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ORIGINAL ARTICLE

Increase in soluble CD95L during subacute phases after human spinal cord injury: a potential therapeutic target

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Study design: A pilot study measuring the levels of serum-soluble CD95 ligand (CD95L) in eight spinal cord-injured patients.
Objectives: To determine the soluble concentration of CD95L in spinal cord injury (SCI) patients after trauma.
Methods: We collected blood samples from eight patients with acute traumatic SCI. Soluble CD95L serum levels were determined using an enzyme-linked immunosorbent assay. American Spinal Injury Association (ASIA) was determined according to ASIA classification. The patients were monitored, and venous blood was drawn after arrival at the hospital on the 1st and 3rd day and during the 1st, 2nd, 4th, 8th and 12th weeks after trauma.
Results: The average patient age was 48.1 years (18–86 years). Three patients were paraplegic (two incomplete, one complete), five were quadriplegic (one complete, four incomplete). The serum concentration of soluble CD95L (sCD95L) decreased during the 1st week (41 ng l^{-1}) and increased after the 2nd week in all eight patients. It peaked during the 4th week (68.5 ng l^{-1}) and reached a plateau during the 12th week (76.2 ng l^{-1}). There are many possible explanations for not being able to detect a statistical significance, one of course being the small sample size.
Conclusion: Promising results for anti-CD95L therapy have already been documented in lab studies with rodents. Anti-CD95L blocks the pro-apoptotic and proinflammatory activity of membrane-bound CD95L during the acute phase of SCI. We observed that sCD95L levels are elevated during the subacute and intermediate phases of SCI. It would be of great interest to study a larger group of patients to determine whether higher sCD95 levels are correlated with improved or impaired neurological outcome or with increasing levels of autoimmune components in peripheral blood.
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Keywords: spinal cord injury; CD 95; paraplegy; quadriplegy; ASIA

INTRODUCTION

Spinal cord injury (SCI) is a devastating condition with high medical, psychological, social and economic consequences for patients and their families. SCI remains a challenge of paramount importance for modern medicine. Improvements to palliative treatments have been made in recent years; however, there are still no effective treatment options to reverse neurological deficiency after SCI.[1]

Current investigations seek to characterize the complex secondary damage as well as regeneration through inflammatory and apoptotic reactions after primary trauma lesions. One of the best researched inducers of apoptosis is the FAS ligand receptor system. CD95 ligand (CD95L) is a surface protein of the FAS ligand receptor system and mediates apoptosis through binding to the CD95 receptor and uncontrolled inflammation.[2]

Attempts have been made to target this protein. Letellier *et al.*[3] demonstrated promising results with anti-CD95L therapy in mice, showing that the treatment blocked the pro-apoptotic and proinflammatory activity of membrane-bound CD95 ligand (mCD95L) during the acute phase of SCI. Our investigation sought to determine whether such a therapy might be applicable to human SCI patients.

Directly following traumatic injury to the vertebra and consequent SCI there is an immediate destruction of neurons proceeded by a cascade of secondary damage with both necrotic and apoptotic cell death.[4] The secondary damage is characterized by ischemia, inflammation, glutamate excitotoxicity, oxidative stress and production of free radicals.[5] Soluble CD95L (sCD95L) is activated by cleavage of mCD95L by the serine matrix metalloproteinases MMP-7 and MMP-3.[6] sCD95L cannot trigger apoptosis, but induces the production of proinflammatory chemokine in neutrophils.[7] Therapeutic neutralization of CD95L significantly decreased apoptotic cell death after SCI.[8] Rodents treated with CD95L-specific antibodies were capable of initiating active hind-limb movements several weeks after injury. Similar results were presented in 2010 by Letellier *et al.*[3]

After SCI, blockade of CD95 leads to decreased neuronal apoptosis in rats. However, intrathecal application of soluble CD95-Fc protein,[9] systemic administration of antibodies directed against CD95L,[8] and deletion in the genome of mutant CD95 mice[10] lead to decreased apoptosis and improved functional and histopathological outcome after acute SCI.[8] In addition to pro-apoptotic effects, CD95 also has physiological functions. For example, it is involved in the branching

