Neuron, Vol. 36, 45–56, September 26, 2002, Copyright ©2002 by Cell Press

Hsp27 Upregulation and Phosphorylation Is Required for Injured Sensory and Motor Neuron Survival

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Summary

Peripheral nerve transection results in the rapid death by apoptosis of neonatal but not adult sensory and motor neurons. We show that this is due to induction and phosphorylation in all adult axotomized neurons of the small heat shock protein Hsp27 and the failure of such induction in most neonatal neurons. In vivo delivery of human Hsp27 but not a nonphosphorylatable mutant prevents neonatal rat motor neurons from nerve injury-induced death, while knockdown in vitro and in vivo of Hsp27 in adult injured sensory neurons results in apoptosis. Hsp27's neuroprotective action is downstream of cytochrome c release from mitochondria and upstream of caspase-3 activation. Transcriptional and posttranslational regulation of Hsp27 is necessary for sensory and motor neuron survival following peripheral nerve injury.

Introduction

The survival of primary sensory and motor neurons in response to a peripheral nerve injury depends on developmental age. Injured adult sensory and motor neurons show delayed and restricted cell loss (Ma et al., 2001; Tandrup et al., 2000), while peripheral nerve injury performed in the early postnatal period results in rapid and extensive neuronal death (Oliveira et al., 1997). This age-dependent difference may be related to the programmed cell death that immature sensory and motor neurons undergo from embryonic age 13 (E13) until shortly after birth (Henderson, 1996; Sendtner et al., 2000). That immature neurons are more prone to the activation of apoptotic pathways than mature neurons may reflect age-dependent differences in the presence or absence of extrinsic survival and death factors and in the balance of intrinsic pro- and antiapoptotic factors (Pettmann and Henderson, 1998).

Exogenous growth factors, either alone or in combination, have survival promoting effects on embryonic motoneurons in vitro and/or in vivo (Sendtner et al., 2000) and can rescue motor neurons from neonatal axotomy-induced death (Oppenheim, 1996). However, it is unclear if these effects are physiological or pharmacological (Oppenheim, 1996), since rescue effects are only transient (Vejsada et al., 1995), and not all motor neurons are lost by null mutations of single genes encoding neurotrophic factors or their receptors (DeChiara et al., 1995; Garces et al., 2000; Liu and Jaenisch, 2000; Oppenheim et al., 2001).

The dynamic regulation of intrinsic death-inducing or survival-promoting factors also contributes to developmental neuronal cell death. The p75 low-affinity neurotrophin receptor (p75NTR) is expressed in developing motor neurons and promotes cell death (Wiese et al., 1999). Fas/Apo-1/CD95, a member of the TNF-death receptor family, is involved in death of motor neurons following growth factor deprivation (Raoul et al., 1999), and the interplay between the proapoptotic factor Bax and the antiapoptotic factors Bcl-2 and Bcl-x_L may regulate the fate of motor neurons during development (Martinou et al., 1994; Parsadanian et al., 1998), following nerve injury (Dubois-Dauphin et al., 1994; Yamada et al., 2001), or growth factor withdrawal (Li et al., 2001). The inhibitor of apoptosis (IAP) family comprised of NAIP, XIAP, HIAP1, and HIAP2 (Deveraux and Reed, 1999), delay the death of neonatal motor neurons after nerve injury (Perrelet et al., 2000), most likely via caspase inhibition (Deveraux et al., 1998), while Reg-2 acts as an extrinsic motor neuron survival factor (Livesey et al., 1997) by stimulating the PKB/Akt survival signaling pathway (Nishimune et al., 2000). Treatment with BRX-220, an inducer of the large heat shock proteins Hsp70 and 90, protects neonatal motor neurons from axotomy-induced cell death (Kalmar et al., 2002).

Another potential intrinsic neuronal survival factor is the small heat shock protein Hsp27. Only the subset of neonatal primary sensory neurons that upregulate Hsp27 following neonatal nerve injury survive (Lewis et al., 1999), whereas in the adult, all primary sensory and motor neurons upregulate Hsp27 after peripheral nerve injury (Costigan et al., 1998), and all survive for 2–3 months (Tandrup et al., 2000). In addition, Hsp27 expression protects cultured neonatal sensory and sympathetic neurons from apoptosis induced by NGF withdrawal (Lewis et al., 1999) or retinoic acid treatment (Wagstaff et al., 1999). Null mutations of Hsp27 result in death of embryonic stem cells (Mehlen et al., 1997), while Hsp27 expression has been shown to confer resistance to cell death in nonneuronal cells in response to multiple stresses (Arrigo and Landry, 1994; Benjamin and McMillan, 1998). Hsp27 in nonneuronal cells interferes with apoptosis by preventing caspase activation

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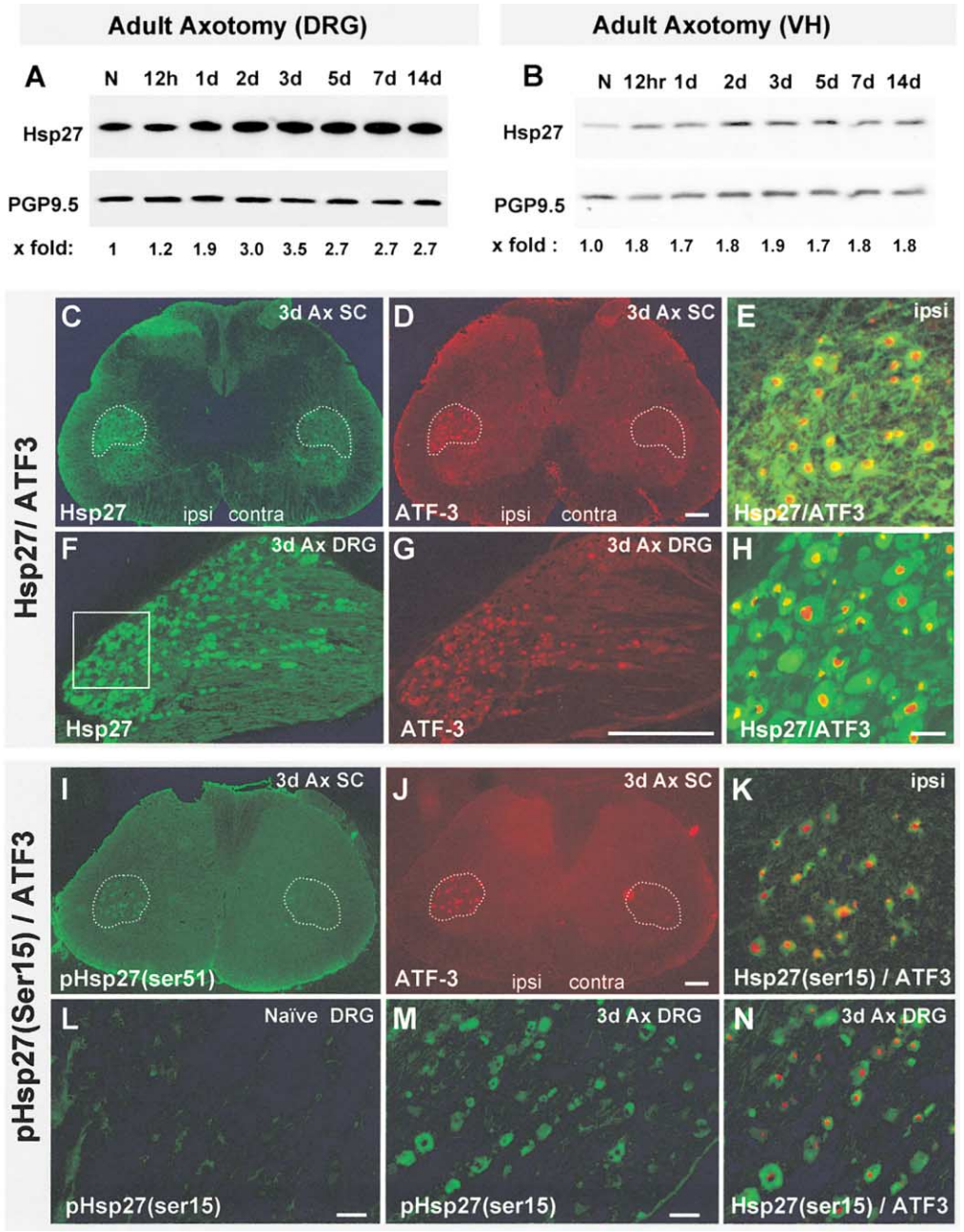

