A neurodegenerative disease mutation that accelerates the clearance of apoptotic cells

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Frontotemporal lobar degeneration is a progressive neurodegenerative syndrome that is the second most common cause of early-onset dementia. Mutations in the progranulin gene are a major cause of familial frontotemporal lobar degeneration [Baker M, et al. (2006) *Nature* 442:916–919 and Cruts M, et al. (2006) *Nature* 442:920–924]. Although progranulin is involved in wound healing, inflammation, and tumor growth, its role in the nervous system and the mechanism by which insufficient levels result in neurodegeneration are poorly understood [Eriksen and Mackenzie (2008) *J Neurochem* 104:287–297]. We have characterized the normal function of progranulin in the nematode *Caenorhabditis elegans*. We found that mutants lacking *pgrn-1* appear grossly normal, but exhibit fewer apoptotic cell corpses during development. This reduction in corpse number is not caused by reduced apoptosis, but instead by more rapid clearance of dying cells. Likewise, we found that macrophages cultured from progranulin KO mice displayed enhanced rates of apoptotic-cell phagocytosis. Although most neurodegenerative diseases are thought to be caused by the toxic effects of aggregated proteins, our findings suggest that susceptibility to neurodegeneration may be increased by a change in the kinetics of programmed cell death. We propose that cells that might otherwise recover from damage or injury are destroyed in progranulin mutants, which in turn facilitates disease progression.

Alzheimer's disease | apoptosis

Frontotemporal lobar degeneration (FTLD) comprises a group of neurodegenerative syndromes that are characterized by behavioral changes, progressive aphasia, and/or semantic language deficits (1). Pathologically, the syndromes are characterized by frontal and temporal lobe atrophy and by intraneuronal deposition of aggregated tau, TDP-43, or FUS proteins (2–4). Several genetic mutations have been associated with the development of FTLD, including mutations affecting *MAPT*, *VCP*, *CHMP2B*, *FUS*, and *progranulin* (2, 5–11).

Progranulin mutations are present in 5% to 10% of all patients with FTLD (11, 12). Unlike mutations affecting tau, APP, α -synuclein, and huntingtin, which result in decreased protein solubility and formation of toxic oligomers (13), FTLD-associated progranulin mutations are not gain-of-function mutations, and progranulin itself is not found in neuronal inclusions. Instead, disease-associated progranulin mutations result in nonsense-mediated decay of progranulin mRNA or an inability to secrete the mutant protein (14). Individuals with these mutations exhibit less than half the normal blood and cerebrospinal fluid levels of progranulin (15–17). As the disease phenotype is inherited in an autosomal-dominant fashion, progranulin mutations appear to result in haploinsufficiency. In addition, variations in the human progranulin gene may also increase the risk for developing ALS, Alzheimer's disease, and Parkinson disease (18–21).

Progranulin (also known as GRN, acrogranin, proepithelin, granulin-epithelin precursor, and PC cell-derived growth factor) is a highly conserved glycoprotein involved in a variety of biological processes, including embryogenesis, inflammation, and

wound healing (22). Increased progranulin secretion is associated with more aggressive forms of breast, brain, renal, and other tumors (22). Progranulin is secreted by many cell types including neurons and glia, and can be cleaved into 6-kDa cysteine-rich granulins whose functions are unclear, although they may oppose the activity of the proprotein (23, 24). In cultured neurons and neuronal cell lines, progranulin knockdown can adversely affect neurite outgrowth and long-term neuronal survival under serum-deprived conditions (25, 26).

The toxic insults to neurons that ultimately result in neuronal death and neurodegeneration in humans are generally thought to occur in a cell-autonomous fashion, although evidence is accumulating that microglia, the resident phagocytic and immune effector cells of the CNS, may be involved in neuronal killing (27). Here, by using *Caenorhabditis elegans* and ex vivo macrophage studies, we describe an unexpected role for progranulin. We find that loss of progranulin accelerates the clearance of apoptotic cells in both worms and mammals. Others have shown that the apoptotic cell-clearance machinery can remove cells that are dying but not yet dead, raising the possibility that loss of progranulin causes damaged neurons to be engulfed before they can be repaired.

