. This pattern was reproducible but was not observed when cells were split directly into FCS-free medium.

When ScGT1-trk cells were treated with NGF for 5 to 10 days, their viability was increased (Table 2, short-term treatment). After discontinuing NGF treatment, the viability of the ScGT1-trk cells decreased to the levels found for the untreated group. Treatment of ScGT1-trk cells with NGF for approximately 3 months improved viability to a level approaching that of uninfected GT1-trk cells (Table 2, long-term treatment). NGF was protective also for ScGT1-trk cells when viability was tested in serum-free medium (data not shown). The effect of NGF on uninfected control cells was only marginal. These findings indicate that the protective effects of NGF are readily reversible.

**Neurodegeneration in ScGT1-trk cells.** Within a few weeks after inoculation with prions, the ScGT1-trk cells showed alterations in their morphology (Fig. 5). The ScGT1-trk cells grew in clusters in which some of the cells no longer formed a monolayer; the morphology of the individual ScGT1-trk cells was difficult to evaluate because of these clusters (Fig. 5B). Interestingly, Trypan blue staining revealed that the majority of cells in the clusters were still viable. These changes stand in striking contrast to the morphology of the GT1-trk cells which also grew in clusters but formed uniform monolayers (Fig. 5A). Treatment of the ScGT1-trk cells with NGF for 48 h reversed the morphologic changes and yielded cells that resembled the control GT1-trk cells (Fig. 5C).

To assess the differences in morphology and viability observed between infected and control cells at the ultrastructural level, GT1-trk and ScGT1-trk cells were compared at three time points during a 7-month period. The GT1-trk cells possessed a normal heterochromatic nucleus, a well-developed Golgi system, intact mitochondria, small secretory granules, and regularly sized dense lysosomes (Fig. 6A). In contrast, a substantial proportion of the ScGT1-trk cells exhibited cytopathologic changes, including numerous autophagic vacuoles, large secondary lysosomes containing myelin-like membrane structures, and many giant "swollen" vacuoles (Fig. 6B). Less frequently, clumping of dense chromatin and nuclear pyknosis were found. These ultrastructural changes resemble a subtype of alterations seen in cells undergoing apoptotic cell death (12, 49). Immunogold electron microscopy showed an intense accumulation of immunoreactive material typical of $PrP^{Sc}$ in endosome-related structures and secondary lysosomes (26) (Fig. 6C). Morphometric analysis revealed that in ~22% of ScGT1-trk cells vacuolation was detectable compared to ~2% for control cells (Table 3). Similarly, signs of autophagy with giant dense bodies as well as chromatin condensation and nuclear fragmentation which correlate with apoptosis were significantly increased in ScGT1-trk cells compared to uninfected controls (Table 3). There was also a striking increase in autophagy over time.

Preliminary data suggest that after treatment with NGF for 2 to 3 months, the ultrastructural changes, including nuclear condensation and fragmentation and autolysosomal structures in the ScGT1-trk cells, were substantially reduced (Fig. 6D). Overall, the effects of NGF on viability and morphology were paralleled by a significant reduction of the ultrastructural alterations observed in ScGT1-trk cells.

**Apoptosis in ScGT1-trk cells is reversed by NGF.** DNA fragmentation, which is a hallmark of apoptosis, was a constant feature of ScGT1-trk cells (Fig. 7). Oligomeric DNA fragments of about 180 nucleotides (nt) in length are indicative of internucleosomal DNA degeneration (16, 63). No signal was visible in extracts from untreated or NGF-treated GT1-trk, N2a, or ScN2a cells (data not shown). Interestingly, NGF diminished the DNA fragmentation. Treatment of confluent ScGT1-trk cells with 100 ng of NGF per ml for 24 h substantially reduced DNA fragmentation (Fig. 7, lanes 2 and 3), whereas in un-