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of developing neurons in the central nervous system, axonal sprouting of dorsal root ganglion neurons, migration of malignant glioma cells and the differentiation of neuronal stem cells.[11]

Unfortunately, almost all previous studies are based on experiments in rodents; clinical studies in humans are rare.[12] Nevertheless, our previous investigation in bone regeneration have shown that peripheral serum blood analysis may be a valid method for detecting complex human biochemical activities.[13] Therefore, we used a protocol that we established previously in our research on bone regeneration to determine whether sCD95L serum levels were upregulated in SCI patients.

MATERIALS AND METHODS

Data were obtained from eight patients (five male, three female) suffering from acute, traumatic SCI who were admitted to the Berufsgenossenschaftlische Unfallklinik Ludwigshafen (BG Trauma Centre). The average patient age was 48.1 years (range of 18–86 years). Three patients were paraplegic (two incomplete, one complete) and five patients were quadriplegic (one complete, four incomplete). The demographic and clinical patient profiles are shown in Table 1. None of the patients received methylprednisolone or nonsteroidal antiphlogistics. Oxygesic was given for pain management.

The study was approved by the Ethics Commission of the Landesärztekammer Rheinland-Pfalz (No. 837.266.09). All participants provided written informed consent before enrollment. The control patients were healthy medical doctors randomized among the staff of the BG Trauma Centre in Ludwigshafen (10 males, 2 females; average age of 44.8).

Sample acquisition

Venous blood was collected (7.5 ml monovette, Sarstedt, Nümbrecht, Germany) following admission to the hospital at specific time points: 24 h after injury and on day 3, 7, 14, 28 and 90 after injury. Following 20 min of coagulation, blood was centrifuged at 3000 r.p.m. for 10 min and the serum supernatant obtained from serum samples was aliquoted and stored at $-80\,^{\circ}C$ until analysis of sCD95L.

Measurement of serum sCD95L

We used the Quantikine Human Fas Ligand/TNFSF6 Immunoassay (R&D Systems, Inc, Minneapolis, MN, USA) for sCD95L measurement. This test was performed strictly in accordance with guidelines given by the manufacturer. The lab technician performing the test was blinded to all patients and clinical information. C-reactive protein levels were determined by turbidimetry immunoassays, and creatinine levels were determined by the Jaffé Method (Dimension, Siemens, Munich, Germany). Leukocyte counts were determined with a Coulter Counter LH 750 (Beckmann-Coulter, Krefeld, Germany). Enzyme-linked immunosorbent assay analyses were performed twice for each patient at each time point.

Data analysis

Data analysis was performed by the Institute for Medical Biometry and Informatics at the University of Heidelberg, Germany. Statistical analyses were performed with the standard unpaired Student's *t*-test. No formal test for normality was applied because of the small sample size. Data were tested by calculating Pearson's correlation coefficients.

All data are presented as the mean ± s.e.m. Statistical significance was determined according to the following criteria: significant $^{*}P<0.05$; strongly significant $^{**}P<0.01$ and highly significant $^{***}P<0.001$. All statistical analyses were performed with SPSS software (IBM Deutschland GmbH, Ehningen, Germany).

RESULTS

Serum levels of sCD95L were variable in SCI patients directly after injury (mean of $41\,ng\,l^{-1}$). Following an initial increase in sCD95L levels, we often observed a decrease during the first 14 days after injury. During weeks 3 and 4, the level of sCD95L mostly increased and ultimately peaked between 60 and 90 days after injury (patient G was an exception). In the serum of healthy control patients, the mean sCD95L level was $52.8\,ng\,l^{-1}$. A possible explanation for a lack of statistical significance is the small sample size.

Patient data

Patient A: incomplete paraplegia; American Spinal Injury Association (ASIA) C; Th 12; fracture type A3; age 18; female. The CD95L level was $59\,ng\,l^{-1}$ 1 day (d) after injury, $59\,ng\,l^{-1}$ after 3 days and the same after 7 days. An increase in sCD95L level was first noted after 14 days ($60\,ng\,l^{-1}$). After 21 days, a further increase in the sCD95L level ($67\,ng\,l^{-1}$) was noted. On day 60 after SCI, the sCD95L level was $69\,ng\,l^{-1}$, and after 90 days the level was $106\,ng\,l^{-1}$.

