Human neuropathological and animal model evidence supporting a role for Fas-mediated apoptosis and inflammation in cervical spondylotic myelopathy

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Although cervical spondylotic myelopathy is a common cause of chronic spinal cord dysfunction in humans, little is known about the molecular mechanisms underlying the progressive neural degeneration characterized by this condition. Based on animal models of cervical spondylotic myelopathy and traumatic spinal cord injury, we hypothesized that Fas-mediated apoptosis and inflammation may play an important role in the pathobiology of human cervical spondylotic myelopathy. We further hypothesized that neutralization of the Fas ligand using a function-blocking antibody would reduce cell death, attenuate inflammation, promote axonal repair and enhance functional neurological outcomes in animal models of cervical spondylotic myelopathy. We examined molecular changes in post-mortem human spinal cord tissue from eight patients with cervical spondylotic myelopathy and four control cases. Complementary studies were conducted using a mouse model of cervical spondylotic myelopathy (twy/twy mice that develop spontaneous cord compression at C2–C3). We observed Fas-mediated apoptosis of neurons and oligodendrocytes and an increase in inflammatory cells in the compressed spinal cords of patients with cervical spondylotic myelopathy. Furthermore, neutralization of Fas ligand with a function-blocking antibody in twy/twy mice reduced neural inflammation at the lesion mediated by macrophages and activated microglia, glial scar formation and caspase-9 activation. It was also associated with increased expression of Bcl-2 and promoted dramatic functional neurological recovery. Our data demonstrate, for the first time in humans, the potential contribution of Fas-mediated cell death and inflammation to the pathobiology of cervical spondylotic myelopathy. Complementary data in a murine model of cervical spondylotic myelopathy further suggest that targeting the Fas death receptor pathway is a viable neuroprotective strategy to attenuate neural...
Degeneration and optimize neurological recovery in cervical spondylotic myelopathy. Our findings highlight the possibility of medical treatments for cervical spondylotic myelopathy that are complementary to surgical decompression.

**Keywords:** cervical spondylotic myelopathy; Fas-mediated apoptosis; inflammation

**Abbreviations:** CSM = cervical spondylotic myelopathy; GFAP = glial fibrillary acidic protein; TUNEL = terminal deoxynucleotidyl transferase dUTP nick end labelling

**Introduction**

Cervical spondylotic myelopathy (CSM) is the most common cause of spinal cord impairment in industrialized countries and is characterized by spinal cord compression due to spondylosis, degenerative disc disease or ossification of the posterior longitudinal ligament (Bohlman and Emery, 1988; Swagerty, 1994; McCormack and Weinstein, 1996; Fehlings and Skaf, 1998). The principal neuropathological features of CSM include cystic cavitation, gliosis, Wallerian degeneration of descending and ascending fibre tracts, and anterior horn cell loss (Bohlman and Emery, 1988; Swagerty, 1994; McCormack and Weinstein, 1996; Ohwada et al., 1996; Fehlings and Skaf, 1998). Clinically, CSM is associated with signs of spinal cord impairment including gait disturbance, clumsiness and paraesthesia of the hands, along with signs of pyramidal and posterior column dysfunction (Nakamura et al., 1999). In contrast, there is limited understanding of the biochemical and molecular mechanisms underlying the progressive neural degeneration in this condition. Despite advances in the surgical treatment for CSM, many, if not most, patients are left with substantial neurological disability related to underlying permanent structural injury to the spinal cord. Hence, the development of neuroprotective strategies that could be used as a complementary approach to surgical decompression for CSM would be of great clinical importance.

Increasing evidence in models of neurotrauma and neurodegeneration has demonstrated that Fas, a receptor known to be involved in cell death mechanisms, plays an important role in mediating neural apoptosis and provoking an inflammatory response through the release of proinflammatory cytokines (Felderhoff-Mueser et al., 2000; Desbarats et al., 2003; Demjen et al., 2004; Casha et al., 2005). Genetic Fas deficiency (Yoshino et al., 2004; Casha et al., 2005; Yu et al., 2009b), competitive inhibition of Fas activation with a CD95-Fc reagent (Ackery et al., 2006) or neutralization of Fas ligand with an anti-Fas ligand antibody markedly reduces death of neurons and oligodendrocytes and improves functional recovery of spinal injured animals and stroke (Martin-Villalba et al., 2001; Demjen et al., 2004; Ackery et al., 2006; Yu et al., 2009b). Neutralization of CD95L also reduces the initial infiltration of inflammatory cells, creating an inflammatory response that facilitates recovery of locomotor function after spinal cord injury (Letellier et al., 2010). Neutralization of Fas ligand in traumatically injured wild-type spinal cord cultures reduced the expression of truncated Bid and activation of caspase-9 (Yu et al., 2009a). Moreover, recent results from our laboratory have demonstrated a role for Fas-mediated apoptosis of neurons and oligodendrocytes in twy/twy mice, which develop spontaneous ossification of the ligamentum flavum at C2–C3, followed by progressive cord compression (Yu et al., 2009a). Based on promising results and previous work in models of traumatic spinal cord injury, we hypothesize that Fas-mediated apoptosis and inflammation play a role in spinal cord degeneration in human CSM and in twy/twy mice. In the present study, we provide for the first time, evidence that Fas-mediated apoptosis and inflammation plays a prominent role in the pathobiology of human CSM. We also, for the first time, demonstrate that neutralization of Fas ligand with a function-blocking antibody reduces cell death and inflammation and dramatically enhances functional neurological outcomes in twy/twy mice. Our data strongly suggest that the Fas receptor could be an attractive clinical therapeutic target to attenuate neural degeneration in CSM, a potential complementary treatment to surgical decompression.

**Materials and methods**

**Clinical data for human cervical spondylotic myelopathy**

The clinical and pathological data are summarized in Table 1. The principal causes of death in six patients with CSM were secondary to cardiorespiratory complications occurring within 3–10 days of surgery. One patient died from mesenteric infarction and bowel obstruction and one patient died as a result of cerebral infarction; both 6 weeks postoperatively. The causes of mortality for four control patients (2 male, 2 female: mean age 69.5 ± 6.7, range 56–85) were due to conditions unrelated to the CNS. The patients ranged from 61 to 89 years of age (6 male, 2 female: mean age 73 ± 9.7) and the time from CSM onset to death ranged from 6 months to 50 years. Motor weakness of the upper or lower extremities with spastic paraparesis and gait disturbance was seen in six patients. Sensory disturbances were observed in five patients. Hyper-reflexia of deep tendon stretch reflexes was exaggerated in all patients. CT/myelography or MRI showed multilevel spondylotic changes, with narrowing of spinal canal and cord compression in all patients with CSM.