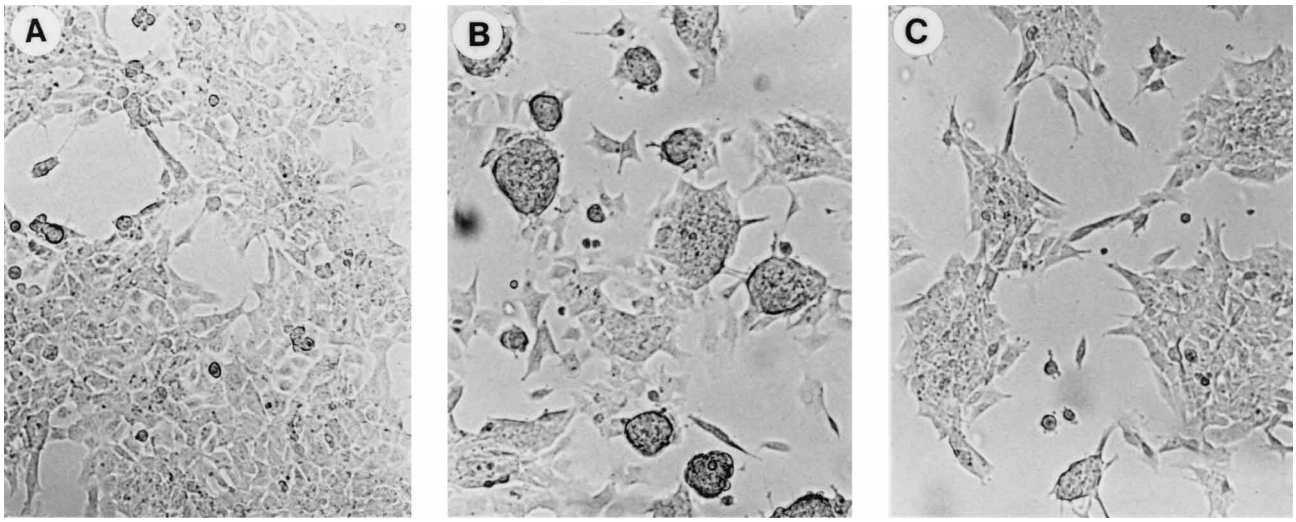


FIG. 5. Cell morphology of GT1-trk (A) and ScGT1-trk cells (B); morphology of GT1-trk and ScGT1-trk cells at light microscopic level. A marked difference in morphology is apparent. Scrapie-infected cells seem to grow in clusters, and the structures of individual cells are difficult to see. (C) NGF treatment of ScGT1-trk cells for 48 h (100 ng/ml) resulted in a reversion of the phenotype to a morphology resembling that of parental cells.

treated cells, the monomeric form of this cleavage process produced a band containing 170 to 180 nt. A concentration of 100 ng of NGF per ml administered with each medium change over 2 to 3 months virtually abolished the DNA laddering found in the ScGT1-trk cells (Fig. 7, lanes 5 and 6). NGF at a concentration of 20 ng/ml reduced DNA laddering by less (Fig. 7, lanes 7 and 8) than that found at the higher concentration. When NGF treatment was stopped, DNA fragmentation reappeared at levels comparable to that of untreated ScGT1-trk cells (Fig. 7, lanes 11 and 12).

To assess the frequency of apoptosis in ScGT1-trk cells, we examined multiple cell clones for morphology, viability, and DNA fragmentation (Table 4). Of 23 attempts to infect GT1 cells (GT1-trk and untransfected GT1-1, GT1-3, and GT1-7 subclones), 16 positive infections were found as assayed for the presence of $PrP^{Sc}$ on immunoblots after at least 12 passages. Altered morphology and viability together with DNA fragmentation were found in 7 of 16 clones (Table 4). Of 12 positive infections using GT1-trk cells, 4 showed alterations characteristic of apoptosis. Of note, these apoptotic changes were usually detectable in cells which exhibited cytopathology early after prion infection. In contrast to results for ScGT1-trk cells, neither cytopathologic alterations nor DNA fragmentation was detected in ScN2a cells.