Patient B: complete paraplegia; ASIA A; Th 5; fracture type A3; age 41; female. The sCD95L level was $32\,ng\,l^{-1}$ 1 day after the injury,

Table 1 Demographic and clinical patient profiles.

Patient	*Gender*	*Age*	*BMI*	*ASIA*	*Paralysis*	*Lesionsite (level)*	*Fracture description*	*Type AO*	*Conus cauda*
A	F	18	18.4	C	Incomplete paraplegia (sensomotoric)	Th 12	Th 12, L 1,2,3, and 5 spinous process fracture L 1 compression fracture L 2 bursting fracture L 3	A3.3	No
B	F	41	20.6	A	Complete paraplegia (sensomotoric)	Th 5	Th 5,6,7 fracture and arch fracture Th 8	A3.3	No
C	m	69	31.9	B	Incomplete paraplegia (sensomotoric)	Th 12	Compression fracture/bursting fracture Th 12 with involvement of the trailing edge and lumbal spinal stenosis, Th 7 fractue, transverse process fracture L 1 to L4 right	C3.2	No
D	M	38	20.8	D	Incomplete tetraplegia	C5	C5/6 rotation/compression fracture, Th 8 end plate deformity without involvement of the trailing edge	C2.2	No
E	m	41	27.5	A	Complete tetraplegia	C4/5	C5 dislocation fracture	B2.3	No
F	m	31	21.3	C	Incomplete tetraplegia	C6	Complete bursting fracture Th 12	B3.2	No
G	W	61	24.5	D	Incomplete tetraplegia	C5	Discoligamentary frakture C5/6	B3.3	No
H	M	86	26.9	C	Incomplete tetraplegia (sensomotoric)	C5	Discoligamentary frakture C4/5	B1.2	No

Abbreviations: ASIA, American Spinal Injury Association; BMI, body mass index.

32 ng l^{-1} after 3 days and after 7 days, we noted an increase in the sCD95L to 56 ng l^{-1}. On day 14, a decrease in sCD95L level to 50 ng l^{-1} was noted. 21 days after SCI, the sCD95L level increased to 66 ng l^{-1}. On day 60, a further increase of the sCD95L level to 93 ng l^{-1} was noted. In contrast to the previous patient, a decrease in sCD95L level to 79 ng l^{-1} was noted 90 days after SCI.

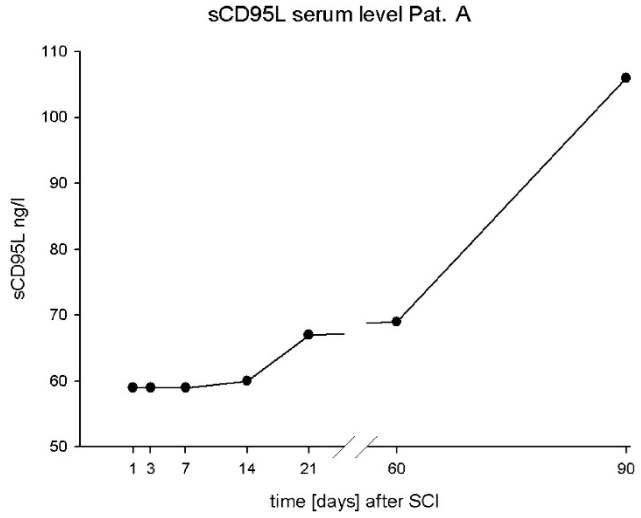
PatientC: incomplete paraplegia; ASIA B; Th 12; fracture type C3; age 69; male. The sCD95L level was 42 ng l^{-1} 1 day after SCI. Three days after SCI, the sCD95L level was 42 ng l^{-1}. Seven days after SCI, sCD95L decreased to 39 ng l^{-1}. On day 14 a further decrease in the sCD95L level to 34 ng l^{-1} was noted. On day 21 the first increase in sCD95L level to 53 ng l^{-1} was noted. After 60 days, the sCD95L level increased further to 68 ng l^{-1}.