Figure 1. Hsp27 Is Upregulated and Phosphorylated in All Injured Adult Primary Sensory and Motor Neurons after Sciatic Nerve Injury

(A) Western blots showing Hsp27 upregulation in the ipsilateral DRG (n = 3, pooled L4 and L5) and (B) ventral horn (VH) in naive animals (N) and 12 hr, 1 hr, 3 hr, 5 hr, 7 hr, and 14 days following sciatic axotomy; PGP9.5 is loading control. Normalized fold increases are relative to naives. (C–E) Adult spinal cord and (F–H) L4 DRG coimmunostained 7 days after sciatic axotomy, with anti-Hsp27 and anti-ATF-3. Composite images of motor (E) and sensory neurons (H) show that all injured (ATF-3 positive) cells express Hsp27. Adult spinal cord (I–K), naive L4 DRG (L), and L4 DRG 3 days following sciatic nerve axotomy (M and N), stained for phosphoHsp27(Ser15) and ATF-3. All injured (ATF-3 positive) neurons are phospho-Hsp27(Ser15) positive. Scale bars, 200 μm (D, G, and J) and 50 μm (E, H, L, and M).

by direct interaction with cytochrome c after its release from mitochondria (Bruey et al., 2000) as well as by interacting with Daxx, preventing caspase-independent apoptosis (Charette et al., 2000).

We now demonstrate a transcriptional and posttranslational regulation of Hsp27 in all injured adult sensory and motor neurons and that Hsp27 is required for survival of these adult neurons when injured. Failure of all neonatal motor neurons to upregulate Hsp27 after nerve injury is shown to be a major determinant for their injury-induced death.

Results

All Injured Adult Sensory and Motor Neurons Upregulate Hsp27

In naive adult animals, a low level of constitutive Hsp27 protein expression is detected in the DRG and ventral

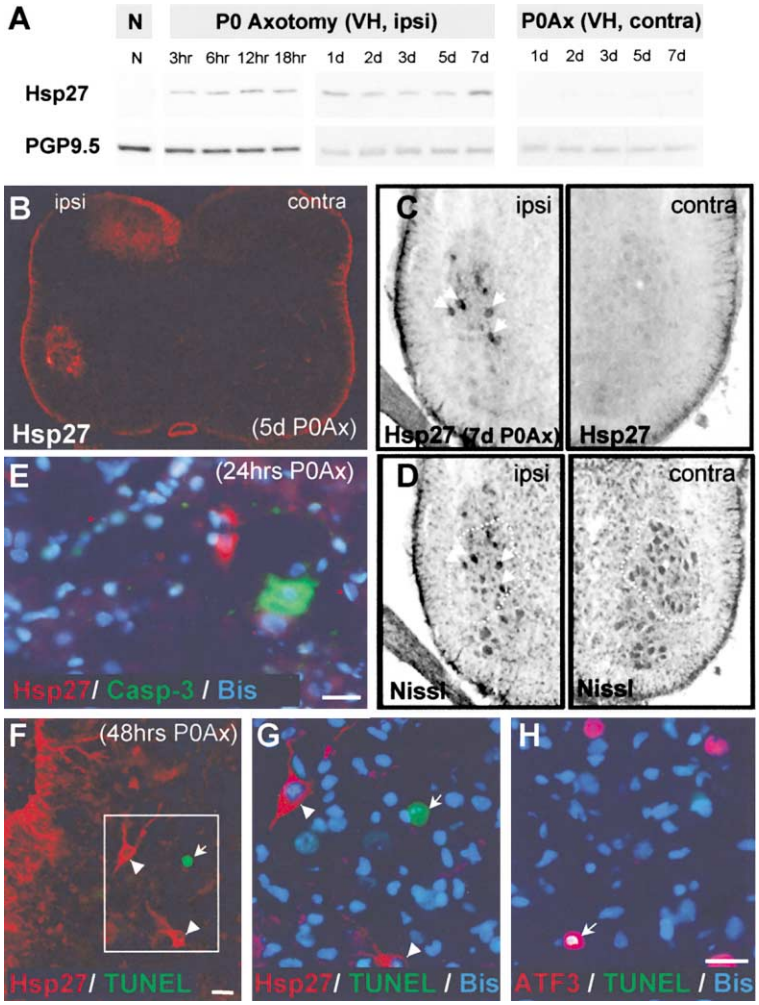

Figure 2. Expression of Hsp27 Correlates with Survival of Motor Neurons following Sciatic Nerve Axotomy at Birth

(A) Western blot analysis of Hsp27 expression in the ipsi- and contralateral ventral horn (VH) in naive animals and following sciatic nerve axotomy at birth (P0). (B) Hsp27 immunostaining in the L4/L5 neonatal spinal cord 5 days following a P0 sciatic axotomy. (C) Hsp27 and (D) Nissl staining in the ipsi- and contralateral L4/L5 ventral horn 7 days after P0 axotomy. Hsp27 is expressed in the subpopulation of surviving injured motor neurons. (E) Triple staining for Hsp27, active caspase-3 (Casp-3), and chromatin (bisbenzimide, Bis) in the L4/L5 ventral cord 24 hr after P0 axotomy. (F) Hsp27 immunostaining combined with TUNEL 24 hr after P0 axotomy. No overlap of Hsp27- and TUNEL-positive profiles was observed. (G) Region outlined in (F), with chromatin staining, shows a TUNEL-positive cell with pyknotic nucleus. (H) ATF-3 combined with TUNEL and chromatin staining shows an injured motor neuron undergoing apoptosis; the triple-labeled nucleus appears white. Arrowheads identify Hsp27-positive motor neurons; arrows identify TUNEL-positive nuclear profiles. Scale bars, 50 μm.

horn (Figures 1A and 1B). After a sciatic nerve transection, Hsp27 protein levels increase considerably in the ipsilateral L4 and L5 DRG and ventral horn (Figures 1A and 1B), with upregulation detectable after 12 hr and Hsp27 levels remaining elevated for at least 2 weeks (Figures 1A and 1B). Double immunocytochemistry for Hsp27 and activation transcription factor ATF-3, a marker for injured neurons (Tsujino et al., 2000), shows that all injured sensory (Figure 1H) and motor neurons (Figure 1E) express high levels of Hsp27.