Results

***C. elegans* PGRN-1 Protein Is Likely Secreted from Neurons and Intestine.** Many human disease proteins have conserved functions in invertebrates. Because of this, and because of the many molecular, genetic, and cell biological approaches available in *C. elegans*, we chose to study the function of progranulin in the nematode. The *C. elegans* progranulin gene (*pgrn-1*) encodes a protein with three predicted granulin domains, compared with the 7.5 granulin domains in human progranulin (Fig. S1). We generated a transgenic *C. elegans* line expressing a fluorescent mCherry protein that acts as a transcriptional reporter for *pgrn-1* expression. In developing *C. elegans* larvae, mCherry protein was present in intestinal cells and select neurons (Fig. 1A–F and Fig. S2) and was excluded from muscle cells in a pattern reminiscent of human progranulin expression (23). We tracked the timing of progranulin expression throughout development. *C. elegans* embryos are laid as eggs, hatch, and proceed through four larval stages to adulthood. The total time from embryo to adulthood takes approximately 3 d. The mCherry transcriptional reporter was first visible in the embryonic intestinal precursor cells at

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Fig. 1. Progranulin expression in the intestine and neurons begins during development. Light (Left) and fluorescent (Right) images of animals expressing a fluorescent mCherry transcriptional reporter ($P_{pgrn-1}::pgrn-1::polycistronic\ mCherry$) (A–F) or a translational RFP reporter ($P_{pgrn-1}::pgrn-1::RFP$) (G–J). (A and B) Confocal images of representative mixed-stage embryos demonstrate that progranulin expression begins at approximately 180 min after the first cleavage event in embryogenesis (at approximately the time programmed cell death begins). Left embryo is at approximately 150 min of development and displays no fluorescent signal. Center embryo is at approximately 180 min of development, during early to mid-gastrulation when intestinal precursor cells are seen clearly in the interior of the embryo. These four intestinal precursor cells contain the mCherry fluorescent protein (dashed line). Right: Comma-stage embryo (~390 min) demonstrates strong mCherry expression (See also Fig. S3 A and B). (C–F) Nomarski and fluorescent micrographs show a representative late larval (L4) animal producing PGRN-1 in the intestine (C and D) and select neurons (E and F). (G–J) Nomarski and fluorescent micrographs of a comma-stage animal (G and H) and day 1 adult animal (I and J) expressing a PGRN-1::RFP translational reporter show the protein diffusing throughout the animal. Arrowheads mark a refractile corpse (G and H) and secreted PGRN-1::RFP taken up by coelomocytes (I and J). (Scale bars: A, B, E–J, 10 μ m; C and D, 100 μ m.)

midgastrulation stage approximately 180 min after the first embryonic cleavage (Fig. 1 A and B and Fig. S3 A and B). We observed neuronal mCherry expression in L1 larvae, and neuronal and intestinal mCherry expression persisted throughout larval development and into adulthood (Fig. 1 C–F).

We also generated a translational reporter with an RFP fluorescent tag fused to PGRN-1. In contrast to the transcriptional reporter, this PGRN-1::RFP fusion protein was not found in discrete tissues. Instead, it was visible in what appeared to be intestinal organelles, in the pseudocoelomic cavity, and in scavenging coelomocytes, suggesting that, as in mammals (14), progranulin is secreted (Fig. 1 G–J and Fig. S3 C–F).

Progranulin Loss-of-Function Mutant Has a Normal Lifespan. The *pgrn-1(tm985)* allele contains a 347-bp deletion encompassing part of the progranulin promoter, all of exon 1, and part of the first intron (Fig. S1). Animals carrying this mutation did not produce progranulin mRNA as assayed by quantitative RT-PCR (Fig. S4A). Thus, *pgrn-1(tm985)* is likely to be a null allele. *pgrn-1* mutants appeared grossly normal, and both the homozygous and heterozygous mutants exhibited a normal lifespan (Fig. S4B and Tables S1 and S2). Likewise, overexpression of progranulin did not significantly alter lifespan (Fig. S4B and Table S3). However, the homozygous mutant had approximately 20% reduction in total number of progeny (Fig. S4C).