**Morphological methods**

In all cases with CSM but one, autopsies were performed between 1 and 24h after death. In the remaining case, the autopsy was conducted at 30h post-mortem. Spinal cords were removed and fixed in 10% neutral buffered formalin. The uninjured caudal sections served as a within-case control. Sections from four non-patients with CSM were used as normal controls. Sections of paraffin tissue, cut at 5μm thickness and placed on positively charged glass slides, were
<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Sex</th>
<th>Years of disease</th>
<th>Level</th>
<th>History of CSM or other significant disorder</th>
<th>Pathological findings in spinal cord</th>
<th>Causes of death</th>
<th>Post-mortem</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>89</td>
<td>F</td>
<td>11</td>
<td>C3-8</td>
<td>Numbness of hand and fingers for 3 months, slight spastic paraparesis. Weakness of right elbow flexors. Eleven years post-cervical laminectomy</td>
<td>Patchy, focal areas of degeneration with demyelination involved corticospinal tract and dorsal roots; degeneration involving ascending posterior columns; gliosis and C5-8 anterior horn cell loss</td>
<td>Mesenteric infarction with bowel obstruction</td>
<td>1 day</td>
</tr>
<tr>
<td>2</td>
<td>61</td>
<td>M</td>
<td>8</td>
<td>C3-6</td>
<td>Marked paraesthesiae and weakness in the upper limbs, with impaired balance</td>
<td>Gliosis with cystic cavitation of central grey matter and posterior columns; moderate anterior horn cell loss; degeneration of lateral and posterior columns, dorsal roots and dorsal root entry zone</td>
<td>Four days post-anterior decompression and fusion: acute myocardial infarction</td>
<td>1 day</td>
</tr>
<tr>
<td>3</td>
<td>75</td>
<td>F</td>
<td>&gt;5</td>
<td>C3-6</td>
<td>Gait ataxia treated by laminectomy 10 years previously</td>
<td>Focal degenerative lesions in lateral and anterior columns</td>
<td>Pneumonia</td>
<td>1 day</td>
</tr>
<tr>
<td>4</td>
<td>83</td>
<td>M</td>
<td>0.5</td>
<td>C3-4</td>
<td>Progressive spastic paraparesis; no surgery</td>
<td>Section showed demyelination and gliosis of the lateral columns</td>
<td>CVA; pituitary infarction</td>
<td>1 day</td>
</tr>
<tr>
<td>5</td>
<td>66</td>
<td>M</td>
<td>3–4</td>
<td>C5-T1</td>
<td>Progressive four limb numbness and weakness (R &gt; L); severe posterior column dysfunction</td>
<td>Marked anterolateral compression and degeneration and demyelination of corticospinal tracts, posterior and dorsal columns, focal in C5-T1; schwannosis; severe anterior horn cell loss</td>
<td>Four days post-laminectomy: acute myocardial infarction</td>
<td>12 h</td>
</tr>
<tr>
<td>6</td>
<td>73</td>
<td>M</td>
<td>4</td>
<td>C4-6</td>
<td>Progressive dysesthesia and stiffness in the shoulders, arms and legs; gait ataxia; diffuse limb hyper-reflexia</td>
<td>Severe loss of myelinated axons in posterior and lateral columns; loss of motoneurons; spongy degeneration of neuropil</td>
<td>One day post-anterior decompression and fusion: pulmonary oedema and cardiac arrest</td>
<td>6 h</td>
</tr>
<tr>
<td>7</td>
<td>63</td>
<td>M</td>
<td>10</td>
<td>C2-7</td>
<td>Progressive gait difficulties and tetraparesis; dorsal column dysfunction; CT: cervical cord compression with severe spongiosification and ossification of posterior longitudinal ligament</td>
<td>Degeneration of the posterior columns and corticospinal tracts; moderate ventral horn cell loss</td>
<td>Six weeks post-anterior decompression and fusion: respiratory compromise and subsequent cardiorespiratory arrest</td>
<td>1 h</td>
</tr>
<tr>
<td>8</td>
<td>74</td>
<td>M</td>
<td>50</td>
<td>C5-T2</td>
<td>Progressive spastic paraparesis 5 years; wasting and weakness of left hand in intrinsic; hyper-reflexia of lower limbs; upgoing plantar responses</td>
<td>Severe anterior horn neuronal loss and gliosis in C7-T1; ascending and descending Wallerian degeneration</td>
<td>Three days post-anterior decompression and fusion: cardiac arrest</td>
<td>1 day</td>
</tr>
<tr>
<td>9</td>
<td>56</td>
<td>M</td>
<td>–</td>
<td>–</td>
<td>Guillain–Barre syndrome</td>
<td>NIL</td>
<td>Respiratory failure</td>
<td>12 h</td>
</tr>
<tr>
<td>10</td>
<td>61</td>
<td>F</td>
<td>–</td>
<td>–</td>
<td>Type I diabetes mellitus</td>
<td>NIL</td>
<td>Right transverse sinus thrombosis post-resection of right vestibular schwannoma</td>
<td>30 h</td>
</tr>
<tr>
<td>11</td>
<td>76</td>
<td>F</td>
<td>–</td>
<td>–</td>
<td>NIDDM, atrial fibrillation, congestive cardiac failure</td>
<td>NIL</td>
<td>Congestive cardiac failure/arrest</td>
<td>8 h</td>
</tr>
<tr>
<td>12</td>
<td>85</td>
<td>M</td>
<td>–</td>
<td>–</td>
<td>Dementia, pleural effusion, atherosclerosis, peptic ulcer, prostatomegaly, nephrosclerosis</td>
<td>NIL</td>
<td>Respiratory failure</td>
<td>12 h</td>
</tr>
</tbody>
</table>

CVA = cerebrovascular accident; L = left; NIDDM = non-insulin-dependent diabetes mellitus; R = right.
retrieved from the tissue archive of the Pathology Department, University Health Network, Toronto, Canada. Two tissue sections per case, taken from both the epicentre of compression and the uninjured caudal region were used for morphological assessment with haematoxylin and eosin, and Luxol fast blue. The remaining sections were used for immunohistochemistry.

