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Temporal expression profile of CXC chemokines in serum of patients with spinal cord injury

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ABSTRACT

Chemokines, a subclass of cytokine superfamily have both pro-inflammatory and migratory role and serve as chemoattractant of immune cells during the inflammatory responses ensuing spinal cord injury (SCI). The chemokines, especially CXCL-1, CXCL-9, CXCL-10 and CXCL-12 contribute significant part in the inflammatory secondary damage of SCI. Inhibiting chemokine's activity and thereby the secondary damage cascades has been suggested as a chemokine targeted therapeutic approach to SCI. To optimize the inhibition of secondary injury through targeted chemokine therapy, accurate knowledge about the temporal profile of these cytokines following SCI is required. Hence, the present study was planned to determine the serum levels of CXCL-1, CXCL-9, CXCL-10 and CXCL-12 at 3–6 h, 7 and 28 days and 3 m after SCI in male and female SCI patients ($n = 78$) and compare with age- and sex-matched patients with non-spinal cord injuries (NSCI, $n = 70$) and healthy volunteers ($n = 100$). ANOVA with Tukey post hoc analysis was used to determine the differences between the groups. The data from the present study show that the serum level of CXCL-1, CXCL-9 and CXCL-10 peaked on day 7 post-SCI and then declined to the control level. In contrast, significantly elevated level of CXCL-12 persisted for 28 days post SCI. In addition, post-SCI expression of CXCL-12 was found to be sex-dependent. Male SCI patients expressed significantly higher CXCL-12 when compared to control and SCI female. We did not observe any change in chemokines level of NSCI. Further, the age of the patients did not influence chemokines expression after SCI. These observations along with SCI-induced CSF-chemokine level should contribute to the identification of selective and temporal chemokine targeted therapy after SCI.

1. Introduction

Traumatic spinal cord injury (SCI) is one of the most leading causes of disability and mortality. SCI initiates a variety of inflammatory and/or immune responses including the infiltration of leukocytes such as monocytes, macrophages, T-cells and NK cells, into the injured area (Chaitanya et al., 2009). The infiltrated immune cells and their secretory inflammatory molecules are the key factors of the secondary damage occurring subsequent to SCI. It is also well established that the inflammatory response in SCI is preceded by the expression of chemokines.

Chemokines are pleiotropic cytokines with more than one biological function. They serve as chemoattractants of leukocytes which secrete neurotoxic compounds under certain conditions at the site of injury (Giulian et al., 1993). Hence, infiltration of inflammatory cells to the site of trauma amplifies the secondary damage cascades in CNS injuries. Activation and migration of immune cells to the site of injury is orchestrated by changes in the expression of chemokines. CXCL-1, CXCL-9, CXCL-10 and CXCL-12, the chemokines studied in the present report are pro-inflammatory and are also under the control of inflammatory cytokines like TNF-α,

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IFN-γ and IL-6. CXCL-1 secreted by activated T cells attracts monocytes, NK cells and immature B cells and dendritic cells. CXCL-10 (IP-10) acts as a potent T-lymphocyte recruiter by binding to the chemokine receptor CXCR3, preferentially on T lymphocytes of the Th1 subtype; CXCL-9 is closely related to CXCL-10 and is also a T-cell chemoattractant and CXCL-12 is a stromal cell derived factor and is chemotactic for lymphocytes (Ara et al., 2003). In addition, chemokines also attract stem cells to the site of injury and promotes their survival and differentiation enhancing remyelination after CNS trauma like SCI.