## DISCUSSION

Prion-infected mouse neuroblastoma cells have provided a system for dissecting many aspects of prion biology. In a search for other cell lines that could be infected with prions, we examined a variety of cultured cells, including several of glial origin. A recently developed neuronal cell line responsive to NGF seemed to be a particularly attractive cell line for prion infection since NGF had been shown to cause such profound changes in cell metabolism (64). GT1 cells are GnRH-secreting neurons, generated by targeted tumorigenesis in transgenic mice, using immortalization with SV40 TAg under the control of the GnRH promoter (31). A subclone was stably transfected with the *trkA* gene (GT1-trk, previously named GT1-1-trk9) (64). GT1-trk cells are responsive to NGF and react with enhanced neuronal differentiation. Interestingly, these cells still divide upon NGF treatment. We found that GT1 cells can be persistently infected with scrapie prions. GT1-trk as well as other GT1 subclones are highly susceptible for propagation of prions. In sharp contrast to the widely used N2a/ScN2a system, we observed phenotypic alterations in a substantial proportion of infection experiments.

Here we describe the long-term follow-up of one of these cell populations (ScGT1-trk), which provided a number of unprecedented features. First, cytopathological changes were produced for the first time in a cell line persistently infected with prions. The changes closely mimicked the spongiform degeneration seen in vivo in prion diseases. Second, the form of cell death described here closely resembles an autophagic subtype of apoptosis that to our knowledge has not been described in vitro. Third, there was release of prion infectivity into the culture supernatant, a result that differs from earlier cell line studies (7, 40, 42). Finally, NGF significantly reduced the morphological and biochemical features of autophagic cell death and improved cellular viability. ScGT1-trk cells might therefore represent an unprecedented cell culture model for the study of neurodegeneration in prion diseases and of the potential interference of neurodegeneration with neurotropic factors.

**CNS cells propagating prions with phenotypic alterations.** The hypothalamic ScGT1-trk cells represent the first prion-infected cell line derived from CNS cells. From histopathologic studies, the hypothalamus is known to contain high levels of $PrP^{Sc}$. Establishment of prion-propagating cell lines proved to be extremely difficult, and loss of prion production was observed in several cell culture systems (11, 28, 56). It is not known what is responsible for this restriction of infectivity and prion propagation in vitro although in vivo many cells of the brain seem to propagate infectious prions. In contrast to the scrapie-infected PC-12 and ScN2a cells, the ScGT1-trk cells showed marked morphologic alterations presumably caused by prion infection. The viability, proliferation rate, and survival in serum-free medium of ScGT1-trk cells were reduced compared to those of parental GT1-trk cells. A comparison of ScN2a and uninfected N2a cells did not reveal any such differences. These findings argue that GT1-trk cells respond differently to prion infection than do N2a cells.