**Immunohistochemical analysis for human cervical spondylotic myelopathy**

After deparaffinization in xylene and rehydration through a series of graded ethyl alcohols for human sections, sections from the epicentre of compression and from caudal regions of CSM and control cases were treated by microwaving in 10 mM citrate buffer pH 6.0 for 10 min and rinsed three times for 5 min in phosphate-buffered saline. Endogenous peroxidase from deparaffinized sections from human CSM was inactivated by treatment with 0.3% H2O2 in phosphate-buffered saline for 30 min. Sections were blocked with 1% bovine serum albumin and 5% non-fat milk with 0.3% Triton X-100 for 1 h. Slides were incubated in blocking solution overnight at 4°C with the following primary antibodies: rabbit anti-Fas and anti-Fas ligand (1:100; Santa Cruz Biotechnology), anti-β-amyloid precursor protein (β-APP; 1:4000, Chemicon International Inc) to label damaged axons; anti-PG-M1 (CD68: 1:50, Dako), directed against CD68, a lysosomal protein expressed by phagocytic macrophages of microglial and mononuclear origin; anti-ionized calcium binding adaptor molecule 1 (Iba1), expressed in both ramified and activated microglia (polyclonal, 1:300 Wako Pure Chemical Industries); anti-CD3 (1:200, Dako) recognizing human mature T lymphocytes; and anti-myeloperoxidase (MPO; 1:50, Dako), a marker of all neutrophils, and matrix metalloproteinase-9 (MMAP-9; 1:100, Chemicon), a proinflammatory protease, activated caspase-3 (1:200, Cell Signaling Technology), activated caspase-7 and -9 (1:150 and 1:1500 Novo Nordisk) recognizing human activated caspases; anti-PG-M1 (CD68: 1:50, Dako), directed against CD68; anti-ionized calcium binding adaptor molecule 1 (Iba1), expressed in both ramified and activated microglia (polyclonal, 1:300 Wako Pure Chemical Industries); anti-CD3 (1:200, Dako) recognizing human mature T lymphocytes; and anti-myeloperoxidase (MPO; 1:50, Dako), a marker of all neutrophils, and matrix metalloproteinase-9 (MMAP-9; 1:100, Chemicon), a proinflammatory protease, activated caspase-3 (1:200, Cell Signaling Technology), activated caspase-7 and -9 (1:150 and 1:1500 Novo Littleton, respectively). Following extensive rinsing in 0.1 M phosphate-buffered saline, sections were incubated in biotinylated goat anti-mouse or anti-rabbit antibody (diluted 1:200, Vector Laboratories) for visualization of antibody binding and to void autofluorescence in human materials. Images were captured using Nikon Eclipse 8000 light and Zeiss LSM 510 META confocal microscopes (Thornwood).

**Twy/twy mouse model of cervical spondylotic myelopathy**

Twy/twy mice were obtained from a breeding colony of the Central Institute for Experimental Animals, Kanagawa, Japan. Mutant twy/twy mice were maintained by brother–sister mating of heterozygous Institute of Cancer Research mice (+/twy) (Uchida et al., 1998; Yu et al., 2009a). These mice, which harbour an autosomal recessive mutation in the NPPS gene, develop progressive spinal cord dysfunction secondary to extradural calcified deposits at C2–C3 with cervical cord compression.

**Administration of anti-Fas ligand antibody in twy/twy mice**

Homozygous twy/twy mice were identified by a characteristic tip-toe walking pattern that typically commences at 3 weeks of age and profound motor parasthesis that progresses between 4 and 7 months. Four-month-old twy/twy mice received the anti-Fas ligand antibody (CD178; anti-Fas ligand) (MFL3; BD Bioscience) as functional grade purified antibody, which is host/isotype from Armenian hamster IgG, 50 μg intra-peritoneally twice weekly for 4 weeks; plain control mice (saline), received artificial CSF twice weekly for 4 weeks as the vehicle control and IgG control mice (IgG) received 50 μg hamster...
IgG1-isotype monoclonal antibody intra-peritoneally twice weekly for 4 weeks. All treatments were performed in a double-blind manner.

**Footprint analysis**

Functional neurological recovery was assessed by quantitative footprint analysis at weekly intervals for the 4-week treatment. Footprint analysis was modified from the method described by de Medinaeli et al. (1982). Briefly, the animals’ forepaws and hindpaws were immersed in non-toxic red (forepaw) and green dyes (hindpaw). The mice (n = 12/group) were then permitted to walk across a narrow wooden board, with dimensions ~1 m long and 7 cm wide, from a brightly illuminated starting box leading to a darkened box containing their familiar housing mates. Before collecting the footprints, each mouse was allowed to freely explore the runway for 5 min. Footprint recording took place when the mouse was able to run along the runway at a steady pace and in a straight line. To collect the footprints, a fresh sheet of white paper was placed on the floor of the runway for each mouse. Toe spread was measured as the distance between the first and fifth toe in the forepaw and hindpaw as expression in centimetre, while interlimb coordination was measured as the distance between the ipsilateral forepaw (centre of pad) and hindpaw (centre of pad) and is expressed in centimetre. Stride length (the mean distance between each footprint) was determined by drawing a line between each ipsilateral forepaw footprint and measuring its length; stride width (the mean distance between left and right footprints) was determined by measuring the perpendicular distance of a given footprint to the line connecting its opposite preceding and proceeding footprints (Carter et al., 1999; Pallier et al., 2009). For each paw of each mouse, a set of four consecutive footprints was used (those from the initiation and finishing of the run were ignored). The mean value of stride length, stride width, toe spread and inter-limb coordination was averaged to a single value per mouse (from 3 to 5 samples) and then divided by the values from the first week of treatment and expressed as the percentage of change. The mean of the percentage of change for each mouse (n = 12/group) was used for statistical comparison. Values are expressed as the mean ± SD.

**Body weight measurements**

As a measure of general health, body weights were assessed at the beginning and end of the 4-week treatment period.