The multifunctional property of chemokines that intervene at different stages of post SCI makes the chemokine-targeted therapy a more complex and time-dependent process. However, a general chemokine inhibitor may not be a suitable therapeutic approach to inflammatory CNS pathology. Hence, multi chemokine targeted timely planned therapeutic approach will help SCI patients by suppressing immune cell mediated secondary injury and preserving the chemokine mediated neurite outgrowth and vasculogenesis at the same time. Therefore, studies on the spatiotemporal expression of various cytokines after SCI will greatly help reaching that target (Knerlich-Lukoschus et al., 2011). Hence, the present study is aimed to determine the serum chemomine levels (CXCL-1 (GRO-α), CXCL-9 (Mig), CXCL-10 (IP-10) and CXCL-12 (SDF-1)) in SCI patients at different time points after SCI and analyze the differential expression pattern of the chemokines based on the time after injury, age and sex between SCI patients and age- and sex-matched non-SCI (NSCI) patients and controls. The outcome should help designing time-dependent specific chemokine targeted therapy to SCI.

2. Material and methods

2.1. Subjects

All the studies with SCI patients ($n = 78$) and NSCI patients ($n = 70$) and healthy volunteers ($n = 100$) were approved by Rafsanjan University of Medical Sciences ethical committee. Written consent forms were filled out prior to the blood sample collection. SCI patients of the present study were from Khorasan and Kerman, the two biggest east provinces of Iran. The study period ranged from 2008–2011. Expert neurologist and neurosurgeon were involved in the validation of SCI based on the clinical findings (MRI and CT scan) and paraclinical manifestations. Age- and sex-matched healthy control volunteers from the same population were also included in the study. Patients with no injury to CNS were recruited (age- and sex-matched to SCI cohort) to study the effect of NSCI on chemokine levels. NSCI group ($n = 70$) included 37 arm injuries and 33 leg injuries.

2.2. ELISA assay

Blood samples were collected by an approved technician in sterile empty tubes without any anticoagulant. Serum was quickly separated, aliquoted and snap frozen in liquid nitrogen till further analysis. Repeated freeze–thaw cycles were avoided. The serum level of chemokines; CXCL-1, CXCL-9, CXCL-10 and CXCL-12 were quantitated by using the commercially available ELISA kits (R&D systems, UK). Inter- and intra-assay assessments of reliability of the kit were also done in the present study. All the assays were performed according to the procedures suggested by the manufacturer.

2.3. Statistical analysis

Kolmogorov test was used to assess normal distribution of serum chemokine levels. CXCL-9 and CXCL-12 had normal distributions and the statistical analysis between their serum levels at different time points was performed by Repeated Measure test. CXCL-1 and CXCL-10 were not normally distributed and hence Friedman test was used which gave the mean ranks for different time points after SCI. Statistical tests were performed in '*R*' statistical environment. Statistical comparisons between data sets were made based on the representation of mean + SEM of data. Statistical analyses were performed using two-way repeated measurements analysis of variance (ANOVA) with Tukey HSC post hoc test for multiple comparisons. Significance level of p-value <0.05 was adopted.

3. Results

3.1. Demographic data

Data on the age of SCI patients and the cause and nature of the trauma are given in Table 1. Statistical analysis of demographic parameters showed that, the mean age, gender, and socio-economic status of the participants were not significantly different between the groups (data not given). The mean age of the surviving patients was 33.3 ± 1.6 years. Most of the patients (74.35%) encountered SCI from automobile accidents and the rest from falling. 21.79% of the patients had cervical SCI and the rest had SCI at all other levels of the spine. We have recorded 12.82% mortality of SCI patients during the study period. The mean age of the deceased was 50.3 ± 6.2 years. NSCI patients were recruited to age- and sex-matched with the SCI cohort. NSCI group had mean age 38.44 ± 13.75 years (32 female and 38 male) and included 37 arm injuries and 33 leg injuries.