Hsp27 Is Phosphorylated in All Adult Injured Sensory and Motor Neurons

Constitutively expressed Hsp27 in sensory and motor neurons in naive adult animals is not detected by an antibody that only recognizes phospho-Hsp27(ser15) (Figure 1L). However, injured (ATF-3 positive) adult DRG (Figure 1M) and motor neuron cell bodies become immunopositive for phospho-Hsp27(ser15) (Figure 1K) after a sciatic nerve transection. Sciatic nerve injury increases Hsp27 immunoreactivity in the superficial laminae of the dorsal horn as a result of the protein being transported from the DRG to the central axonal terminals of sensory neurons (Figure 1C), but this transported Hsp27, unlike that located in the injured cell bodies, is not phosphorylated (Figure 1I).

Hsp27 Expression in Motor Neurons after Neonatal Nerve Injury

Hsp27 protein expression is absent or negligible in uninjured motor neurons in naive neonatal rats (aged 1 week or younger) (Figures 2A and 2B). Sciatic nerve injury performed at birth (P0) results in an increase in Hsp27 in some motor neurons of the ventral horn soon after the injury (Figures 2A and 2B). Hsp27 immunostaining (Figure 2C) combined with Nissl staining at P7 after a P0 axotomy (Figure 2D) indicates that, while the majority of injured sciatic motor neurons die, those that survive are immunopositive for Hsp27.

Negative Correlation between Hsp27 Expression and Injury-Induced Neuronal Apoptosis

To assess if there is a differential susceptibility to cell death of those neonatal motor neurons that express Hsp27 and those that do not, TUNEL and immunoreactivity for Hsp27 and activated (cleaved) caspase-3 were analyzed in sections of the spinal cord 24 hr after a P0 axotomy (Figure 2E). Activated caspase-3 is never found in Hsp27 immunopositive profiles (Figure 2E) (15 to 23 sections analyzed per animal, $n = 3$). Furthermore, Hsp27 immunopositive motor neurons after a P0 axotomy do not exhibit either DNA strand breaks (detected by TUNEL) or show chromatin condensation/fragmenta-

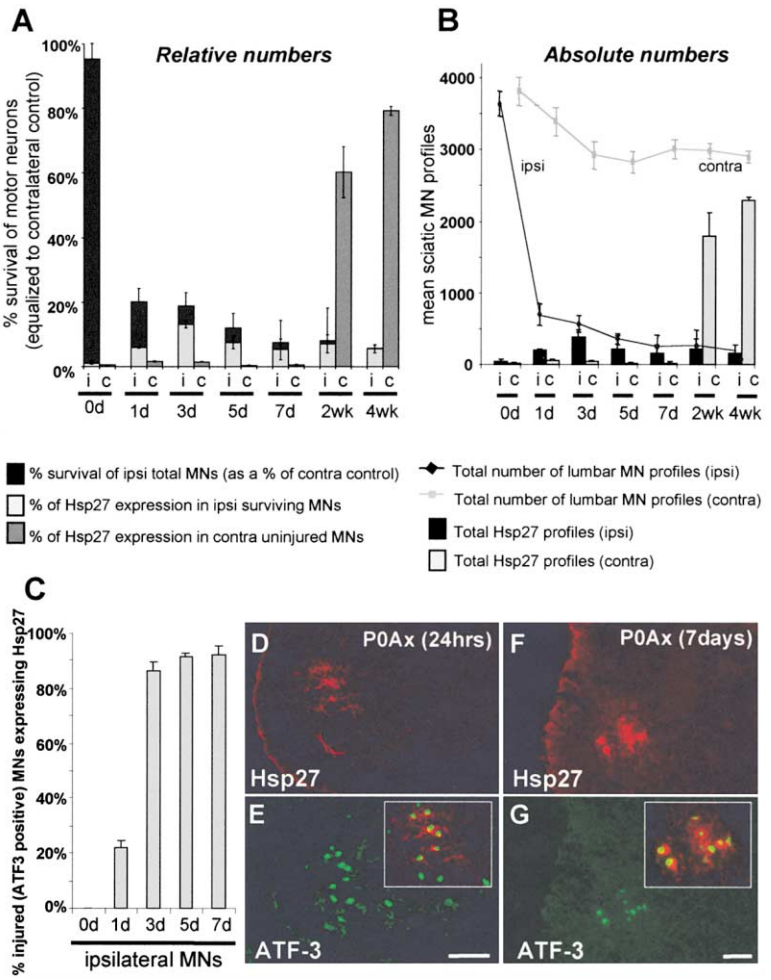

Figure 3. Hsp27 Is Expressed in Surviving Injured Neonatal Motor Neurons

(A) *Relative numbers* of ipsilateral (i) sciatic motor neurons and the proportion expressing Hsp27, both represented as percentage of contralateral sciatic motor neurons (c) in uninjured animals (0d) and after P0 sciatic axotomy (n = 3–5/group). (B) *Absolute numbers* of sciatic motor neurons indicating axotomy-induced loss relative to normal developmental motor neuron death compared with total number of Hsp27 expressing sciatic motor neurons. (C) Proportion of Hsp27 expressing injured (ATF-3 positive) motor neuron profiles, indicated by ATF-3 immunoreactivity 1–7 days after P0 axotomy (n = 4–5/group). Double immunostaining of Hsp27 and ATF-3 and merged inset 24 hr (D and E) and 7 days (F and G) after P0 sciatic axotomy. Not all injured neurons express Hsp27 at 24 hr, but all surviving ones at 7 days do. Scale bar, 100 μm.

tion (bisbenzimide staining) (Figures 2F and 2G) (65 consecutive 10 μm sections per animal, n = 3 at 24 hr and 48 hr post axotomy). In contrast, injured motor neurons that do not stain for Hsp27 do undergo apoptosis, as indicated by TUNEL in ATF-3 double-labeled profiles (Figure 2H).

Neonatal Nerve Injury Results in the Death of the Majority of Injured Motor Neurons and Those that Survive Express Hsp27

To see if Hsp27 expression and motor neuron survival after a P0 axotomy correlate, the number of motor neurons expressing Hsp27 was counted in the ipsilateral and contralateral sciatic motor neuron pool 1 day to 1 month after injury at birth. The number of motor neurons ipsilateral to the sciatic nerve lesion is decreased 1 day after injury at birth and then further declines, although to a lesser extent, from 1 to 7 days following the injury (Figures 3A and 3B). Beyond 7 days, no further loss occurs, and the number of surviving motor neurons in the lumbar motor neuron pool is <10% of the number present in the intact contralateral pool. Although there is substantial upregulation of Hsp27 within hours after sciatic nerve injury (Figure 2A), the upregulation only occurs in a small subpopulation of injured motor neurons (<5% of the total preinjury number) 24 hr after injury (Figures 3A and 3B). As the injured sciatic motor neurons die, the number of Hsp27-positive motor neurons, although remaining small in absolute terms, becomes an increasingly large proportion of the surviving motor neurons (Figures 3A and 3C). Of the ipsilateral sciatic motor neurons remaining 7 days after injury, 71.9% ± 3.3% express Hsp27 compared with 2.5% ± 0.3% in the contralateral pool (Figure 3A). The percentage of the injured motor neuron population, identified by ATF-3 immunoreactivity, that expresses Hsp27 increases from 22.2% ± 2.2% at 1 day to 86.6% ± 3.0% 3 days after the P0 axotomy (Figure 3C). When examined in absolute terms, the number of Hsp27 immunoreactive motor neurons peaks 3 days after the P0 axotomy (Figure 3B), while the contralateral population of lumbar motor neurons only begins to express Hsp27 constitutively 2 weeks after birth (Figure 3B).