**Immunohistochemical analysis for twy/twy mice**

Twy/twy mice were perfused transcardially with 4% paraformaldehyde solution under deep anaesthesia with sodium pentobarbital (50 mg/kg Somnotol; MTC Pharmaceuticals). Cervical spinal cord segments containing the compressed region were dissected, post-fixed using 10 and 20% sucrose solutions and then embedded within Tissue-Tek Optimal Cutting Temperature compound (Sakura Finetek). Serial transverse sections of 14 μm thickness were cut and mounted onto gelatin-subbed slides and stored at ~80 °C. The site of injury was defined by Luxol fast blue and haematoxylin and eosin staining of the tissue sections at 500 μm intervals. Sections were blocked in a blocking solution (0.3% Triton X-100, 5% milk and 1% bovine serum albumin in phosphate-buffered saline) for 1 h and incubated with astrocyte-selective rabbit anti-GFAP (1:100; Sigma-Aldrich), macrophage/microglia-selective rat anti-F4/80 (Santa Cruz Biotechnology) and rabbit anti-ionized calcium binding adaptor molecule 1 (Iba1, Wako Chemicals USA) antibodies in blocking solution overnight at 4 °C. The slides were washed in phosphate-buffered saline three times and incubated with fluorescent Alexa 594 or 488 anti-mouse, anti-rabbit or anti-rat secondary antibodies (1:200; Sigma-Aldrich) for 1 h. Staining specificity was determined both by omitting the primary antibody and by competing the primary antibody with its corresponding peptide prior to incubation.

**Western blotting in twy/twy mice**

Spinal cord samples of 1 cm length centred at the injury site were extracted from twy/twy mice (n = 5 per group) for total proteins. Spinal cords were individually homogenized in a buffer (5 mM Tris-HCl, 4 mM EDTA, 1 μM peptatin, 100 μM leupeptin, 100 μM phenylmethylsulphonylfluoride and 10 μg/ml aprotinin) at 4 °C. All protein samples were resolved (20–50 μg per lane) in a 12% sodium dodecyl sulphate–polyacrylamide gel at 200 V. Proteins were transferred to a nitrocellulose membrane. Membranes were blocked with 5% non-fat milk in Tris–buffered saline–TWEEN-20 for 1 h and incubated with: (i) rabbit anti-NF200 (1:1000, Sigma); (ii) mouse anti-microtubule associated protein-2 (MAP2, 1:200, Sigma); (iii) mouse anti-β-III tubulin (1:400, Chemicon); (iv) rabbit anti-caspase-9 (Cell Signalling Technology); (v) Bcl-2 (1:200, Cell Signalling); (vi) rabbit anti-GFAP (1:1000, Chemicon) and (vii) rabbit anti-Iba1 antibody (1:400, Wako Chemicals USA). Membranes were washed and then incubated for 1 h in the presence of anti-mouse (1:4000) or anti-rabbit (1:2000) secondary antibody conjugated to horseradish peroxidase. Reaction products were visualized using an ECL western blot detection kit (Amersham Biosciences Inc) and exposed to film. To quantify the amount of protein, the bands were determined to be within the linear range of the radiographic film, and optical densities were then determined by measuring the integrated optical density across the band using Gel Pro analysis software (Media Cybernetics). Densitometric values were normalized to those of β-actin (1:400; Sigma). Western blot analysis confirmed Iba1, GFAP, Caspase-9, NF200 and anti-β-III tubulin expression derived from Institute of Cancer Research, saline, anti-Fas ligand (n = 5/group) and IgG (n = 6 groups) mice. The mean value of each sample from 3 to 5 repeat experiments was averaged to a single value per mouse for Iba1, GFAP, Caspase-9, NF200 and β-III tubulin expression to statistical comparison and expressed as the mean ± SD ratio.

**Cell quantification for human cervical spondylotic myelopathy**

All digital images were acquired in a double-blind manner. Digital images were captured from two random fields per section from the epicentre of compression and uncompRESSED caudal region of cases with CSM, and control cases using a Nikon Eclipse E800 light microscope ×20 objective for CD68 and Iba1-positive cells and ×10 objective for Fas, Fas ligand and MAP2-positive neurons. We counted digital images of CD68, Iba1, Fas, Fas ligand and MAP2-positive cells in two fields per section using ImageJ software (developed at the National Institute of Health). Values from two fields were averaged to a single value per case for CD68, Iba1, Fas, Fas ligand and MAP2. The results were expressed as the number of CD68 and Iba1-positive cells and the number of Fas, Fas ligand and MAP2-positive neurons per ×20 or ×10 field. To assess the number of TUNEL-positive cells, digital images were captured in five random fields per section from the epicentre of compression and uncompressed caudal regions of cases with CSM, and control cases in spinal cord grey and white matter at ×20 objective using a Nikon Eclipse E800 light microscope. We then counted the number of TUNEL-positive cells and expressed as total values per case in grey matter and white matter, respectively.
Statistical analysis

Significant differences in cell counts were analysed with repeated measures ANOVA and t-test using the Statistical Package for the Social Sciences (SPSS SigmaStat 3.0) (Aspire Software International). All data are expressed as mean ± SD. The criterion for significance was set at P < 0.05.

Results

Clinical data

The clinical and pathologic data are summarized in Table 1. The ages of the patients ranged from 61 to 89 years (mean age 73 years), and time from CSM onset to death ranged from 6 months to 50 years.

Histopathological findings in human cervical spondylotic myelopathy

We analysed cross-sections of the cervical cord obtained from eight autopsied patients whose post-mortem interval ranged between 1 and 30 h. Luxol fast blue staining revealed uniformly myelinated tracts of white matter in control cases (Fig. 1). However, flattening and indentation of the spinal cord and severe anterior and posterior horn atrophy, neuronal loss, axonal loss and swellings, myelin pallor, gliosis and vacuolations were observed in the compressed epicentre from the six most severely damaged spinal cords of patients with CSM. Demyelination was observed in the compressed epicentre and severe myelin pallor in the white matter at the compressed epicentre of the spinal cords from patients with CSM (Fig. 1J and K). Between 4 and 10 years after the onset of CSM, Fas-positive motoneurons were marked loss of neurons in the compressed epicentre were observed when compared with control cases.