3.2. Chemokines level

In the present study, we have quantitated the serum level of chemokines, CXCL-1, CXCL-9, CXCL-10 and CXCL-12 of SCI patients at 3–6 h, 7 and 28 days, and 3 months (Late) after the trauma. The data are given in Table 2 and Figs. 1 and 2. In the SCI patients, the serum level of all the chemokines significantly increased 3–6 h following the trauma. However, there was no change in the serum chemokine in NSCI patients. Control individuals had the following range of serum chemokines; CXCL-1: 122.67 ± 13.55; CXCL-9: 212.57 ± 12.66; CXCL-10: 75.57 ± 4.21; and CXCL-12: 180.69 ± 16.22. Regardless of the nature of trauma, the observed range of chemokine levels in the SCI patients was; CXCL-1:131.57 ± 10.10–475.03 ± 116.3, CXCL-9: 160.78 ± 14.29–628.60 ± 92.63, CXCL-10:158.60 ± 0.80–517.76 ± 71.18 and CXCL-12:1064.63 ± 87.17–1639.70 ± 133.76. The increase in the serum level of CXCL-1, CXCL-9 and CXCL-10 reached a peak on day 7 after SCI. Chemokine CXCL-12 also peaked on day 7 post SCI, however; the significantly elevated level persisted up to 28 days (Fig. 1A–D). After that period, the level of chemokines returned back to the control level. In general all the chemokines except CXCL-12 reached the level of control on 7 days post-SCI, while CXCL-12 level remained significantly higher till 28 days post SCI. There was no correlation between the age and the level of CXCL-12 in both the male and female patients. In both the sexes, the expression pattern of CXCL-12 was similar in different age groups. From the data analysis, it can also be inferred that there exists a significant difference in the serum level of CXCL-12 between the male and female SCI patient.

There was a significant difference between the serum levels of CXCL-9 and CXCL-12 at different time points (3–6 h, 7 and 28 days) following SCI measured by Repeated Measure test ($p < 0.0001$). There was also a significant difference between the mean ranks of CXCL-1 and CXCL-10 serum levels at different time points (3–6 h, 7 and 28 days) following SCI ($p < 0.0001$). Higher mean rank demonstrated higher serum chemokine level which peaked at 7 days after SCI for both CXCL-1 and CXCL-10 (Table 2).

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Table 1
Demographics of SCI patients ($n = 78$) involved in the present study.

Factors		Number of Patients			Age year (mean ± SD)
		Male	Female	All	
Cause of injury	Vehicle accident	30	18	58	33.5 ± 2
	Falling	18	2	20	36.7 ± 3
Site of injury	Neck	9	8	17	30.7 ± 2.9
	Others	49	12	61	35.3 ± 2
Outcome	Death	8	2	10	50.3 ± 6.2
	Survived	48	20	68	33.3 ± 1.6

Table 2
Serum CXCL-chemokine levels of SCI patients ($n = 78$) at various conditions and time points (mean ± SEM).

Conditions	Variable	Time	CXCL-1 (GRO)	CXCL-9 (Mig)	CXCL-10 (IP-10)	CXCL-12 (SDF-1α)
Cause of trauma (SCI)	Vehicle accident	3–6 h	199.22 ± 25.74	317.87 ± 33.30	224.84 ± 25.98	1064.63 ± 87.17
		7 days	293.75 ± 30.79	576.37 ± 46.55	328.06 ± 28.78	1479.26 ± 85.29
		28 days	131.57 ± 10.10	191.63 ± 22.35	162.56 ± 10.43	1334.46 ± 85.44
	Falling	3–6 h	160.04 ± 41.72	278.33 ± 60.07	285 ± 57.27	1453.45 ± 152.8
		7 days	275.73 ± 63.73	607.68 ± 108.5	517.76 ± 71.18	1433.2 ± 151.78
		28 days	149.47 ± 30.66	195.33 ± 33.71	174.84 ± 17.74	1507.95 ± 144.06
Site of trauma (SCI)	Neck	3–6 h	276.77 ± 66.30	332.34 ± 66.34	242.15 ± 59.65	1423.98 ± 152.04
		7 days	327.53 ± 62.28	510.55 ± 57.81	268.45 ± 58.59	1546.21 ± 124.51
		28 days	146.44 ± 26.86	402.84 ± 61.65	159.63 ± 20.57	1374.01 ± 124.88
	Others	3–6 h	165.86 ± 20.68	300.88 ± 32.43	239.27 ± 26.41	1091.10 ± 88.04
		7 days	278.92 ± 31.27	604.98 ± 53.87	377.39 ± 33.81	1446.57 ± 87.92
		28 days	134.46 ± 12.03	160.78 ± 14.29	166.94 ± 9.97	1381.76 ± 84.43
Outcome of SCI	Death	3–6 h	101.31 ± 46.29	333.78 ± 96.05	343.02 ± 122.1	1639.70 ± 133.76
		7 days	475.03 ± 116.3	628.60 ± 92.63	391.97 ± 117.3	1594.40 ± 151.03
		28 days	76.85 ± 32.95	Dead	158.60 ± 0.80	1551.40 ± 76.20
	Survived	3–6 h	196.57 ± 23.23	305.56 ± 30.57	231.52 ± 24.25	1123.09 ± 81.66
		7 days	274.18 ± 28.13	580.71 ± 47.18	374.16 ± 30.38	1457.48 ± 79.05
		28 days	138.25 ± 11.16	192.75 ± 18.45	166.06 ± 9.24	1375.04 ± 75.81
NSCI	Survived	3–6 h	144.23 ± 14.65	213.16 ± 9.60	77.02 ± 4.25	165.09 ± 8.47
		7 days	161.67 ± 12.97	235.48 ± 7.80	99.38 ± 3.70	160.91 ± 5.85
		28 days	133.27 ± 12.34	219.75 ± 8.80	98.33 ± 5.89	141.29 ± 3.81