Human Hsp27 but Not a Nonphosphorylatable Mutant Rescues Neonatal Motor Neurons In Vivo

To test if Hsp27 can prevent neonatal (P3) motor neuron death in vivo, the sciatic motor neurons were infected at the time of nerve transection with an adenovirus expressing human Hsp27 (Adv-hHsp27) (Figure 4A). Fluorogold was used to retrogradely label the sciatic motor

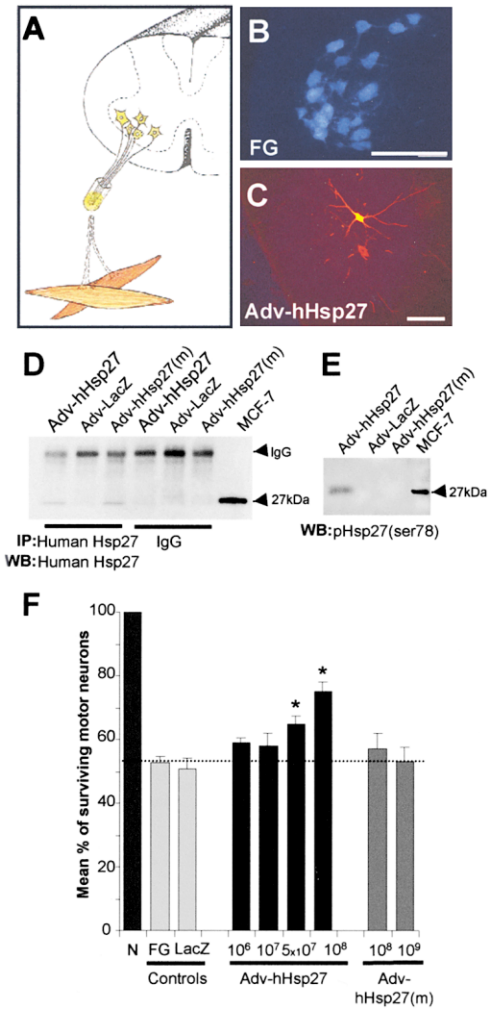

Figure 4. Hsp27 Promotes the Survival of Neonatal Motor Neurons after Sciatic Nerve Injury In Vivo

(A) Delivery of adenoviral vectors and fluorogold for retrograde transport to neonatal motor neurons. (B) Fluorogold (FG) labeled sciatic motor neurons from P3 injured animals. (C) Immunostaining for GFP 7 days after coadministration of Adv-GFP and Adv-hHsp27. (D) Human Hsp27 protein expression in ventral horn after treatment with adenovirus expressing human Hsp27 (Adv-hHsp27) or a nonphosphorylatable mutant of hHsp27 [Adv-hHsp27(m)] but not β-galactosidase (Adv-LacZ). MCF-7 cell line is a positive control. (E) Adv-hHsp27 but not Adv-LacZ or Adv-hHsp27(m) are immunoreactive for pHsp27(ser78). (F) Fluorogold (FG) and Adv-LacZ administration at 10^8 ($n = 9$ and 6) results in a 50% loss of motor neurons 7 days after P3 sciatic nerve injury compared with uninjured naive (N). Administration of Adv-hHsp27 at 10^6, 10^7, 5×10^7, and 10^8 viral particles/ml ($n = 3$, 4, 6, and 7, respectively) rescues motor neurons (*$p < 0.05$ versus fluorogold control). Scale bars, 100 μm.

neuron pool so that the numbers of surviving motor neurons could be determined (Figure 4B) (Perrelet et al., 2000). Viruses expressing β-galactosidase (Adv-LacZ) or GFP (Adv-GFP) were used as controls. GFP expression is detectable in injured sciatic motor neurons of animals coinfected with Adv-GFP and Adv-hHsp27 1 week after a P3 sciatic nerve transection (Figure 4C), indicating successful retrograde transport of adenovirus and survival of the infected neurons. The appearance of immunoreactivity for human Hsp27 in the ventral horn after the application of Adv-hHsp27 indicates that the protein is expressed in infected motor neurons (Figure 4D). Infection with Adv-GFP alone at P3 results in few GFP-labeled neurons at P10, presumably because most of the infected cells have died by this time in the absence of hHsp27 expression.

The mean number of fluorogold-positive sciatic motor neurons 1 week following a P3 sciatic nerve lesion in controls (fluorogold only) or after infection at the time of injury with the β-galactosidase adenovirus (Adv-LacZ) is 912 ± 64 and 953 ± 33, respectively (Figure 4F). At this age, the sciatic motor neuron population is 1800 to 1900 in uninjured animals (Perrelet et al., 2000). This indicates that a P3 sciatic lesion results in a loss of 50%–60% of the total sciatic motor neuron population, a significant loss although less than after a P0 nerve injury. Infection of the motor neurons with Adv-hHsp27 applied to the injured nerve at titers of 5×10^7 or 10^8 virus particles/ml results in an increase in the number of surviving motor neurons (Figure 4F). Following infection with 10^8 Adv-hHsp27 virus particles/ml, the number of fluorogold-labeled motor neurons increased by 25% compared with controls where either fluorogold alone or fluorogold together with Adv-LacZ was applied to the sciatic nerve (Figure 4F). As a measure of the efficiency of adenovirus motor neuron infection, the percentage of the total fluorogold-positive motor neuron profiles that were also positive for β-galactosidase after Adv-Lac-Z infection or for GFP after Adv-GFP treatment was 20%–25% in P11 rats. P11 rats were used to estimate adenoviral infectivity because motor neuron loss no longer occurs after a sciatic nerve injury at this time (Perrelet et al., 2000). Absolute infectivity at P3 cannot be measured directly because the sciatic nerve procedure used to introduce the viral particles results in a loss of the motor neurons. Assuming that infectivity at P3 is the same as at P10, this would imply a close to complete rescue of all motor neurons infected with Adv-hHsp27; however, if infectivity were greater at P3 than P10, the proportion rescued would be lower. Nevertheless, the fact that significantly more cells survive after Adv-hHsp27 than Adv-GFP, Adv-Lac-Z, or AdvhHsp27(m) (see below) shows that human Hsp27 has a survival effect on neonatal injured motor neurons.

The contribution that phosphorylation of Hsp27 plays in neuronal survival was assessed with a human Hsp27 mutant adenovirus [Adv-hHSP27(m)] in which the three N-terminal serine residues (Ser15, Ser78, Ser82) are changed to alanine, producing a nonphosphorylatable Hsp27 protein (Rogalla et al., 1999). Human Hsp27 immunoreactivity is detected in these samples, indicating that the mutant protein is expressed, but absence of any signal for immunoreactive phospho-human Hsp27(ser78) 7 days after treatment with Adv-hHsp27(m) (Figure 4E) confirms that the mutant human Hsp27 protein is not phosphorylated. Adv-hHSP27(m) unlike Adv-hHsp27 does not rescue injured motor neurons (Figure 4F).