Programmed Cell Death Is Altered in *pgrn-1* Mutants. *C. elegans* development is characterized by an invariant pattern of cell division and cell death (28). The initiation of PGRN-1 production and secretion coincided temporally with the first programmed cell deaths in *C. elegans* (Fig. 1 A and B). Because of this temporal link between PGRN-1 production and the onset of programmed cell death, and because progranulin haploinsufficiency has been linked to human neurodegenerative disease, we investigated whether *pgrn-1* mutants exhibited defective regulation of programmed cell death. In developing nematodes and the adult nematode germ line, cells dying via the process of apoptosis can be visualized by Nomarski microscopy as refractile bodies or “corpses.” We counted apoptotic corpses in embryos at several developmental stages and found significantly fewer apoptotic bodies in *pgrn-1* mutants compared with control animals (Fig. 2A). To verify that the cell death defect was a result of progranulin deficiency, we reintroduced the *C. elegans* progranulin gene and observed partial rescue of the mutant phenotype (Fig. S4D). We speculate that the incomplete rescue may be because expression of *pgrn-1* from a transgene does not fully mimic the expression of the endogenous gene. In addition to cell deaths that occur as part of the developmental program, apoptosis can be triggered in the germ line by UV irradiation. We found that *pgrn-1* mutations did not affect UV-induced germ cell death (Fig. 2B).

Reduced numbers of apoptotic bodies in embryos could be caused by a failure of cells to die or by an increase in the rate of apoptotic cell clearance. To distinguish these possibilities, we used fluorescent markers that label the sisters of apoptotic cells that survive. These fluorescent markers are known to label “undead” cells in *ced-3* caspase mutants in which cell death is blocked. If we saw “extra” cells, this would suggest that some cells normally programmed to die did not. First, we used an ODR-1::RFP reporter that normally is present in AWB, AWC, I1, and ASI neurons (29). I1 and ASI have sister cells that die (28). The same number of ODR-1::RFP fluorescent cell bodies were observed in *pgrn-1* mutants and controls, suggesting that cell death occurs normally in the I1 and ASI lineage (Fig. 2C). We also used a LIN-11::GFP reporter which is seen in approximately five extra ventral cord motor neurons in strong *ced-3(lf)* mutants (30). Once again, *pgrn-1* mutants had similar numbers of LIN-11::GFP fluorescent cell bodies compared with controls (Fig. 2D). Finally, we looked for extra cells in the anterior pharynx, where, in strong *ced-3(lf)* mutants, approximately 12 extra cells are found (31). Once again, *pgrn-1* mutants had the same number of cells in the anterior pharynx as control worms

Fig. 3. Loss of progranulin accelerates the clearance of apoptotic cells in *C. elegans*. With the use of four-dimensional Nomarski time-lapse video microscopy, the time to clearance and time to die were determined for early deaths in the AB lineage in control animals and *pgrn-1(tm985)* mutants ($n = 3$ embryos per strain). (A) Diagram depicting approximate relative timing of events in time to die and time to clearance. (B) Results for time to clearance are shown in a scatterplot in which each circle represents a single cell-death observation. Harmonic means were 10.7 min for control and 5.7 min for *pgrn-1*-mutant animals (Mann–Whitney t test, $P = 0.0036$). (C) Results for time to die are depicted in a scatterplot in which each circle represents a single cell-death observation. Mean times to die were 20.2 min for control animals and 21.7 min for *pgrn-1* mutants (harmonic means, Mann–Whitney t test, $P = 0.3$). (D) Frequency distribution histogram for time to die. The distribution of deaths in control animals was Gaussian, whereas in *pgrn-1* mutants it was not (D’Agostino–Pearson omnibus normality test, $P = 0.05$ and $P < 0.0001$, respectively).