4. Discussion and conclusion

In the present study, we reported for the first time that the temporal expression profile of CXC chemokines in SCI patients to occur in a phase-dependent manner. Expression of inducible inflammatory chemokines increased immediately after SCI (within 3–6 h) and their elevated level was seen for 7 days post SCI. However, the increased expression of CXCL-12 was persistent up to 28 days after SCI. Chemokines were observed in both the CSF and serum of patients with chronic inflammatory diseases. However, it is difficult to draw a definite correlation between the chemokine levels in the serum and CSF, since available reports reveal both positive and negative correlations between serum and CSF chemokine level. John et al. (2008) found no correlation between serum and CSF chemokines. In amyotrophic lateral sclerosis, the chemokine RANTES did not show any correlation between serum and CSF levels (Rentzos et al., 2007). The correlation between the level of chemokines in the serum and CSF is determined by several factors including the nature of the disease. For example, a pronounced increase of CSF-CXCL10 over serum-CXCL was observed in tick borne encephalitis. This differential expression was attributed to the local synthesis and compartmentalization of chemokines (Zajkowska et al., 2011). Likewise, significant higher concentration of CSF-CXCL recorded in the chronic inflammatory demyelinating polyneuropathy patients was attributed to the intrathecal synthesis of chemokines (Mahad et al., 2002) (Rosler et al., 1998). Additionally, during neuroinflammation, the CSF-CXCL is increased mainly due to the B-cell recruitment to the CNS compartment (Kowarik et al., 2012).

It has been previously demonstrated that the chemokines are up-regulated in several CNS pathologies including SCI (Azin et al., 2012). Hence, antibody mediated neutralization of CXCL-10 pre-SCI has been shown to be neuroprotective in SCI mice (Gonzalez et al., 2007). However, post-treatment strategy is practical only when the temporal expression pattern of chemokines is known. The data from the present study suggests a suitable time window for CXCL targeted antibody therapy in treating SCI patients.

Chemokines play a major role in the recruitment of lymphocytes to the site of SCI that helps enhanced tissue preservation and functional outcome after SCI. Chemokines like CXCL-10 also have angiostatic property. However, enhanced angiogenesis is a prerequisite of functional recovery after SCI (Rong et al., 2012). Hence, pre-SCI antibody mediated neutralization of CXCL-10 has been shown to be neuroprotective in SCI mice through increased angiogenesis and neovascularization (Gonzalez et al., 2007). On the other hand, CXCL-10 also activates microglia to express CXCR-3, the specific receptor of CXCL-10. Activated glial cells secrete a wide variety of reactive oxygen and nitrogen species and neurotoxins that participate in the secondary degenerative cascade following SCI. Thus the dramatic elevation of the inducible pro-inflammatory CXC chemokines in immediate early phases (3–6 h) actively contributes to the induction of degenerative processes following SCI. Neuronal CXCL-10 elevation precedes the astroglial induction (de Haas et al., 2007) and therefore, we suggest that short term quenching of CXCL-10 immediately after SCI with antibody will help dampening degenerative process while long-term suppression will also affect angiogenesis. However, the speculation needs more experimental support.