Patient D: incomplete tetraplegia; ASIA D; C5; fracture type, C2; age 38; male. The sCD95L level was 28 ng l^{-1} 7 days after SCI and 22 ng l^{-1} after 14 days. Twenty-one days after SCI, sCD95L level increased to 42 ng l^{-1}. On day 60, the level further increased to 67 ng l^{-1}.

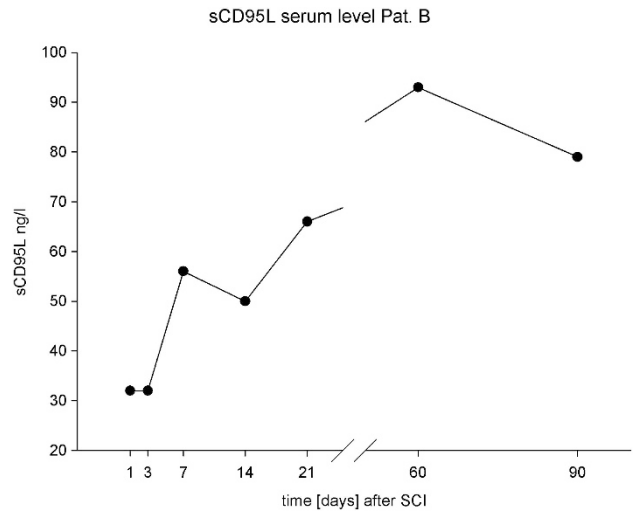
Patient E: complete tetraplegia; ASIA A; C4/5; fracture type B2; age 41; male. The sCD95L level was 31 ng l^{-1} 7 days after SCI and increased to 61 ng l^{-1} after 14 days. A further increase in sCD95L level to 63 ng l^{-1} was noted on 21 days. On day 60, the level further increased to 69 ng l^{-1}. This sCD95L level was also measured 90 days after SCI.

Patient F: incomplete tetraplegia; ASIA C; C6; fracture type B3; age 31; male. The sCD95L level was 77 ng l^{-1} 3 days after SCI. Seven days after SCI, sCD95L level decreased to 39 ng l^{-1}. On day 14, the level had increased to 72 ng l^{-1}. On day 21 after SCI, the sCD95L level was 77 ng l^{-1}, and the level remained constant until 60 days after SCI. The highest sCD95L level was detected on day 90 (91 ng l^{-1}).

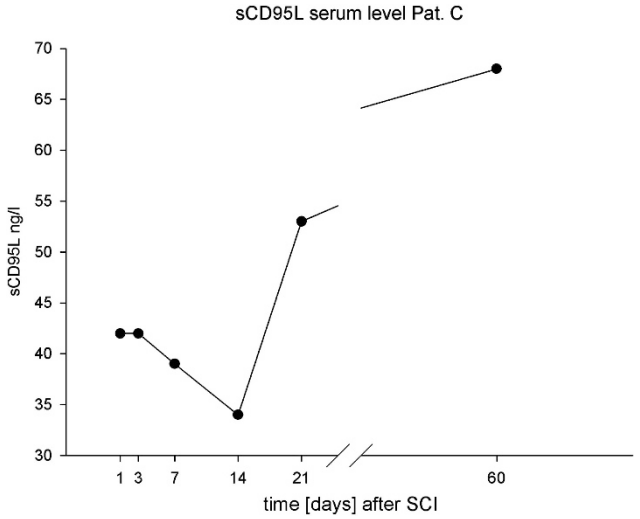
Patient G: incomplete tetraplegia; ASIA D; C5; fracture type B3; age 61; female. The sCD95L level was 84 ng l^{-1} 1 day after SCI, which was higher than that in the previous patient. Three days after SCI, sCD95L level decreased to 42 ng l^{-1}, and after 7 days it further decreased to 33 ng l^{-1}. On day 21, we noted an increase in the sCD95L level to 39 ng l^{-1}. Sixty days after SCI, the sCD95L level was 55 ng l^{-1}, and on day 90 the level slightly decreased to 53 ng l^{-1}.