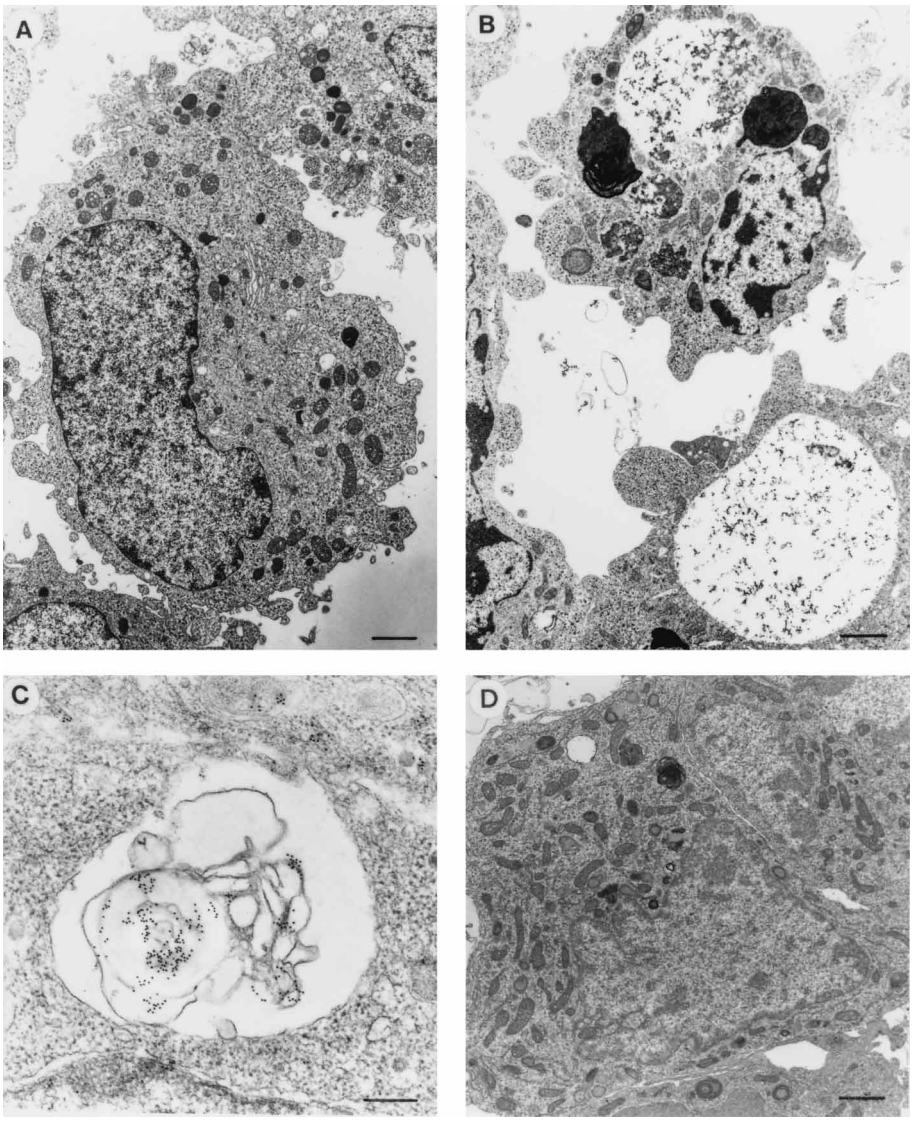


FIG. 6. Ultrastructural signs of autophagic vacuolation and apoptosis in ScGT1-trk cells. (A) An uninfected (GT1-trk) cell showing a normal heterochromatic nucleus, well-developed Golgi system, intact mitochondria, small secretory granules, and regular-size dense lysosomes. (B) Electron micrograph demonstrates typical cytopathologic changes in ScGT1-trk cells (present in about 20% of cells). Note the cytoplasmic accumulation of autophagic vacuoles representing different stages of degeneration, giant, swollen vacuoles, and peripheral clumping of dense chromatin in the nucleus. Bars in panels A, B, and D, 1 μm. (C) Immunogold particles corresponding to $PrP^{Sc}$ show significant accumulation in a giant secondary lysosome containing membrane remnants. Bar, 0.2 μm. (D) Long-term NGF treatment reduced the ultrastructural features of autophagic apoptotic cell death in ScGT1-trk cells. Compared to untreated ScGT1-trk cells (panel B), the extent of heterochromatin condensation and nuclei fragmentation was much reduced. Autophagocytosis was still present, but lysosomal structures were significantly reduced in size.

**Features of autophagic vacuolation and apoptosis.** Neuronal cell death and vacuolation are in vivo hallmarks of spongiform degeneration in prion diseases. How neurons degenerate in these diseases is unknown, but it has been suggested that apoptosis may feature in this process (1, 22, 36). Observations that treatment of certain CNS-cultured cells with peptides corresponding to regions of Aβ and PrP results in apoptotic cell death (5, 6, 15, 27) suggest that this form of cell death may be relevant to neurodegeneration. Our ScGT1-trk cells exhibit both the ultrastructural and biochemical features of the autophagic subtype of apoptotic cell death, closely mimicking that in vivo. Autophagy is an important process in the intracellular turnover of proteins and organelles. It is known to occur in normal cells (e.g., neurons), but the autophagic lysosomal compartment is markedly increased in cells undergoing involution in vertebrates and invertebrates (23). The autophagic giant vacuoles in ScGT1-trk cells are likely to represent an extreme form of autophagocytosis and may play a role in cellular destruction by causing lysosomal rupture and neuronal death (12). It is important that only infected cells show this autophagic phenotype. Furthermore, the ultrastructural plasticity of the neurosecretory ScGT1-trk cells might facilitate the analysis of individual stages of pathological changes as well as prion protein trafficking and the cellular compartments involved in the conversion of $PrP^{C}$ into $PrP^{Sc}$.