Knockdown of Hsp27 Induces Apoptosis in Adult Sensory Neurons

To establish if Hsp27 expression is necessary for injured adult sensory neuron survival, the effect of Hsp27 pro-

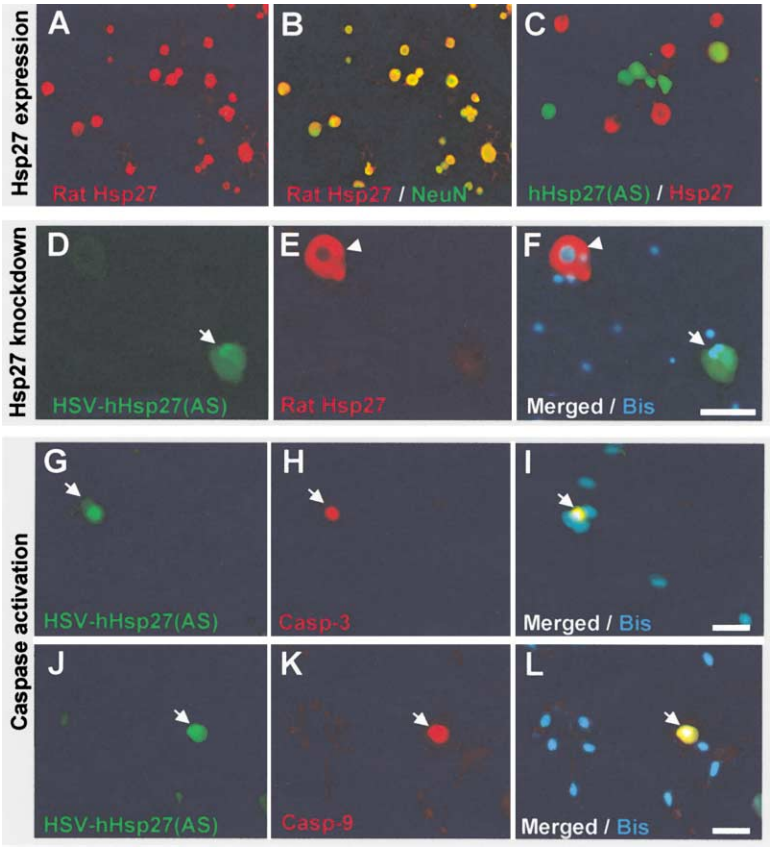

Figure 5. Antisense Knockdown of Hsp27 Induces Apoptosis in Adult DRG Neurons In Vitro

(A) Rat Hsp27 expression, combined with NeuN (B), in 24 hr cultured adult sensory neurons, showing all neurons are Hsp27 positive. (C) Knockdown of rat Hsp27 12 hr after infection with HSV amplicon vector expressing antisense human Hsp27 [HSV-hHsp27(AS)]. (D) GFP localization (small arrow) to indicate HSV-hHsp27(AS) infection combined in (E) with rat Hsp27 immunostaining (arrowhead) and merged in (F) with chromatin staining (bisbenzimide, Bis) 24 hr postplating. (G and J) HSV-hHsp27(AS) infected cells stain for active caspase-3 (Casp-3) (H) or active caspase-9 (Casp-9) (K). Chromatin staining (I and L) shows that HSV-hHsp27 infected cells coexpressing activated caspases have condensed chromatin and pyknotic nuclei. Experiments performed in triplicate and repeated more than two times. Scale bars, 50 μm.

tein knockdown was investigated using Hsp27 antisense delivery with a Herpes-Simplex virus amplicon vector [HSV-hHsp27(AS)]. Hsp27 is expressed by 24 hr in all cultured adult DRG neurons, a process which necessarily injures their axons. This was identified by the colocalization of Hsp27 with the neuronal marker NeuN (Figures 5A and 5B). The HSV amplicon vector and a control HSV amplicon expressing GFP alone (HSV-GFP) both infect 60%–70% of cultured adult sensory neurons. In 24 hr DRG cultures infected with HSV-hHsp27(AS), rat Hsp27 expression is reduced to a level where it cannot be detected by immunostaining (Figure 5C), but there is no loss of rat Hsp27 immunoreactivity in those cells infected with HSV-GFP (300 cells analyzed per group). All noninfected cells remain Hsp27 immunoreactive (Figure 5E). HSV-hHsp27(AS) infected cells show condensed chromatin (Figures 5D–5F) and are immunoreactive for active caspase-3 (Figures 5G–5I) and active caspase-9 (Figures 5J–5L) (200 cells analyzed per group). These changes are not detected in HSV-GFP infected control cells. Cell counts reveal that 76.2% ± 4.9% of cultured adult sensory neurons are lost 24 hr after infection with HSV-hHsp27(AS) at 10 m.o.i. (multiplicity of infection), compared with a 19.1% ± 5.4% loss in sensory neurons infected with HSV-GFP at the same concentration.

In vivo delivery of HSV-hHsp27(AS) into the sciatic nerve in adult rats at the time of injury produced similar results, although with a low yield, which may partly reflect death induced in the infected cells. After 8 days, 18 (75%) out of 24 HSV-hHsp27(AS) infected DRG neurons detected have no rat Hsp27 immunoreactivity and are immunopositive for active caspase-3, indicating that they are undergoing apoptosis (Figures 6A–6C). In contrast, all but one of the 63 HSV-GFP infected DRG neurons express rat Hsp27 and show no activation of caspase-3 (Figures 6D–6G). Noninfected injured adult DRG neurons are also never immunopositive for activated caspase-3.

Hsp27 Acts Downstream of Cytochrome c Release in Protecting Neonatal Sensory Neurons

The intrinsic pathway of apoptosis involves the release of cytochrome c from mitochondria, enabling it to complex with Apaf-1 in the cytoplasm and recruit and activate caspase-9 to form an apoptosome, which eventually leads to activation of caspase-3 (Li et al., 1997). To investigate how Hsp27 inhibits neuronal apoptosis, the effect of human Hsp27 on the apoptotic pathway was studied in neonatal sensory neurons. P0 dissociated primary DRG cell cultures were infected with HSV amplicon vectors expressing sense orientation of human Hsp27 [HSV-hHsp27(S)] or HSV-GFP. After NGF withdrawal, the majority of noninfected or HSV-GFP infected cells die by apoptosis, and this involves the release of cytochrome c from mitochondria. Induction of apoptosis in noninfected cells or cells infected with HSV-GFP is indicated by a diffuse staining pattern for cytochrome c and positive staining for active caspase-3 (Figures 7C and 7D). Cells not undergoing apoptosis (identified by absence of activated caspase-3) all have a punctate cytochrome c staining, indicating that cytochrome c has not been released from mitochondria, labeled by CMXRos (Figures 7A and 7B). Caspase-3 is not activated

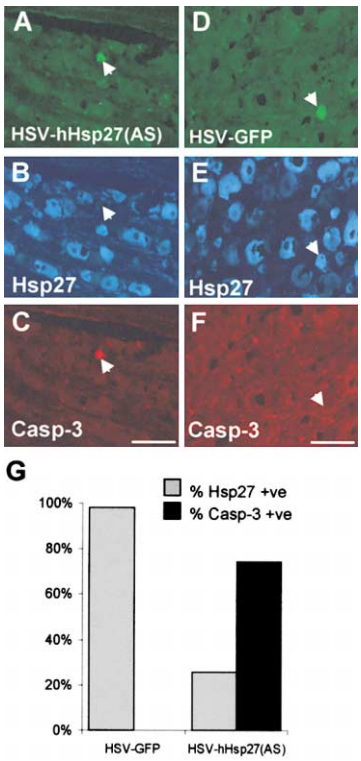

Figure 6. Antisense Knockdown of Hsp27 In Vivo in Injured Adult DRG Neurons Induces Apoptotic Cell Death