The demographic analysis of the data shows that the mean age of the SCI patients of automobile accident and falling were 33.5 ± 2 and 36.7 ± 3 years old, respectively, which is lower than that has been reported from different geographic regions (DeVivo and Chen, 2011). It may be due to the difference in life style and

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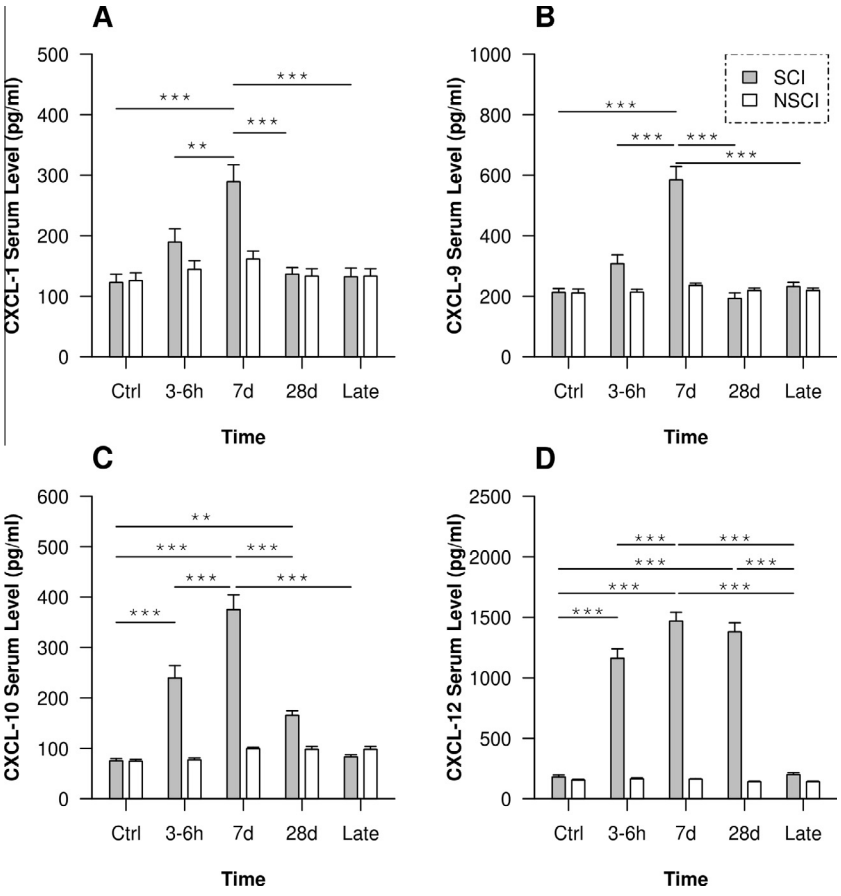

Fig. 1. Stem height (mean ± SEM) of serum chemokine levels following spinal cord injury (SCI) (n = 78) and non-spinal cord injuries (NSCI) (n = 70). Serum from SCI patients and control individual (NSCI and healthy volunteers) were collected 3–6 h, 7, 28 days and at a later stage (>30 days) after a sustained SCI (late). Circulating chemokine (CXCL-1, 9, 10 and 12) levels were determined using ELISA. All the chemokines tested show significant increase over the control during the initial period post SCI (7 days; $p < 0.001$). However, there is no significant difference in the levels of tested chemokines between SCI patients and control 28 days after the trauma. Data are represented as mean ± SEM. n; SCI: 78, NSCI: 70, Control: 100, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (two-way ANOVA test and Tukey HSD post hoc analysis). There is no change in tested chemokines in NSCI patients after the injury.