Patient A



Patient B



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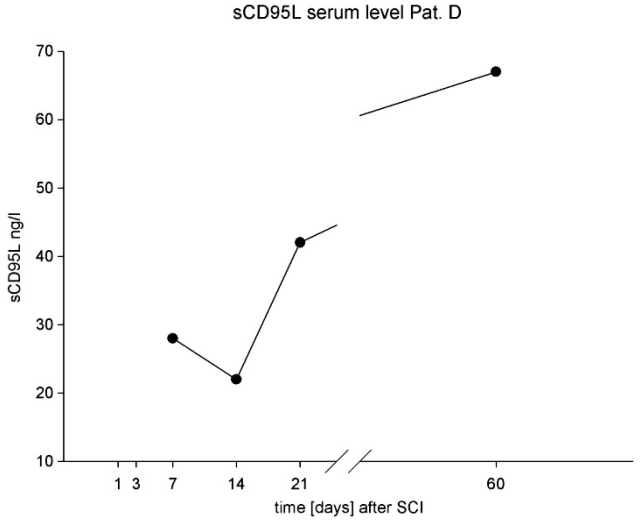
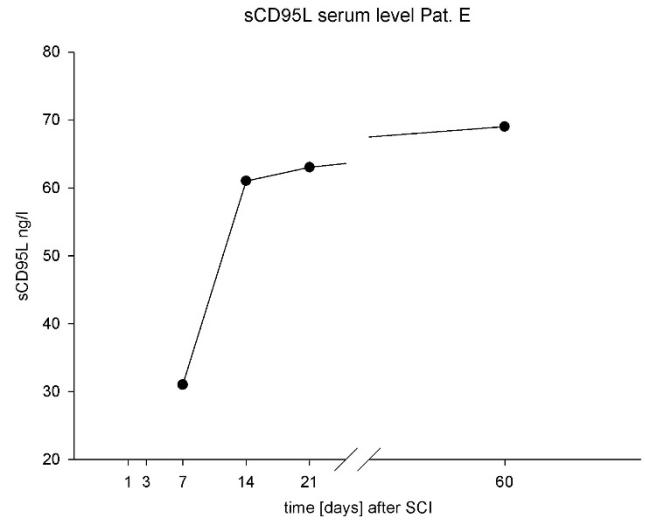
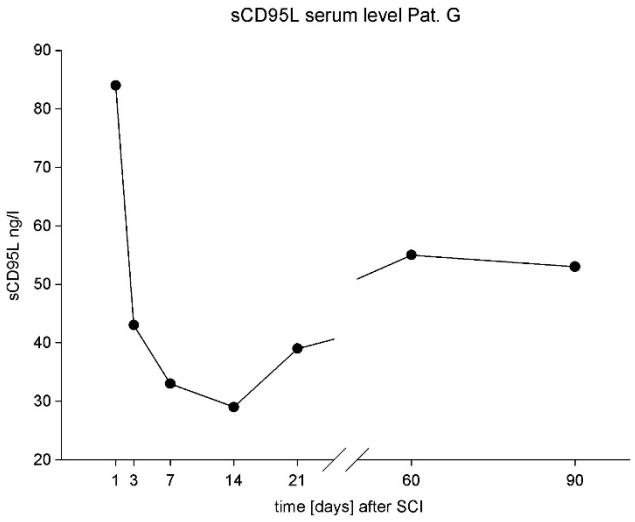