Expression of Fas ligand in the compressed epicentre of human cervical spondylotic myelopathy

To determine whether chronic spinal cord compression induces Fas expression, we performed immunohistochemistry with an anti-Fas antibody. A small number of Fas-positive cells were observed in control spinal cords (Fig. 2A) and in the caudal spinal cord of cases with CSM (Fig. 2B). In contrast, we observed many Fas-positive cells in the grey and white matter at the epicentre of all CSM spinal cords. Double-labelling with Fas and cell-specific markers revealed Fas immunoreactivity in neurons (Fig. 2C), astrocytes (Fig. 2D), microglia/macrophages (Fig. 2E) and oligodendrocytes (Fig. 2F) in the compressed epicentre of spinal cords from cases with CSM. Between 6 months and 4 years after the onset of CSM, Fas-positive cells mainly consisted of macrophages and astrocytes at the epicentre of compression in CSM spinal cords. Between 10 and 50 years after the onset of CSM, Fas-positive motoneurons could still be observed in the epicentre of CSM spinal cords but not in the uninjured caudal regions of CSM spinal cords. Furthermore, the number of Fas-positive neurons (10.2 ± 5.76) was significantly higher in the injury epicentre when compared with control spinal cords and the caudal spinal cord of cases with CSM (1.5 ± 0.75, P = 0.002) (Fig. 2L).

Expression of Fas ligand in the compressed epicentre of human cervical spondylotic myelopathy

To determine whether chronic spinal cord compression induces Fas ligand expression, we employed immunohistochemistry with an anti-Fas ligand antibody. We found little Fas ligand immunoreactivity in control spinal cords (Fig. 2G). However, in the non-compressed caudal regions of spinal cords from cases with CSM, there was a slight increase in the number of Fas ligand-positive cells (Fig. 2H). By 6 months after CSM, we observed many Fas ligand-positive cells in the compressed spinal cord of cases with CSM. By double labelling with Fas ligand and cell-specific markers, we determined that Fas ligand immunoreactivity was mainly expressed in neurons (Fig. 2I) and astrocytes (Fig. 2J) in the epicentre of CSM spinal cords. Between 4 and 50 years after the onset of CSM, despite markedly reduced...
expression of Fas ligand-positive astrocytes when compared with 6 months, we noted a moderate increase in the number of activated microglia/microphages in degenerated white matter (Fig. 2K). Furthermore, the number of Fas ligand-positive neurons was significantly higher in the compressed epicentre (5.85 ± 3.38) in all eight cases of CSM when compared with control spinal cords or the caudal spinal cord from cases with CSM (P = 0.001) (Fig. 2M).

Neural apoptosis is prominent in the compressed cervical cord in human cervical spondylotic myelopathy

To determine whether the chronic non-traumatic spinal cord injury caused by CSM induces apoptosis, we employed TUNEL labelling. We observed no TUNEL-positive cells in control spinal cords (Fig. 3A)
and only a small number of TUNEL-positive cells in the caudal spinal cord of cases with CSM (Fig. 3B). We observed that most apoptotic cells were randomly distributed in the epicentre of compression in CSM spinal cords at 6 months after CSM diagnosis. Remarkably, 50 years after the onset of CSM, TUNEL-positive cells could still be seen in the cervical cord of cases with CSM. Quantitative analysis showed a significant increase in the number of TUNEL-positive cells in white matter and grey matter in the lesion epicentre of cases with CSM when compared with caudal regions of the cord from cases with CSM or the cervical cord from control cases \( (P < 0.05) \), Fig. 3D). We performed double labelling with cleaved caspase-3, TUNEL and cell specific markers to determine which cell types were undergoing apoptosis. We confirmed the presence of myelin basic protein-positive oligodendrocytes that co-expressed cleaved caspase-3 (Fig. 3E), TUNEL-positive neurons (Fig. 3F) and microglia/macrophages (Fig. 3G) in the lesion epicentre of cases with CSM. Quantification with cell specific labelling revealed that 12% of the TUNEL-positive cells were neurons, 19% were oligodendrocytes and 29% were macrophages. Clear double labelling could not be identified in the remaining 40% of cells, potentially due to progressive degradation of cellular integrity due to pre-mortem biological effects or due to length of storage in paraffin blocks/pathology archive. Furthermore, we also found activated caspase-3 positive cells at 6 months (Fig. 3H) and 50 years (Fig. 3I) and activated caspase-9 (Fig. 3J) and -7 (Fig. 3K) positive cells in the epicentre of CSM spinal cords.

**Fas/Fas ligand-mediated apoptosis in human cervical spondylotic myelopathy**

To confirm that Fas expression is associated with apoptotic cell death, we performed double-labelling experiments with TUNEL and anti-Fas antibody. We observed that Fas-positive cells...
Figure 3 Apoptosis and Fas mediated apoptosis in the spinal cords of humans with CSM. No TUNEL-positive cells were observed in control cases and a few could be seen in caudal to CSM lesion (A and B). We observed that most apoptotic cells were distributed in the epicentre of CSM spinal cords (C) at 6 months. The quantification of apoptotic cells showed a significant increase in the number of TUNEL-positive cells in CSM when compared with caudal to CSM lesion (n = 8) and (n = 4) control cases (P < 0.05, D) (white bar = white matter; black bar = grey matter). Double labelling with cell-specific markers and cleaved caspase-3 or TUNEL was used to demonstrate TUNEL-positive oligodendrocytes (E), neurons (F) and microglia/macrophages (G) in the epicentre of compression of CSM. We observed activated caspase-3 positive cells (H and I) and activated caspase-9 (J) and -7 (K) positive cells in the epicentre of CSM spinal cords. To confirm that Fas expression is associated with apoptotic cell death, we performed double-labelling experiments with TUNEL (M) and anti-Fas antibody (L). We observed that Fas-positive cells co-labelled with TUNEL in the white matter of spinal cords from patients with CSM (N).
co-labelled with TUNEL (Fig. 3N) in the white matter of spinal cords from patients with CSM.