**NGF and apoptosis.** Although neuronal cell death is one of the pathologic hallmarks of prion infection in vivo, there is limited insight into the molecular basis of neuronal cell death (36). Wyllie and colleagues have pointed to two distinct morphologies that characterize dying cells: necrosis and apoptosis

TABLE 3. Ultrastructural signs of neurodegeneration by electron microscopy

| Cells and mo after infection | Vacuolation | | Intense autophagy | | Giant dense bodies | | Chromatin condensation and/or nuclear fragmentation | |
|---|---|---|---|---|---|---|---|---|
| | No. positive cells/ no. cells analyzed | % | No. positive cells/ no. cells analyzed | % | No. positive cells/ no. cells analyzed | % | No. positive cells/ no. cells analyzed | % |
| GT1-trk | | | | | | | | |
| 5 | 5/333 | 1.5 | 23/333 | 6.9 | 4/333 | 1.2 | 3/333 | 0.9 |
| 7 | 7/333 | 2.1 | 27/333 | 8.1 | 6/333 | 1.8 | 5/333 | 1.5 |
| 11 | 6/334 | 1.8 | 29/334 | 8.7 | 6/334 | 1.8 | 4/334 | 1.2 |
| Mean | | <2 | | 8.0 | | <2 | | <2 |
| ScGT1-trk | | | | | | | | |
| 5 | 65/333 | 19.5 | 107/333 | 32.1 | 63/333 | 18.9 | 42/333 | 12.6 |
| 7 | 81/333 | 24.3 | 134/333 | 40.2 | 76/333 | 22.8 | 47/333 | 14.1 |
| 11 | 77/334 | 23.0 | 161/334 | 48.2 | 98/334 | 29.3 | 45/334 | 13.5 |
| Mean | | 22.0 | | 40.0 | | 23.7 | | 13.4 |
| *P* | | <0.001 | | <0.01 | | <0.01 | | <0.001 |

(63). Apoptosis is present in normal development and during tissue homeostasis including neuronal development (12, 41, 49, 58, 63); it is a regulated process and is characterized by certain biochemical hallmarks (16, 63). It has been hypothesized that apoptotic cell death may play a role in the pathogenesis of neurodegenerative diseases (1). In vivo detection of apoptosis is difficult, since apoptotic cells are rapidly removed via phagocytosis. However, DNA fragmentation in vivo has been found in scrapie-infected sheep brains (14). Some specific insults have been associated with apoptotic cell death in cultured neurons (15, 21, 22, 27, 29, 49).

NGF is known from a number of in vivo and in vitro models to be neuroprotective (4, 13, 18, 19, 50). We had already shown that survival of parental GT1-trk cells in serum-free medium is improved with NGF (64); it was then reasonable to look for similar protective effects of NGF in scrapie prion-infected GT1-trk cells. Application of NGF dramatically reduced neurodegeneration. Long-term NGF application improved viability to levels found for parental cells, reverted the morphology to a phenotype similar to that of uninfected GT1-trk cells, and caused changes with ultrastructural and biochemical features of apoptosis. Internucleosomal DNA cleavage was barely detected, the ultrastructure of the nuclei was drastically improved, and the degree of autophagocytosis was reduced. The degree of reduction of DNA fragmentation was found to be dependent on the permanent presence of high amounts of NGF.

It was possible that we selected for a subpopulation of ScGT1-trk cells during the long-term NGF experiments and this was responsible for the observed changes. To test this hypothesis, we stopped NGF treatment and looked for reversibility of a number of the alterations. Indeed, viability decreased to the levels of untreated cells, and DNA fragmentation again significantly increased. These findings argue that the protective effects of NGF were not due to selection of a resistant subpopulation of cells and that the effects of NGF are reversible. Based on the experimental results reported here, it seems that NGF might provide a molecular switch that regulates diverse features of prion pathogenesis.