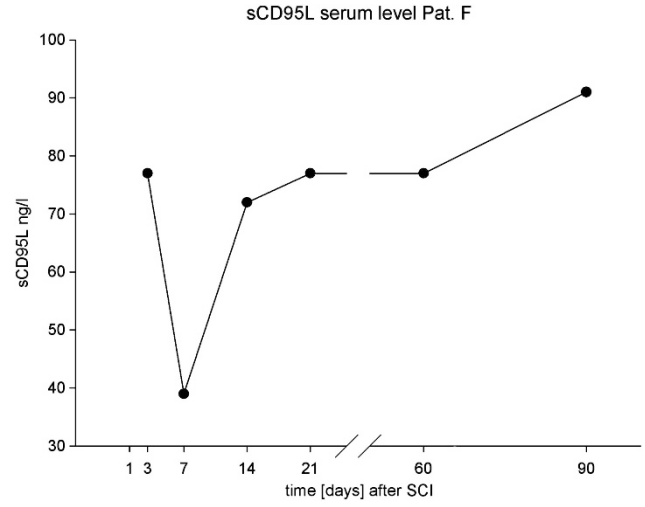
Patient D



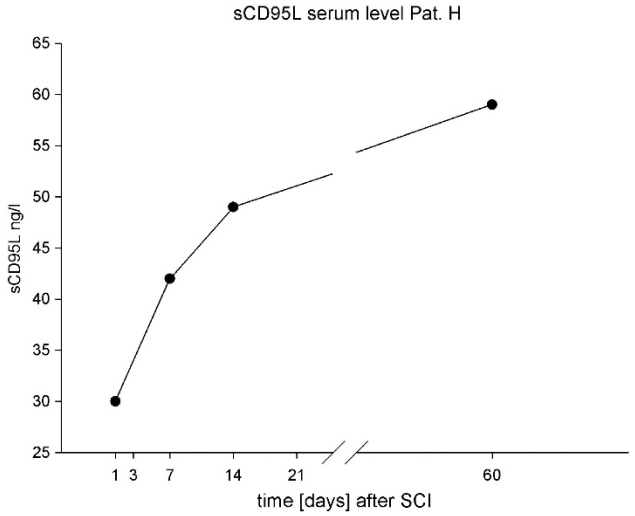
Patient E



Patient G



Patient F



Patient H

Patient H: incomplete tetraplegia; ASIA C; C5; fracture type B1; age 86; male. One day after SCI, the sCD95L level was 30 ng l^{-1}, which was the lowest level observed in our patient group at that point. Seven days after SCI, the sCD95L level increased to 42 ng l^{-1}. On day 14, the level further increased to 49 ng l^{-1}. After 60 days, the sCD95L level was 59 ng l^{-1}.

sCD95L and levels of leukocytes, C-reactive protein, hemoglobin and creatinine

In our patient group, no significant correlation between sCD95L levels and leukocyte, C-reactive protein, hemoglobin or creatinine levels was determined. We observed varying levels of leukocytes, C-reactive protein, hemoglobin and creatinine among our patients. These characteristics were quite heterogeneous, not comparable between patients, and not correlated with sCD95L levels.

DISCUSSION

In the current scientific literature, fluctuations of CD95 over time in SCI rodents and humans have already been documented, but not in SCI patients *in vivo*. A recent publication concerning Fas/FasL levels in patients post mortem showed that Fas-mediated apoptosis of neurons and oligodendrocytes occurred in the acute and subacute phases of SCI, but not in the chronic phase nor in the control group.[14] In a similar study, researchers performed an immunohistochemical analysis of nine SCI patients in subacute and acute phases post mortem.[15] Results showed that neutrophiles and microglia may possess destructive activity within the first days of SCI. Both studies showed an increase in Fas/FasL in the acute and subacute phases. These findings, however, cannot be compared with ours, because researchers used differing methods, that is immunochemical analysis on spinal cord samples vs serum enzyme-linked immunosorbent assay and post mortem vs *in vivo*. Nevertheless, similar to our own study these studies concern themselves with a possible therapy for SCI patients.

A recent animal experiment demonstrated that CD95L on the surface of peripheral myeloid cells triggers their recruitment to the inflammatory site through the activation of Syk kinase [3]. The authors also found that a pharmacological blockade of CD95L leads to improved functional and histopathological outcomes in rodents. Similar to the previous studies mentioned above, the temporal

fluctuations in CD95L levels in rodents were not identical to our findings with SCI patients. This discrepancy is unclear; however, we suspect that this occurred because we measured only sCD95L and not a combination of sCD95L and mCD95L. Perhaps this suggests that the expression of sCD95L is higher than that of mCD95L, but a separate study is needed before one can draw any meaningful conclusions.