**Time course and Fas-mediated inflammatory response in human cervical spondylotic myelopathy**

To determine the role of Fas ligand in the modulation of immune reactions involving macrophages, immunohistochemistry was undertaken with antibodies to Iba1 (resident microglia/macrophages), CD68 (activated macrophages), CD3 (T-lymphocytes), myeloperoxidase (neutrophils) and MMP9 (matrix metalloproteinases). We demonstrated that Iba1 and CD68 positive microglia/macrophages was significantly greater in the epicentre of CSM spinal cords when compared with controls ($P = 0.003$ and $P = 0.002$, respectively, D) ($n = 8$ cases of CSM and $n = 4$ control cases). No MMP9 positive cells were observed in control cases and caudal to CSM lesion (E). However, MMP9-positive cells were also observed in the epicentre of compression (F). There are few CD3 and myeloperoxidase (MPO) positive cells in control case (G and J) and caudal (H and K) to CSM lesion. However, many myeloperoxidase (I) positive cells and CD3 (L) positive lymphocytes were found throughout the lesioned spinal cord parenchyma in a human subject with a 6 month history of CSM.
degenerated white matter of spinal cords 6 months after the onset of CSM. Four years after CSM, many Iba1 and CD68 positive ramified microglia could be seen. At 10 and 50 years, despite the reduced expression of CD68 positive, lipid-laden, ‘foamy’ macrophages, a moderate increase in the number of activated microglia/macrophages was observed in areas of axonal degeneration in the white matter. The number of CD68 positive microglia/macrophages was significantly higher in the compressed epicentre (47.14 ± 23.41) of CSM spinal cords when compared with caudal regions (14 ± 8.51) of CSM and normal spinal cords (2.25 ± 1.71) (P = 0.003, Fig. 4D). We found that the number of Iba1-positive microglia/macrophages was significantly higher at the epicentre of CSM spinal cords when compared with the number of CD68 positive microglia/macrophages (P = 0.002, Fig. 4D). Furthermore, we also observed several MMP9-positive neurons and macrophages in the epicentres of CSM spinal cords at 6 months (Fig. 4F) but not in control cases (Fig. 4E). To confirm our findings with regards to Fas-mediated inflammation, we carried out double-labelling with CD68, Fas and Fas ligand antibodies. We identified the colocalization of Fas with CD68 (Fig. 2E) and Fas ligand with CD68 (Fig. 2K) in the compressed epicentre of CSM spinal cords. We found no myeloperoxidase-positive cells in control cases or in caudal regions of CSM spinal cords (Fig. 4G and H). However, at 6 months after CSM, we observed numerous myeloperoxidase-positive cells distributed throughout the spinal cord parenchyma, including phagocytic foamy macrophages and many intensely stained ramified and activated microglia/macrophages (Fig. 4I). Four years after CSM, many myeloperoxidase positive-activated microglia were distributed in the spinal cord parenchyma. At 50 years of CSM, only scattered solitary myeloperoxidase-positive cells were seen in the spinal cord parenchyma. Furthermore, there were few CD3-positive cells in control cases and caudal regions (Fig. 4J and K) of CSM spinal cords. Six months after CSM, however, many CD3-positive lymphocytes were seen; mostly throughout the spinal cord parenchyma (Fig. 4L). Four and 10 years after CSM, small groups of CD3-positive cells were observed to be randomly distributed in the spinal cord parenchyma, near blood vessels, and in the perivascular space at the margin of the cystic cavity. Fifty years after CSM, we detected scattered solitary CD3-positive cells in the spinal cord parenchyma.

**Macrophage/microglial infiltration in twy/twy mice**

We observed numerous Iba1-positive microglia/macrophages distributed in the twy/twy compressed epicentre (Fig. 5B saline and 5C IgG) in comparison with normal control mice (Fig. 5A normal). Neutralization of Fas ligand by a function-blocking antibody in twy/twy mice reduced the number of Iba1-positive macrophages and reactive microglia at the lesion sites relative to saline controls by immunohistochemistry (Fig. 5D). Western blot analysis demonstrated a reduction in levels of Iba1-protein expression in twy/twy mice treated with a function-blocking antibody relative to saline control and IgG controls in twy/twy mice (Fig. 5I and J).

**Glial fibrillary acidic protein-positive gliosis in twy/twy mice**

We have previously reported a significant increase in the number of the GFAP-positive astrocytes, which were conspicuously present within the grey matter of the twy/twy lesioned area (Yu et al., 2009a) in comparison to normal control mice (Fig. 5E, normal). In the present study, we found an increased number of GFAP-positive astrocytes (Fig. 5F saline and 5G IgG) of twy/twy mice. However, neutralization of Fas ligand by a function-blocking antibody in twy/twy mice reduced the number of the GFAP-positive astrocytes (Fig. 5H anti-Fas ligand) and levels of GFAP protein expression when compared with saline control of twy/twy mice by immunohistochemistry and western blot analysis (Fig. 5I and K).

**Apoptosis in twy/twy mice**

This is consistent with previous results obtained from our laboratory showing a significant increase in activation of caspase-9 expression in twy/twy mice compared with normal control mice (Yu et al., 2009a). Of note, neutralization of Fas ligand in the twy/twy mouse model of CSM resulted in a reduction in caspase-9 activation (Fig. 5L and M) and increase in Bcl-2 expression (Fig. 5L and N) compared with IgG controls by western blot analysis.

**Changes of neurons and axons in twy/twy mice**

Levels of NF200, consistent with previous results obtained from our laboratory, showing expression in twy/twy mice were significantly decreased relative to normal control mice (Yu et al., 2009a). Neutralization of the Fas ligand with a function-blocking antibody in twy/twy mice reduced loss of neurons and axons as determined by western blot analysis with NF200 (Fig. 5O and P) and β-III tubulin (Fig. 5O and Q) antibodies compared with IgG controls of twy/twy mice.

**Neurological dysfunction in twy/twy mice**

Consistent with previous results obtained from our laboratory, footprint analysis of Institute of Cancer Research mice revealed highly coordinated forelimb and hindlimb foot placements (Fig. 6A and F). In contrast, twy/twy mice showed increased ipsilateral forelimb–hindlimb distance (interlimb coordination) when compared with normal control mice (Yu et al., 2009a). Neutralization of Fas ligand by a function-blocking antibody in the twy/twy mouse model of CSM reduced changes in inter-limb coordination at 4 weeks when compared with saline (P < 0.001) and IgG controls (P < 0.001) (Fig. 6F). Anti-Fas ligand treatment in twy/twy mice reduced per cent stride length changes when compared with IgG mice (forepaws, P < 0.003 at 2 weeks; P < 0.009 at 4 weeks) and saline (forepaw, P < 0.001 at 2 weeks; P < 0.011 at 4 weeks; Fig. 6G). Anti-Fas ligand treatment in twy/twy mice also prevents the progression of locomotor dysfunction relative to saline of twy/twy mice as measured by per cent change of toe spread at 3 weeks (forepaw: P < 0.003;
Furthermore, anti-Fas ligand treatment in twy/twy mice limited changes in per cent stride width in the forepaws when compared with saline-treated animals \( (P < 0.016 \text{ at 3 weeks; } P < 0.003 \text{ at 4 weeks}) \) and IgG treatment \( (P < 0.025 \text{ at 4 weeks}) \) (Fig. 6I). While the toe spread and stride width of the hindpaws in anti-Fas ligand treatment mice did not differ from that of their saline and IgG littermates. All data are from Institute of Cancer Research, saline, IgG and anti-Fas ligand treatment in twy/twy mice \( (n = 12 \text{ mice/groups}) \). Moreover, neutralization of Fas ligand by a function-blocking antibody in the twy/twy mouse model of CSM attenuated weight loss \( (P < 0.042) \) and improved functional neurological recovery relative to IgG control \( (P < 0.042) \) of twy/twy mice. The data suggest that intervention to inhibit the Fas pathway may substantially improve outcome and prevent the progression of locomotor dysfunction.