microglia, the phagocytic cells of the CNS (37). We cultured peritoneal macrophages from WT (i.e., $+/+$) and progranulin-KO littermates in heat-inactivated serum for several days and then exposed them to one of several targets. First, we exposed them to FITC-labeled polystyrene beads. We found that, compared with WT macrophages, progranulin-KO macrophages engulfed latex beads more rapidly (Fig. 4A). We then tested substrates likely internalized via receptor-mediated endocytosis, yeast cell wall β -glucan particles, and, most significantly, apoptotic thymocytes. Compared with *Pgrn* $^{+/+}$ controls, *Pgrn* $^{-/-}$ macrophages had increased levels of phagocytosis of yeast cell wall particles and apoptotic thymocytes (Fig. 4B–D). Together, these data suggest that, as in *C. elegans*, loss of progranulin influences phagocytosis by macrophages, including engulfment of apoptotic cells.

Discussion

We have identified a function for progranulin in modulating the kinetics of programmed cell death that may be relevant for neuron loss in neurodegenerative diseases. We showed that lack of progranulin caused apoptotic cells to be cleared more rapidly in *C. elegans*. This type of change in the kinetics of programmed cell death would have been difficult to observe in mammals. However, we have extended this finding to mouse macrophages, which also exhibit enhanced engulfment of latex beads, yeast cell wall, and, importantly, apoptotic cells. A defect in programmed cell death kinetics could gradually contribute to neuronal loss over the lifetime of human progranulin-mutation carriers. A neurodegenerative phenotype was not observed in *C. elegans*, but this may be because, unlike vertebrates, adult worms do not exhibit programmed cell death in somatic tissues.

Fig. 4. Loss of progranulin accelerates phagocytosis in mouse macrophages. Peritoneal macrophages were cultured from *Pgrn* $^{+/+}$ and *Pgrn* $^{-/-}$ animals and incubated with FITC-labeled polystyrene beads (A), yeast cell-wall particles (zymosan) (B), or pHrodo-labeled apoptotic thymocytes (C and D) in the presence of 10% heat-inactivated FBS. (A) The average number of beads engulfed by each macrophage after 90 min incubation was determined ($n \geq 100$ cells per condition). Error bars represent SD (Student t test, $***P < 0.0001$). (B) Average increase in zymosan uptake by colorimetric assay after 90 min of incubation. Error bars represent SEM of two independent experiments (Student t test, $*P < 0.05$). (C and D) The average number of apoptotic thymocytes engulfed by each macrophage after 90 min of incubation was determined. Representative fields are shown in C (with pHrodo-labeled apoptotic thymocytes in red and CD-11b-FITC-labeled macrophage membranes in green) and average counts shown in D ($n \geq 100$ cells per condition). Error bars represent SEM (Student t test, $***P < 0.0001$).

Tokyo Women's Medical University (Tokyo, Japan) and out-crossed four times to our laboratory's N2 strain. Other published strains were obtained from the *Caenorhabditis* Genetics Center at the University of Minnesota. *SI Materials and Methods* provides additional strain information.

Assays. Somatic cell and germ line corpses were visualized directly on a Zeiss Axioplan 2 microscope fitted with Nomarski optics as described previously (28). *C. elegans* embryos, larvae, and adults were mounted on 2% agarose pads in M9 with 2.5 mM levamisole. Apoptotic corpses were counted in a blinded fashion. To measure the kinetics of cell corpse formation and clearance, four-dimensional time-lapse Nomarski microscopy was performed as described previously (50). Results are shown in scatterplots and histogram form. *SI Materials and Methods* provides additional details on phagocytosis assays.