In vivo experiments in non-SCI patients showed that the serum of patients with certain hematological malignancies contained elevated sCD95 levels.[16] And more recent studies have shown a correlation between CD95L levels and several human pathologies such as systemic lupus erythematosus,[17] toxic epidermal necrosis, acute pancreatitis,[18] acute hepatic failure[19] and burn injuries.[20]

The influence of the initial phase of acute SCI on recovery or prognosis has not yet been researched. Recent studies have only concentrated on the prevention or alleviation of the non-acute phase of injury.[3] We, however, believe that our results will be useful in better understanding the importance of the initial phase after injury and perhaps lead to further studies investigating therapeutic measures.

In our pilot study, we investigated sCD95L in serum in patients with SCI. The goal of this investigation was to detect an interaction between post-traumatic serum levels of sCD95L and the time after injury in SCI patients. We observed decreased sCD95L serum levels between the time of the initial injury and the second week after SCI. After the first week, the mean sCD95L level was $48.7\,ng\,l^{-1}$, which increased after the second week and peaked during week 4 at $68.5\,ng\,l^{-1}$.

Thus, it can be concluded from our results that an elevation in sCD95L levels was highest during the subacute and chronic phases after injury. In contrast to the results of Letelier *et al.*,[3] we measured only sCD95L levels and found that they did not increase in the first days post injury. This can perhaps be attributed to the elevation of mCD95L and neutrophils during this phase. mCD95L is primarily believed to be responsible for the inflammatory activity of CD95L[7] and its absence may reduce apoptotic activity in rodents.[21] Shortly after SCI, mCD95L and apoptotic markers are elevated.[8] Recent studies have demonstrated that sCD95L promotes an autoimmune reaction;[21] however, it is still unclear whether autoimmune reactions are beneficial or if a 'protective autoimmunity' exists.[22] In other words, there is not enough evidence to suggest that mCD95L is associated with neurological damage and sCD95L is associated with beneficial processes following SCI. Nevertheless, this is an interesting question and we hope that researchers will pursue it in future studies.

We believe that it would be of great interest to study a larger number of patients to determine whether higher sCD95L levels are correlated with an improved or impaired neurological outcome or with increasing levels of autoimmune components in peripheral blood. Further investigations involving larger study populations and comparison groups (patients with spine injuries without neurological deficits) are urgently needed to develop a therapeutic strategy based on serum levels of sCD95L.

DATA ARCHIVING

There were no data to deposit.

CONFLICT OF INTEREST

All authors have disclosed all financial and personal relationships with other people or organizations that could inappropriately influence their work. Examples of potential conflict of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations and grants or other funding.