**Figure 5** Neutralization of Fas ligand in the twy/twy mice reduces inflammation, apoptosis and neurodegeneration. There is an increased number of Iba1 positive microglia/macrophages in saline (B) and IgG (C) of twy/twy mice when compared to Institute of Cancer Research (ICR) control mice (A). We also found increased the number of GFAP-positive astrocytes in saline (F) and IgG (G) of twy/twy mice when compared to ICR control mice (E). However, anti-Fas ligand treatment in twy/twy mice reduced the number of Iba1-positive microglia/macrophages (D) and GFAP-positive astrocytes (H) when compared with saline and IgG of twy/twy mice, as demonstrated by immunohistochemistry. Using western blotting with Iba1, GFAP, caspase-9, Bcl-2, NF200 and β-III Tubulin antibodies, anti-Fas ligand (FasL) treatment in twy/twy mice reduces densitometry of Iba1 (I and J), GFAP (I and K), caspase-9 activation (L and M) expression and increases Bcl-2 expression (L and N), promotes the preservation of neurons and axons \( (O, P \text{ and } Q) \) as compared with saline and IgG in twy/twy mice. Densitometric values were normalized to those of β-actin \( (1:400; \text{Sigma}) \). Western blot analysis confirmed Iba1, GFAP, caspase-9, NF200 and Tubulin expression derived from ICR saline, anti-Fas ligand \( (n = 5 \text{/group}) \) and IgG \( (n = 6 \text{ groups}) \) mice. The mean of each sample from several \( (3–5) \) experiments was used for statistical comparison and expressed as the mean ± SD ratio.
Discussion

Our data provide strong evidence implicating a role for Fas-mediated apoptosis in the pathobiology of CSM. The human neuropathology data point to the association of Fas expression with ongoing neuronal and oligodendroglial apoptosis, which is further characterized by persisting inflammation, demyelination and neuronal/axonal loss. Moreover, blocking Fas ligand with a Fas function-blocking antibody in a murine model of CSM dramatically improves neurobehavioural function, attenuates Fas-mediated neural apoptosis and reduces the neuropathological consequences of cord compression. These data strongly suggest that targeting the Fas pathway is an attractive therapeutic target to treat patients with CSM, which is complementary to existing surgical decompressive strategies.
A growing body of evidence demonstrates that apoptosis plays an important role in determining neurological outcome after spinal cord injury based on studies in humans and clinically relevant animal models (Li et al., 1996, 1999; Crowe et al., 1997; Emery et al., 1998; Lou et al., 1998; Springer et al., 1999; Casha et al., 2001; Keane et al., 2001). The death receptor Fas has gained widespread recognition as a critical regulator of apoptosis (Felderhoff-Mueser et al., 2000; Desbarats et al., 2003; Demjen et al., 2004; Casha et al., 2005) following spinal cord injury. We and others have shown that neuronal, oligodendrocytic and microglial apoptosis, through activation of the Fas death receptor pathway, is a key event following spinal cord injury (Casha et al., 2001; Zurita et al., 2001; Yamaura et al., 2002; Demjen et al., 2004; Yoshino et al., 2004) and in animal models of CSM (Yu et al., 2009a). Fas deficiency (Yoshino et al., 2004; Casha et al., 2005), the neutralization of endogenous Fas ligand (Demjen et al., 2004; Yu et al., 2009a) and deletion of Fas ligand in myeloid cells (Letellier et al., 2010) have been shown to decrease apoptosis in neurons and oligodendrocytes and to greatly decrease the number of neutrophils and macrophages infiltrating the injured spinal cord and improve functional recovery following spinal cord injury. Furthermore, there are significant histopathological and pathophysiological similarities between CSM and traumatic spinal cord injury (Fehlings and Skaf, 1998). We and others have shown that apoptosis of neurons and oligodendrocytes caused by activation of death-promoting caspases is observed at the most compressed site of twy/twy mice, an animal model of CSM, as well as in case reports of patients with CSM (Yamaura et al., 2002; Yu et al., 2009a). Apoptosis plays a central role in delayed demyelination and axonal dysfunction, leading to the development of neurological deterioration in CSM. In the present study, we observed a large number of apoptotic oligodendrocytes and neurons in the spinal cords of all cases with CSM. Specifically, we observed 19% neuronal apoptosis in the epicentre of compression in CSM spinal cords with a time course from 6 months to 50 years when compared with control cases and caudal regions of CSM spinal cords. Our data provide evidence that a significant consequence of spinal cord compression is the loss of cells due to apoptosis, leading to spinal cord dysfunction, which is associated with loss of neurons and oligodendrocytes, demyelination and reactive astrogliosis. There are several factors that may contribute to oligodendrocytic and neuronal apoptosis 6 months to 50 years after the onset of CSM. First, the decreases in spinal cord blood flow and ischaemia that result from cervical spinal stenosis may be an important event that initiates apoptosis (the sensitivity of neurons and oligodendrocytes) in CSM. Second, there is a significant increase in Fas/Fas ligand-positive neurons in compressed epicentre when compared with control cases and caudal regions of CSM spinal cords. We also confirmed many activated caspase-3, 7 and 9 positive neurons in the compressed epicentre of CSM spinal cords and the co-localization of Fas and TUNEL in the degenerated white matter of CSM spinal cords, which suggests that Fas-mediated apoptosis plays an important role in neuronal degeneration in CSM. Furthermore, the neutralization of Fas ligand resulted in a reduction of caspase-9 activation, demyelination, upregulation of the anti-apoptotic factor Bcl-2, and improved functional neurological recovery in twy/twy mice. These findings represent the first evidence suggesting that the targeting of a death receptor pathway is a viable neuroprotective strategy, and demonstrate that neurons and glia (specifically, oligodendrocytes) are targets for neuroprotection in CSM. Our findings may lead to better therapeutic strategies to improve neuronal and oligodendrocyte survival, and thus may improve neurological outcome in patients suffering from CSM.