**Neurotropic factors and therapeutics in prion diseases.** Several experimental model systems for Alzheimer's disease showed that elevated levels of neurotropic factors like NGF can restore a normal neuronal phenotype of certain vulnerable

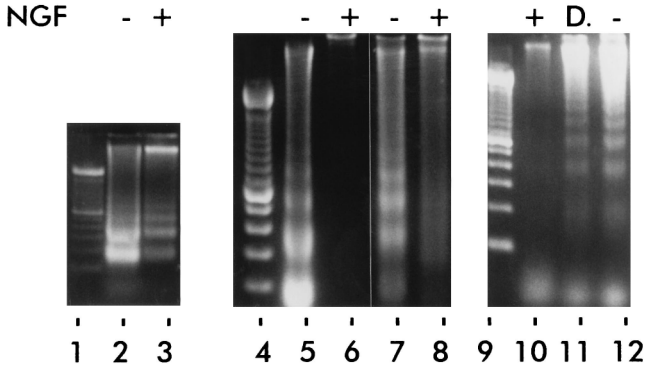


FIG. 7. Effect of NGF on DNA fragmentation in ScGT1-trk cells. Cytosolic DNA corresponding to $2 \times 10^6$ cells per lane was loaded on 1.9% agarose gels and stained with ethidium bromide. The 100-bp ladders are shown as molecular size markers (lanes 1, 4, and 9). NGF was added at 100 ng/ml for 24 h to confluent cells. Lane 2 shows the DNA ladder of non-NGF-treated cells. Notably, the monomeric form of the DNA fragment is pronounced (about 170 to 180 nt). After NGF treatment, this form was reduced to a more uniform DNA ladder, and high molecular bands were enriched (lane 3). After long-term NGF treatment (freshly added with each medium change over 2 to 3 months), DNA fragmentation was almost undetectable (lane 5 versus lane 6 and lane 10 versus lane 12). When the NGF concentration in the medium was decreased from 100 to 20 ng/ml and medium was changed less frequently, the effect was less marked but still present (lanes 7 and 8). The effect of discontinuation of NGF treatment for 3 weeks is shown in lane 11. DNA fragmentation was detectable at levels comparable to that of untreated cells (lane 12), which indicates NGF actions are reversible. DNA fragmentation was not detected in untreated or NGF-treated GT1, N2a, or ScN2a cells (data not shown).

TABLE 4. Rate of positive prion infections, DNA fragmentation, and cell viability

| Cell line | Positive inoculations[a] | | DNA fragmentation | | Reduced viability[b] | |
|---|---|---|---|---|---|---|
| | No. | % | No. | % | No. | % |
| GT[c] | 16/23 | 70 | 7/16 | 44 | 8/16 | 50 |
| GT1-trk[d] | 12/14 | 86 | 4/12 | 33 | 5/12 | 42 |
| N2a | 2/4 | 50 | 0/0 | 0 | 0/0 | 0 |

[a] Using purified mouse RML prion preparations; PK-resistant PrP after 3 months.

[b] More than 5% reduction in infected cell populations compared to uninfected cells.

[c] GT1-1 (3), GT1-3 (3), GT1-7 (3), and GT1-trk (14) subclones.

[d] 14 GT1-trk subclones out of a total of 23 GT cell lines (line 1) assayed.

neuronal populations. Therefore, NGF and other neurotropic factors might be useful in preventing CNS dysfunction and death of neurons in neurodegenerative diseases (32). Although long-term treatment with high NGF concentrations of ScGT1-trk cells was able to normalize ultrastructural and biochemical features of neurodegeneration, it did not bring about the loss of $PrP^{Sc}$ (data not shown). Furthermore, the restricted pattern of *trkA* expression in the CNS argues that tropic factors in addition to NGF would be needed to abrogate the neurodegeneration found in prion diseases.

The results presented here which mimic closely some hallmarks of neurodegeneration observed in vivo together with features of apoptotic cell death describe a new cell culture model for studies of prion diseases. Understanding the molecular processes responsible for neurodegeneration and the mechanisms underlying the cellular dysfunction in the prion diseases might facilitate the development of effective therapeutic approaches. With the possible spread of prions from cattle with bovine spongiform encephalopathy to humans (10, 61), development of effective therapies has taken on a new urgency. Furthermore, the ability to detect carriers of lethal mutations of the PrP gene decades in advance of the onset of CNS dysfunction disease also makes development of an effective therapy for these illnesses an important goal.

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