1 Rowland JW, Hawryluk GW, Kwon B, Fehlings MG. Current status of acute spinal cord injury pathophysiology and emerging therapies: promise on the horizon. *Neurosurg Focus* 2008; **25**: E2.
2 Takahashi T, Tanaka M, Inazawa J, Abe T, Suda T, Nagata S. Human Fas ligand: gene structure, chromosomal location and species specificity. *Int Immunol* 1994; **6**: 1567–1574.
3 Letellier E, Kumar S, Sancho-Martinez I, Krauth S, Funke-Kaiser A, Laudenklos S *et al.* CD95-ligand on peripheral myeloid cells activates Syk kinase to trigger their recruitment to the inflammatory site. *Immunity* 2010; **32**: 240–252.
4 Beattie MS. Inflammation and apoptosis: linked therapeutic targets in spinal cord injury. *Trends Mol Med* 2004; **10**: 580–583.
5 Diaz-Ruiz A, Salgado-Ceballos H, Montes S, Guizar-Sahagun G, Gelista-Herrera N, Mendez-Armenta M *et al.* Delayed administration of dapsone protects from tissue damage and improves recovery after spinal cord injury. *J Neurosci Res* 2011; **89**: 373–380.
6 Chakraborti S, Mandal M, Das S, Mandal A, Chakraborti T. Regulation of matrix metalloproteinases: an overview. *Mol Cell Biochem* 2003; **253**: 269–285.
7 Shudo K, Kinoshita K, Imamura R, Fan H, Hasumoto K, Tanaka M *et al.* The membrane-bound but not the soluble form of human Fas ligand is responsible for its inflammatory activity. *Eur J Immunol* 2001; **31**: 2504–2511.
8 Demjen D, Klussmann S, Kleber S, Zuliani C, Stieltjes B, Metzger C *et al.* Neutralization of CD95 ligand promotes regeneration and functional recovery after spinal cord injury. *Nat Med* 2004; **10**: 389–395.
9 Ackery A, Robins S, Fehlings MG. Inhibition of Fas-mediated apoptosis through administration of soluble Fas receptor improves functional outcome and reduces posttraumatic axonal degeneration after acute spinal cord injury. *J Neurotrauma* 2006; **23**: 604–616.
10 Casha S, Yu WR, Fehlings MG. FAS deficiency reduces apoptosis, spares axons and improves function after spinal cord injury. *Exp Neurol* 2005; **196**: 390–400.
11 Corsini NS, Sancho-Martinez I, Laudenklos S, Glagow D, Kumar S, Letellier E *et al.* The death receptor CD95 activates adult neural stem cells for working memory formation and brain repair. *Cell Stem Cell* 2009; **5**: 178–190.
12 Nunamaker DM. Experimental models of fracture repair. *Clin Orthop Relat Res* 1998; **355**(Suppl): S56–S65.
13 Moghaddam A, Muller U, Roth HJ, Wentzensen A, Grutzner PA, Zimmermann G. TRACP 5b and CTX as osteological markers of delayed fracture healing. *Injury* 2010; **42**: 758–764.
14 Yu WR, Fehlings MG. Fas/FasL-mediated apoptosis and inflammation are key features of acute human spinal cord injury: implications for translational, clinical application. *Acta Neuropathol* 2011; **122**: 747–761.
15 Fleming JC, Norenberg MD, Ramsay DA, Dekaban GA, Marcillo AE, Saenz AD *et al.* The cellular inflammatory response in human spinal cords after injury. *Brain* 2006; **129**(Part 12): 3249–3269.
16 Tanaka M, Suda T, Haze K, Nakamura N, Sato K, Kimura F *et al.* Fas ligand in human serum. *Nat Med* 1996; **2**: 317–322.
17 Tinazzi E, Puccetti A, Gerli R, Rigo A, Migliorini P, Simeoni S *et al.* Serum DNase I, soluble Fas/FasL levels and cell surface Fas expression in patients with SLE: a possible explanation for the lack of efficacy of hrDNase I treatment. *Int Immunol* 2009; **21**: 237–243.
18 Endo S, Inoue Y, Fujino Y, Yamada Y, Sato N, Wakabayashi G *et al.* Soluble Fas and soluble Fas L levels in patients with acute pancreatitis. *Res Commun Mol Pathol Pharmacol* 2000; **108**: 179–186.
19 Singhal S, Chakravarty A, Das BC, Kar P. Tumour necrosis factor-alpha and soluble Fas ligand as biomarkers in non-acetaminophen-induced acute liver failure. *Biomarkers* 2009; **14**: 347–353.
20 Yamada Y, Endo S, Nakae H, Makabe H, Sato N, Wakabayashi G *et al.* Examination of soluble Fas (sFas) and soluble Fas ligand (sFasL) in patients with burns. *Burns* 2003; **29**: 799–802.
21 LA OR, Tai L, Lee L, Kruse EA, Grabow S, Fairlie WD *et al.* Membrane-bound Fas ligand only is essential for Fas-induced apoptosis. *Nature* 2009; **461**: 659–663.
22 Popovich PG, Longbrake EE. Can the immune system be harnessed to repair the CNS? *Nat Rev Neurosci* 2008; **9**: 481–493.