The inflammatory response following neurotrauma is a complex phenomenon involving the activation of resident microglia and the infiltration of neutrophils, monocytes/macrophages and lymphocytes into the lesion from the systemic immune system (Popovitch et al., 1997; Hausmann, 2003). This leads to tissue damage, demyelination and neurological dysfunction (Giulian and Corpuz, 1993; Jones et al., 2005) as well as enhancement of Fas-mediated apoptosis of neurons and oligodendrocytes after spinal cord injury (Spanaues et al., 1998; Badie et al., 2000; Pouly et al., 2000; O’Connell et al., 2001). Fas-induced inflammatory responses in astrocytes can exacerbate Fas-mediated tissue damage by increasing the proliferation and migration of immune cells into the CNS (Choi et al., 2003). Apoptotic cells may release neurotoxic molecules and cytokines that stimulate microglia and the infiltration of neutrophils, monocytes/macrophages and lymphocytes into the lesion from the systemic immune system (Popovitch et al., 1999; Mabon et al., 2000), leading to further tissue damage, demyelination and neurological dysfunction (Giulian and Corpuz, 1993; Jones et al., 2005). Fas-induced inflammatory responses in astrocytes can exacerbate Fas-mediated tissue damage by increasing proliferation and migration of immune cells into the CNS (Choi et al., 2003). Identifying the key triggers of apoptosis in CSM, as we have shown here, and developing approaches to block these cell-death programmes, are major challenges in the field. However, the involvement of Fas activation in the initiation of apoptosis and inflammation in the setting of CSM has been less clear prior to these results. We detected Fas and Fas ligand-positive astrocytes at the epicentre of compression in the spinal cords of patients with CSM, from 6 months to 4 years after onset. This is in agreement with previous findings that have shown that astrocytes may be resistant to Fas-mediated cell death (Becher et al., 1998) and instead produce proinflammatory cytokines and chemokines upon Fas ligation, which can induce Fas-mediated neuronal and oligodendrocytic apoptosis, possibly leading to neurodegeneration and demyelination. Exclusive deletion of CD95L on myeloid cells reduced the number of neutrophils and macrophages infiltrating the injured spinal cord, reduced the death of neurons and oligodendrocytes, and improved functional recovery following spinal cord injury (Letellier et al., 2010). Evidence has shown that proinflammatory and pro-apoptotic cytokines, including TNF-a, IL-1b and Fas ligand regulate cellular events (Martin-Villalba et al., 1999) and downregulate proinflammatory cytokines, neutralization of IL-6 and IL-1 improve functional recovery after spinal cord injury (Okada et al., 2004; Akuzawa et al., 2008). We found that the neutralization of Fas ligand reduced the infiltration of macrophage and reactive microglia along with reducing glial scar formation in twy/twy mice. The depletion of macrophages/microglia (Popovitch et al., 1999; Mabon et al., 2000), reduction of myeloperoxidase activity (Hamada et al., 1996) and MMP-9 null mice (Goussev et al., 2003) result in the reduction of secondary
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demyelination and axonal loss, as well as in improved functional recovery after spinal cord injury, respectively. Fleming et al. (2006) reports that 5 days after spinal cord injury in humans, activated microglia and macrophages were the most common inflammatory cells in the spinal cord for up to 4 months (Buss et al., 2007), and a year after spinal cord injury. They also found myeloperoxidase expression for up to 1–3 days and T-cells in peak numbers between 3 and 7 days post-injury, remaining in the spinal cord for as long as 10 weeks post-spinal cord injury (Popovich et al., 1997).

In the present study, we found that activated microglia/macrophages were the predominant inflammatory cell type in all cases of CSM from 6 months to 50 years. We also observed that myeloperoxidase-positive cells and CD3-positive lymphocytes (T-cells) were seen throughout the grey and white matter in four cases of CSM from 6 months to 11 years after onset. Furthermore, we identified that Fas ligand and CD68, as well as Fas and CD68, are colocalized in the epicentre of CSM spinal cords. It is also possible that mechanical compression and ischaemia of the cervical cord segments increased the levels of Fas ligand, leading to Fas-mediated neuronal and oligodendrocytic apoptosis. Fas-induced inflammatory responses as witnessed by microglia/macrophages after the onset of CSM from 6 months to 50 years can exacerbate the Fas-mediated tissue damage by increasing inflammatory cell infiltration with consequent release of proinflammatory cytokines. In contrast to these tissue-destructive effects, microglia/macrophages and T lymphocytes also participate in the removal of injured tissue debris following spinal cord injury and in the release of protective cytokines that promote neuronal regeneration, wound healing and tissue repair (McTigue et al., 1998; Herx et al., 2000; Jones et al., 2005). This is significant because T lymphocytes can allow for early contact of the immune system with cellular debris, removal of pathogenic agents protecting neurons from degenerative conditions (Kipnis et al., 2002; Schwartz and Kipnis, 2007) and secretion of various trophic factors that are important for axonal regeneration and growth (Moalem et al., 2000). Therefore, further knowledge of the role of inflammatory cell infiltration in pathophysiological, neurochemical and molecular mechanisms of the disease course of CSM is needed.

In conclusion, we report novel evidence showing that Fas/Fas ligand-mediated apoptosis of neurons and oligodendrocytes and inflammation contributes to the pathobiology of spinal cord degeneration and affects neuronal function and survival in CSM. Neutralization of the Fas ligand using a function-blocking antibody reduces cell death, attenuates inflammation, promotes axonal repair and enhances functional neurological outcomes in rodent models of CSM. The targeting of a death receptor pathway is a viable neuroprotective strategy to attenuate neuronal degeneration and optimize neurological recovery in CSM. Our findings will open the door to the possibility of complementary treatments to surgical decompression.

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