(A) In vivo expression of HSV-hHsp27(AS), detected by the expression of GFP, results in knockdown of rat Hsp27 (B) and in the activation of caspase-3, identified by immunostaining for cleaved (active) caspase-3 (Casp-3) (C) 8 days after sciatic nerve axotomy. (D) HSV-GFP expression does not alter Hsp27 (E) or caspase-3 expression (F). Proportion of the total detected HSV-hHsp27(AS) profiles (24 from 16 independent sections) and HSV-GFP profiles (63 from 13 sections) expressing rat Hsp27 and active caspase-3 after cuff application of the appropriate virus to the proximal end of a cut sciatic nerve. Scale bars, 100 μm.

in cells expressing human Hsp27 after infection with HSV-hHsp27(S), demonstrating that this vector rescues the cells by interfering with the apoptotic pathway upstream of caspase-3 (Figures 7E–7J). Rescue was confirmed by cell counts and an MTT assay (data not shown). A subpopulation of HSV-hHsp27(S) infected cells had diffuse cytochrome c staining but were not immunopostive for active caspase-3 (Figures 7E–7J and 7K). This implies that, in these cells, although the apoptotic pathway is initiated leading to cytochrome c release from mitochondria, caspase-3 activation is aborted by the expression of hHsp27. Only HSV-hHsp27(S) infected cells show a combination of extramitochondrial cytochrome c and no caspase activation (Figure7K). HSV-GFP infected and noninfected cells never exhibit this staining pattern; instead, if there is cytochrome release, there always is caspase 3 activation.

Discussion

While Hsp27 is upregulated in all adult sensory and motor neurons after peripheral nerve injury, it is only expressed in a small subpopulation of neonatal sensory (Lewis et al., 1999) and motor neurons after injury at birth. However, all surviving injured neonatal motor and sensory (Lewis et al., 1999) neurons express Hsp27, and those sensory (Lewis et al., 1999) and motor neurons identified as undergoing apoptosis do not express Hsp27. That this correlation reflects an intrinsic survival role for this small heat shock protein is shown by the delivery of human Hsp27 in vivo, which rescues neonatal motor neurons from injury-induced death. As previously reported, neonatal sensory and sympathetic neurons are also rescued in vitro by Hsp27 delivery (Lewis et al., 1999; Wagstaff et al., 1999). Hsp27 is, moreover, not only sufficient but also necessary for survival. Knockdown of endogenous rat Hsp27 in adult sensory neurons in vitro and in vivo by antisense delivery with an HSV amplicon vector induces apoptotic cell death.

Those transcriptional activators, repressors, or modulators of mRNA stability that regulate Hsp27 mRNA and protein levels have yet to be identified, as have the extrinsic signals that switch them on after injury. Injury-induced induction of Hsp27 occurs in all adult neurons with an axon in the peripheral nervous system. A signal universal to peripheral axonal injury must be responsible, one that is though only fully activated some time after birth. Failure of induction of Hsp27 contributes to axonal injury-induced neuronal loss. In the *paralysé* mouse, a model of spinal muscular atrophy with early onset, motor neuron expression of Hsp25 (the mouse homolog of Hsp27) is decreased compared with control mice (Pieri et al., 2001), and this parallels motor neuron cell loss (Houenou et al., 1996), suggesting that loss of constitutive Hsp27 promotes neurodegeneration. The upregulation and phosphorylation of Hsp27 in the hippocampus and in retinal ganglion neurons after injury (Krueger-Naug et al., 2002; Valentim et al., 2001) and its protection of retinal ganglion neurons from ischemia (Yokoyama et al., 2001) indicate that Hsp27's survival role may not be limited to the PNS.

Cell Survival and Hsp27

Nonneuronal cells expressing Hsp27 acquire resistance to heat shock, cytotoxins, and oxidative and chemical stresses as well as inflammatory cytokines (Benjamin and McMillan, 1998). Hsp27's diverse roles, including stabilization of mRNA (Carper et al., 1997), molecular chaperoning (Jakob et al., 1993), and preservation of cytoskeletal stability by capping the ends of actin filaments, preventing their depolymerization (Landry and Huot, 1999), may all contribute to its ability to protect cells from diverse insults. However, Hsp27 also contributes to cell survival by inhibiting apoptosis (Figure 8). Hsp27 interacts with cytochrome c after its release from mitochondria, blocking its interaction with Apaf-1 and procaspase-9, and thereby preventing activation of the downstream caspases and cell death (Bruey et al., 2000). In addition, phosphorylated Hsp27 interacts with Daxx to inhibit Fas-induced caspase-independent apoptosis (Charette et al., 2000). Hsp27 associates with Akt/PKB kinase (Murashov et al., 2001), and this prevents cell death by inhibiting cytochrome c release from the mitochondria as well as by phosphorylating and inactivating procaspase-9 (Cardone et al., 1998). Hsp27 may directly inhibit caspase-3 activation (Concannon et al.,

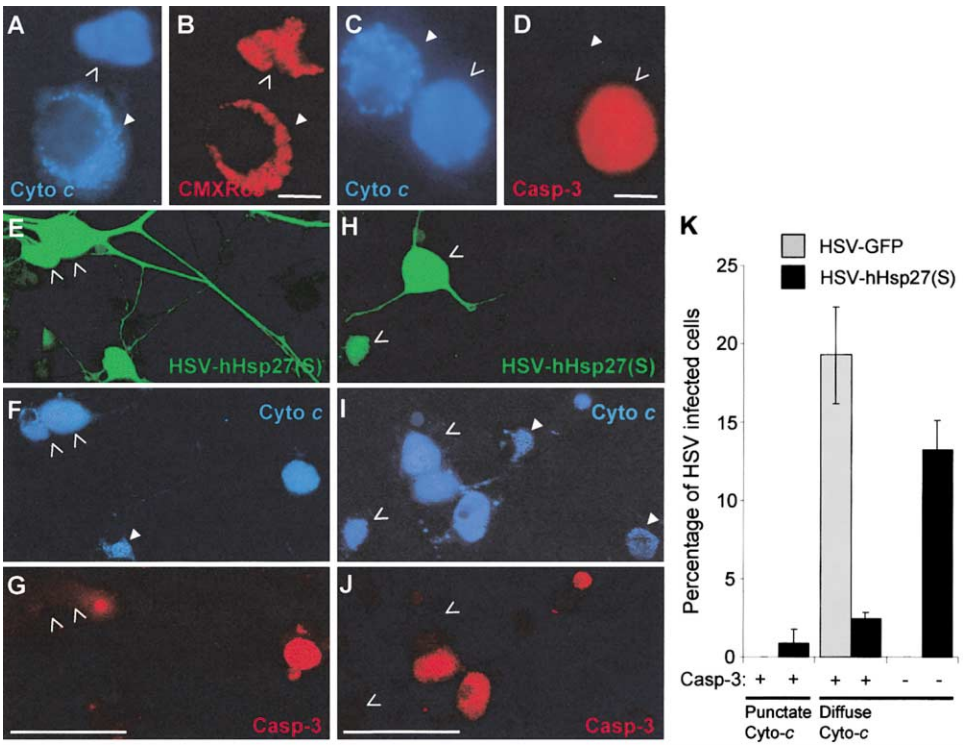

Figure 7. Human Hsp27 Prevents Apoptosis in Neonatal Sensory Neurons In Vitro Downstream of Cytochrome c Release from the Mitochondria and Upstream of Caspase-3