- Neary D, et al. (1998) Frontotemporal lobar degeneration: A consensus on clinical diagnostic criteria. *Neurology* 51:1546–1554.
- Hutton M, et al. (1998) Association of missense and 5'-splice-site mutations in tau with the inherited dementia FTDP-17. *Nature* 393:702–705.
- Neumann M, et al. (2006) Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science* 314:130–133.
- Mackenzie IR, et al. (2010) Nomenclature and nosology for neuropathologic subtypes of frontotemporal lobar degeneration: an update. *Acta Neuropathol* 119:1–4.
- Watts GD, et al. (2004) Inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia is caused by mutant valosin-containing protein. *Nat Genet* 36:377–381.
- Skibinski G, et al. (2005) Mutations in the endosomal ESCRTIII-complex subunit CHMP2B in frontotemporal dementia. *Nat Genet* 37:806–808.
- Kwiatkowski TJ, Jr., et al. (2009) Mutations in the FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis. *Science* 323:1205–1208.
- Vance C, et al. (2009) Mutations in FUS, an RNA processing protein, cause familial amyotrophic lateral sclerosis type 6. *Science* 323:1208–1211.
- Baker M, et al. (2006) Mutations in progranulin cause tau-negative frontotemporal dementia linked to chromosome 17. *Nature* 442:916–919.
- Cruts M, et al. (2006) Null mutations in progranulin cause ubiquitin-positive frontotemporal dementia linked to chromosome 17q21. *Nature* 442:920–924.
- Gass J, et al. (2006) Mutations in progranulin are a major cause of ubiquitin-positive frontotemporal lobar degeneration. *Hum Mol Genet* 15:2988–3001.
- Le Ber I, et al.; French Research Network on FTD/FTD-MND (2007) Progranulin null mutations in both sporadic and familial frontotemporal dementia. *Hum Mutat* 28:846–855.
- Kayed R, et al. (2003) Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. *Science* 300:486–489.
- Eriksen JL, Mackenzie IR (2008) Progranulin: Normal function and role in neurodegeneration. *J Neurochem* 104:287–297.
- Ghidoni R, Benussi L, Glionna M, Franzoni M, Binetti G (2008) Low plasma progranulin levels predict progranulin mutations in frontotemporal lobar degeneration. *Neurology* 71:1235–1239.
- Finch N, et al. (2009) Plasma progranulin levels predict progranulin mutation status in frontotemporal dementia patients and asymptomatic family members. *Brain* 132:583–591.
- Sleegers K, et al. (2009) Serum biomarker for progranulin-associated frontotemporal lobar degeneration. *Ann Neurol* 65:603–609.
- Brouwers N, et al. (2007) Alzheimer and Parkinson diagnoses in progranulin null mutation carriers in an extended founder family. *Arch Neurol* 64:1436–1446.
- Brouwers N, et al. (2008) Genetic variability in progranulin contributes to risk for clinically diagnosed Alzheimer disease. *Neurology* 71:656–664.
- Sleegers K, et al. (2008) Progranulin genetic variability contributes to amyotrophic lateral sclerosis. *Neurology* 71:253–259.
- Viswanathan J, et al. (2009) An association study between granulin gene polymorphisms and Alzheimer's disease in Finnish population. *Am J Med Genet B Neuropsychiatr Genet* 150B:747–750.
- Bateman A, Bennett HP (2009) The granulin gene family: From cancer to dementia. *Bioessays* 31:1245–1254.
- Daniel R, He Z, Carmichael KP, Halper J, Bateman A (2000) Cellular localization of gene expression for progranulin. *J Histochem Cytochem* 48:999–1009.
- Irwin D, Lipka CF, Rosso A (2009) Progranulin (PGRN) expression in ALS: An immunohistochemical study. *J Neurol Sci* 276:9–13.
- Van Damme P, et al. (2008) Progranulin functions as a neurotrophic factor to regulate neurite outgrowth and enhance neuronal survival. *J Cell Biol* 181:37–41.