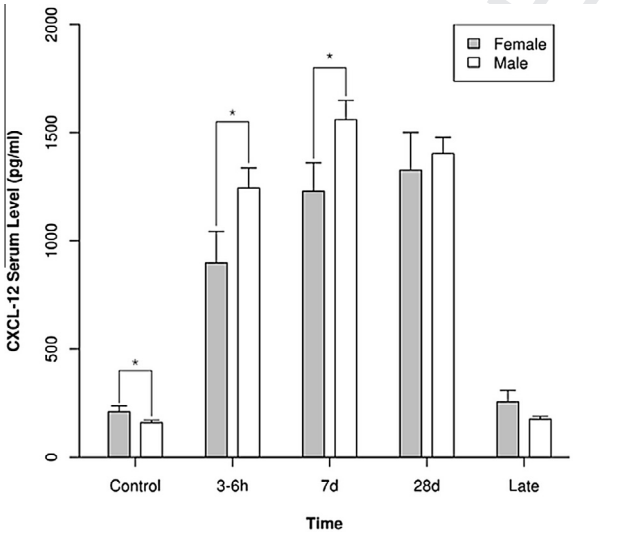

Fig. 2. Stem height (mean ± SEM) of serum expression level of CXCL-12 chemokine, in male (n = 57) and female (n = 21) SCI patients. CXCL-12 expression after SCI is sex dependent. We find significant difference in the CXCL-12 expression between male (n = 57) and female (n = 21) during early time points after SCI. The difference was observed till 7 days after SCI. However, there was no significant difference between the CXCL-12 expression level and sex at 28 days post-SCI and thereafter. Data are represented as mean ± SEM; $^{*}p < 0.05$ (two-way ANOVA test and Tukey HSD post hoc analysis).

socioeconomic status of the geographic area involved in the study. In the present study, we have seen 12.82% mortality in the SCI patients. The mortality rate recorded is much lesser than the published results (Emejulu and Ekweogwu, 2009). The mean age of deceased (50.3 ± 6.2 years) was higher than the surviving patients (33.3 ± 1.6 years). It has been well established that the most important risk factor associated with mortality after SCI is the age of the succumbent next to the type of the SCI incurred (Kawu et al., 2011). It is also obvious that the mortality occurred in patients with complete cervical SCI. The observation corroborates well with the previous reports that most of the fatal SCI are cervical (Emejulu and Ekweogwu, 2009). Further, survival with incomplete SCI imparts hope that neurological and life style recovery is possible.

In the present study, we have seen elevated level of CXCL-12 persisting for 28 days post-SCI. CXCL-12 regulates leucocyte extravasation into the CNS and hence, CXCL-12 antagonist has been shown to exacerbate experimental autoimmune encephalomyelitis in rats (Miljkovic et al., 2011). CXCL-12 also enhances axonal sprouting, neuroprotection and tissue preservation after SCI (Jaerve and Muller, 2012). Hence, increased level of CXCL-12 is an adaptive response to SCI. In the present study we did not find any statistically significant correlation between the clinical outcomes of SCI and the level of CXCL-12 chemokine. Studies on other CNS diseases also fail to conclude if there is a significant correlation between CXCL-12 level and the disease activity (Hansen et al., 2006). However, the validation requires extended study with still higher number of samples. To validate the sexual difference in che-

mokine level, recently, Hall and Korach discovered the existence of estrogen receptor (ER)-CXCL-12-CXCR4 signaling axis (Hall and Korach, 2012). In the present study we have seen significantly decreased level of CXCL-12 in females. This may be due to the fact that SCI disrupts the normal sexual cycle and reproductive physiology culminating in reduced level of estrogen and ER (Shunmugavel et al., 2012).

In conclusion, the data from the present study opens up new perspectives with regard to temporal targeting of the chemokine system in the injured CNS.

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