(A and C) Noninfected cultured P0 DRG neurons not undergoing apoptosis have preserved punctate cytochrome c (Cyto c) staining (filled arrowheads) colocalized with the mitochondrial stain MitoTracker Red (CMXRos) (B), whereas cells undergoing apoptosis (D), identified by active caspase-3 (Casp-3) label, have diffuse (cytoplasmic) cytochrome c immunostaining (empty arrowheads). (E and H) Some HSV-hHsp27(S) infected P0 sensory neurons with diffuse cytochrome c staining (empty arrowheads) (F and I) do not stain for active caspase-3 (G and J). Cells with punctate cytochrome c staining in these fields are indicated with filled arrowheads. (K) Proportion of HSV-hHsp27(S)- and HSV-GFP-infected cells with active caspase-3 staining that have punctate or diffuse cytochrome c staining patterns (199 and 206 cells, respectively, from three individual triplicate experiments). Scale bars, 10 μm (A–D), 50 μm (E–J).

2001; Pandey et al., 2000), although this is controversial (Bruey et al., 2000; Garrido et al., 1999). The capping by Hsp27 of actin can inhibit Bid translocation to the mitochondria and thus mitochondrial cytochrome c release (Paul et al., 2002). This indicates that Hsp27 has multiple antiapoptotic actions that contribute to its survival-promoting effects. We have found that in sensory neurons at least one action of Hsp27 is downstream of mitochondrial release of cytochrome c and upstream of caspase-3 activation. Hsp27 may act in concert with other intrinsic survival factors, such as antiapoptotic members of the Bcl-2 family, IAPs, and other heat shock proteins (Kalmar et al., 2002).

Phosphorylation of Hsp27 and Cell Survival

The capacity of Hsp27 to protect nonneuronal cells from cell death is regulated by phosphorylation (Huot et al., 1996; Rogalla et al., 1999), providing the cell with an immediate and transient response to cellular stress during the delay inherent in the transcription of the many different intrinsic survival proteins. Hsp27 contains three amino terminal serine residues: Ser15, Ser78, and Ser82 (equivalent to Ser86 on the murine homolog Hsp25), which are all phosphorylated in vitro in response to growth factors and heat shock (Rogalla et al., 1999). We find that a mutant Hsp27 that is nonphosphorylatable on all three residues has no survival-promoting activity. This leaves unresolved which residues need to be phosphorylated for this function. The specific role of Ser15 phosphorylation, which is located within a conserved N-terminal hydrophobic motif, may be important for Hsp27 deoligomerization to dimers (Benndorf et al., 1994; Lambert et al., 1999). This dissociation may contribute to Hsp27's protective function, since Hsp27 dimers are the conformation which bind to cytochrome c to prevent caspase-dependent apoptosis (Bruey et al., 2000) and are the principal species involved in intracellular protein relocalization (Rogalla et al., 1999). Ser15 phosphorylation may also be important in mediating amino-terminal interactions of Hsp27 with other proteins, such as PKB/Akt (Konishi et al., 1997), F-actin, or granzyme A (Benndorf et al., 1994). Although we were able to demonstrate phophorylation of Ser15, phosphorylation of Ser82 could also be important, as it results in the destabilization of amino-terminal intramolecular interactions (Lambert et al., 1999) and the subsequent dissociation from large oligomic Hsp27 supramolecular structures to small dimeric and multimer species (Lambert et al., 1999; Rogalla et al., 1999). The role of Ser78 phosphorylation, which is present only on human Hsp27, is not known. Single serine mutants will be required to evaluate this further.

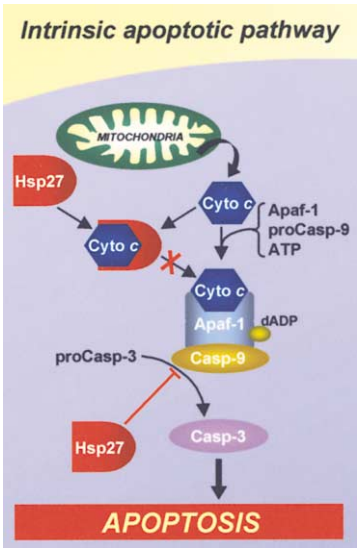

Figure 8. Antiapoptotic Mechanisms of Hsp27

The intrinsic apoptotic pathway induces mitochondrial membrane permeabilization and release of cytochrome c (Cyto c) into the cytosol where it complexes with Apaf-1 to recruit and activate procaspase-9 (proCasp-9) in the so-called apoptosome. Activated caspase-9 (Casp-9) cleaves and thereby activates procaspase-3 (proCasp-3), the downstream executioner caspase. Our data indicate Hsp27 acts to prevent the activation of caspase-3 but not cytochrome c release. Hsp27 may bind with cytochrome c after its release from the mitochondria to prevent its interaction with Apaf-1. Hsp27 may also interfere directly with the activation of caspase-3.

Hsp27 is a substrate for a variety of kinases (Kato et al., 2001), including the stress-activated p38/SAPK2a mitogen-activated kinase (MAPK) and a downstream substrate, MAPKAPK-2 (MAPK-activated protein kinase 2) (Guay et al., 1997). The p38 MAPK signaling cascade regulates Hsp27 phosphorylation after various stressors (Hedges et al., 1999; Landry and Huot, 1999), with evidence of Ser15 and Ser87 phosphorylation by p38 and MAPKAPK-2 in response to cell-damaging stimuli (Guay et al., 1997). There is a coordinated upregulation of p38 and Akt/PKB with Hsp27 in adult motor neurons following sciatic nerve transection (Murashov et al., 2001; Namikawa et al., 2000).

Conclusion

The importance of maintaining neuronal survival after injury requires a redundancy of neuronal survival factors acting by multiple independent mechanisms to prevent activation of apoptosis. We now show that Hsp27 is an intrinsic sensory and motor neuronal survival factor, one that is both sufficient to rescue neonatal injured cells from death and apparently necessary for the survival of adult injured neurons.

Experimental Procedures

Animal Surgery

Sprague-Dawley rats were used following Massachusetts General Hospital Animal Care guidelines. Anesthesia was produced by halothane for adult rats (200–250 g) and hypothermia for postnatal day 0 (P0) and P3 rat pups.

Sciatic Nerve Injury

The left sciatic nerve was exposed at mid thigh level, ligated, and transected distal to the ligation.

Adenovirus Motor Neuron Infection

Postnatal day 3 (P3) neonates were anesthetized, the left sciatic nerve transected, and the proximal stump capped with a capsule containing 2.5 μM recombinant adenovirus vector (Lewis et al., 1999) at 1×10^9, 1×10^8, 5×10^7, 1×10^7, and 1×10^6 particles/ml titers and 2.5 μl of 2.5% fluorogold (Molecular Probes) (Perrelet et al., 2000).