- Ryan CL, et al. (2009) Progranulin is expressed within motor neurons and promotes neuronal cell survival. *BMC Neurosci* 10:130.
- Ilieva H, Polymenidou M, Cleveland DW (2009) Non-cell autonomous toxicity in neurodegenerative disorders: ALS and beyond. *J Cell Biol* 187:761–772.
- Sulston JE, Schierenberg E, White JG, Thomson JN (1983) The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Dev Biol* 100:64–119.
- L'Etoile ND, Bargmann CI (2000) Olfaction and odor discrimination are mediated by the *C. elegans* guanylyl cyclase ODR-1. *Neuron* 25:575–586.
- Reddien PW, Cameron S, Horvitz HR (2001) Phagocytosis promotes programmed cell death in *C. elegans*. *Nature* 412:198–202.
- Shaham S, Reddien PW, Davies B, Horvitz HR (1999) Mutational analysis of the *Caenorhabditis elegans* cell-death gene *ced-3*. *Genetics* 153:1655–1671.
- Hoepfner DJ, Hengartner MO, Schnabel R (2001) Engulfment genes cooperate with *ced-3* to promote cell death in *Caenorhabditis elegans*. *Nature* 412:202–206.
- Kinchen JM, et al. (2005) Two pathways converge at CED-10 to mediate actin rearrangement and corpse removal in *C. elegans*. *Nature* 434:93–99.
- Hurwitz ME, et al. (2009) Abl kinase inhibits the engulfment of apoptotic [corrected] cells in *Caenorhabditis elegans*. *PLoS Biol* 7:e99.
- Hu B, Sonstein J, Christensen PJ, Puntieri A, Curtis JL (2000) Deficient in vitro and in vivo phagocytosis of apoptotic T cells by resident murine alveolar macrophages. *J Immunol* 165:2124–2133.
- He Z, Ong CH, Halper J, Bateman A (2003) Progranulin is a mediator of the wound response. *Nat Med* 9:225–229.
- Lucin KM, Wyss-Coray T (2009) Immune activation in brain aging and neurodegeneration: too much or too little? *Neuron* 64:110–122.
- Albeck JG, et al. (2008) Quantitative analysis of pathways controlling extrinsic apoptosis in single cells. *Mol Cell* 30:11–25.
- Rehm M, Huber HJ, Dussmann H, Prehn JH (2006) Systems analysis of effector caspase activation and its control by X-linked inhibitor of apoptosis protein. *EMBO J* 25:4338–4349.
- Zanocco-Marani T, et al. (1999) Biological activities and signaling pathways of the granulin/epithelin precursor. *Cancer Res* 59:5331–5340.
- Nykjaer A, et al. (2004) Sortilin is essential for proNGF-induced neuronal cell death. *Nature* 427:843–848.
- Hu F, et al. (2010) Sortilin-mediated endocytosis determines levels of the frontotemporal dementia protein, progranulin. *Neuron* 68:654–667.
- Yin F, et al. (2010) Exaggerated inflammation, impaired host defense, and neuropathology in progranulin-deficient mice. *J Exp Med* 207:117–128.
- Ahmed Z, et al. (2010) Accelerated lipofuscinosis and ubiquitination in granulin knockout mice suggests a role for progranulin in successful aging. *Am J Pathol* 177:311–324.
- Yin F, et al. (2010) Behavioral deficits and progressive neuropathology in progranulin-deficient mice: A mouse model of frontotemporal dementia. *FASEB J*, 10.1096/fj.10-161471.
- Fuhrmann M, et al. (2010) Microglial Cx3cr1 knockout prevents neuron loss in a mouse model of Alzheimer's disease. *Nat Neurosci* 13:411–413.
- Clement AM, et al. (2003) Wild-type nonneuronal cells extend survival of SOD1 mutant motor neurons in ALS mice. *Science* 302:113–117.
- Boillée S, et al. (2006) Onset and progression in inherited ALS determined by motor neurons and microglia. *Science* 312:1389–1392.
- Brenner S (1974) The genetics of *Caenorhabditis elegans*. *Genetics* 77:71–94.
- Schnabel R, Hutter H, Moerman D, Schnabel H (1997) Assessing normal embryogenesis in *Caenorhabditis elegans* using a 4D microscope: Variability of development and regional specification. *Dev Biol* 184:234–265.