HSV-Sensory Neuron Infection

Adult rats were anesthetized and 10 μl HSV-hHsp27(AS) or HSV-GFP ($n = 2$) at 10^6–10^7 transducing units/ml applied to the proximal cut sciatic nerve.

Adenovirus Production

Adenovirus expressing human Hsp27 (Adv-hHsp27) was produced (Lewis et al., 1999) and checked for wild-type contamination by PCR for the E1A gene. Viruses expressing a mutated human Hsp27 gene [Adv-hHsp27(m)], where 3 serine residues (Ser15, Ser78, Ser82) were changed to alanine residues by PCR-mediated site-directed mutagenesis, and GFP-expressing recombinant viruses were also constructed. All express LacZ.

Herpes Simplex Virus Amplicon Production

HSV amplicon pHGCX(2) plasmids (expression cassette based on pcDNA2.1) containing a GFP reporter under control of IE4/5 promoter (Saeki et al., 1998, 2001) were constructed to include human Hsp27 cDNA in sense [HSV-hHsp27(S)] or antisense orientation [HSV-hHsp27(AS)] under a human cytomegalovirus promoter. Amplicon vector generation and packaging was as described previously (Saeki et al., 1998, 2001) and titers of 10^6–10^7 transducing units/ml obtained.

Tissue Culture

Primary DRG Cultures

P0 primary DRG cultures were prepared (Lewis et al., 1999) and cultured in F-12 media (GibcoBRL) containing N2-supplement (GibcoBRL), 0.5% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin (Pen-Strep), 100 ng/ml 2.5S NGF (Promega). Adult primary DRG cultures were prepared in the same way, except ganglia were digested with 5 mg/ml collagenase and 1 mg/ml Dispase II (Roche) prior to 0.25% trypsin (GibcoBRL), and triturated cells were centrifuged through a Percoll (Sigma) gradient prior to plating and cultured in Neurobasal media (GibcoBRL) containing B27 supplement (GibcoBRL), Pen-Strep, and 10 μM Ara-c.

HSV Amplicon Infection of Primary DRG Cultures

Adult and P0 dissociated sensory neurons were infected 24 hr after plating with 5 min.o.i. of HSV amplicon vector for 8 hr at 37°C.

NGF Withdrawal of P0 Dissociated DRG Cultures

NGF was withdrawn 24 hr after plating, at the time when the vector-containing media was removed. The replacing media included anti-NGF polyclonal antiserum (1:300). In the control condition, cells were grown in the presence of 100 ng/ml NGF, without anti-NGF antiserum. 12 or 24 hr after NGF withdrawal, the MitroTracker Red (CMXRos) mitochondrial stain was used (Molecular Probes) prior to fixation in 4% paraformaldehyde.

Tissue Preparation

Animals were terminally anesthetized with sodium pentobarbitol. For Western blots, the L4/L5 segments of the spinal cord were rapidly removed on dry ice. For immunocytochemistry, the animals were perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) (PB). Tissue was postfixed and cryoprotected in 20% sucrose in 0.1 M PB.

Immunocytochemistry

Immunofluorescent staining was performed on 10 μm cryosections (Benn et al., 2001) or on primary DRG cultures (Lewis et al., 1999). Rat Hsp27 was detected using anti-Hsp25 antibody (Stressgen) raised against the murine Hsp27 homolog Hsp25, which recognizes both the phosphorylated and unphosphorylated forms of rat Hsp27. Green Flurescein Signal amplification (NEN, Boston) was used for

double labeling of Hsp27 and ATF-3 (Santa Cruz) or active caspase-3 (Cell Signaling) or double immunolabeling with phosphoHsp27 (Ser15) (Calbiochem) and ATF-3. The following antibodies were also used: anti-NeuN (Chemicon), anti-caspase-9 (active form) (Cell Signaling), anti-cytochrome c (Pharmingen), and anti-GFP (Molecular Probes). Antibody labeling was detected with Cy3-conjugated anti-rabbit (Jackson ImmunoResearch), FITC-conjugated anti-mouse (Vector), Cy5-conjugated anti-mouse (Jackson ImmunoResearch), or biotin-conjugated anti-rabbit (Vector), followed by peroxidase detection (ABC kit, Vector) and Nissl counterstaining. Bisbenzimide staining (Hoechst 33342; Calbiochem) was performed at 10 μg/ml in the first wash after the secondary antibody incubation.

Detection of Apoptosis

The ApopTag Fluorescein Kit (Intergen) was used for terminal deoxynucleotide transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) of DNA strand breaks. Following incubation with a FITC-conjugated anti-digoxigenin antibody to detect the inserted dUTP, the tissue sections were processed for immunofluorescent labeling of Hsp27 or ATF-3 and bisbenzimide staining.

Immunoprecipitation

Ventral horn tissue was homogenized in ice-cold RIPA buffer (50 mM Tris [pH 7.2], 150 mM NaCl, 0.1% Triton X-100, 1% sodium deoxycholate, 0.02% sodium azide) containing protease inhibitors (Minicomplete, Roche) and the protein concentration determined using BCA assay (Pierce). Protein lysates were preabsorbed with mouse IgG (Santa Cruz) and protein Sepharose A (Amersham Life Sciences) and immunoprecipitated overnight with antibody specific for human Hsp27 (Stressgen), precipitated with protein A-sepharose beads, reconstituted in lysis buffer (100 mM Tris [pH 6.8], 2% SDS, and 20% glycerol), and processed for Western blot analysis. Parallel samples were immunoprecipitated with mouse IgG for controls.

Western Blot

Ventral horns were homogenized in lysis buffer, boiled, and protein concentration quantified (BCA kit, Pierce). Protein samples (20 μg/sample) were resolved by SDS-PAGE (BioRad) and electrotransferred to PVDF membrane (Millipore) and blotted with anti-Hsp25, anti-human Hsp27, or anti-phospho Hsp27(ser78) (Upstate). Immunoblots were developed with the appropriate peroxidase-conjugated secondary antibodies: anti-rabbit or anti-mouse (Amersham Life Sciences) or anti-sheep (Upstate) and enhanced chemiluminescence (New England Biolabs). Membranes were immunoblotted with mouse anti-PGP 9.5 antibody (Accurate Chemical & Scientific Corp) as an internal standard to monitor protein loading. Densitometric analysis using Photoshop was used to calculate mean fold increase from triplicate experiments.

Data Analysis

Sciatic motor neurons were counted ipsi- and contralateral to the sciatic nerve injury on 10 μm transverse sections through the lumbar spinal cord at 50 μm intervals, beginning at the most caudal section containing the sciatic motor neuron pool (identified by the presence of ATF-3 or fluorogold) and ending with the most rostral positive section (Clarke and Oppenheim, 1995). All counts were performed blind to the treatment. Statistical significance was assessed by ANOVA and t test. Data expressed as mean ± SEM.

Acknowledgments

We wish to thank the National Institutes of Health (National Institute of Child Health and Human Development, HD38533; National Institute of Neurological Disorders and Stroke, NS38253) (C.J.W.) and the Alexander von Humboldt-Foundation (J.S.) for financial support and Drs. Xandra Breakfield, Susan Lewis, and Michael Costigan for technical advice.

Received: January 31, 2002
Revised: September 5, 2002

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