

ORIGINAL ARTICLE

Subclinical atherosclerosis is related to injury level but not to inflammatory parameters in spinal cord injury subjects

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Study design: Cross-sectional.

Objectives: Individuals with spinal cord injury (SCI) exhibit increased carotid intima-media thickness (IMT) and are reported to be exposed to higher circulating levels of inflammatory mediators. This study evaluated the relationship between inflammatory markers and carotid surrogates of cardiovascular risk in subjects with SCI.

Setting: São Paulo, Brazil.

Methods: A total of 65 nondiabetic, nonhypertensive, sedentary, nonsmoker men (34 with SCI; 31 healthy subjects) were evaluated by medical history, anthropometry, routine laboratory tests, analysis of hemodynamic, inflammatory parameters and ultrasound examination of carotid arteries.

Results: Subjects with SCI (18 tetraplegic and 16 paraplegic) had lower systolic blood pressure ($P=0.009$), higher serum C-reactive protein ($P=0.001$), tumor necrosis factor (TNF) receptor-II ($P=0.02$) and TNF receptor-I ($P=0.04$) levels and increased *in vitro* production of interleukin-6 by mononuclear cells ($P=0.04$), compared to able-bodied individuals. No differences in serum interleukin-6, e-selectin, intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and transforming growth factor- β levels, or *in vitro* release of interleukin-10, interleukin-17 and interferon- γ by mononuclear cells, were detected between the studied groups. Common carotid IMT, but not internal carotid resistive index, was significantly higher in subjects with SCI ($P<0.0001$ adjusted for C-reactive protein and TNF receptor-II levels). In addition, tetraplegic subjects exhibited increased IMT ($P=0.002$ adjusted for systolic blood pressure and body mass index), but similar levels of inflammatory mediators compared to paraplegic ones.

Conclusions: Individuals with SCI exhibit a clustering of vascular and inflammatory surrogates of increased cardiovascular risk. Nevertheless, subclinical carotid atherosclerosis is related to injury level but not to increased inflammatory status in these subjects.

Spinal Cord (2010) **48**, 740–744; doi:10.1038/sc.2010.12; published online 16 February 2010

Keywords: spinal cord injury; atherosclerosis; intima-media thickness; inflammation; tetraplegia; paraplegia

Introduction

Cardiovascular disease is commonly seen in subjects with spinal cord injury (SCI) and coronary heart disease is more prevalent in individuals with SCI individuals than in the able-bodied population.^{1,2} Recent data from our group showed that carotid intima-media thickness (IMT) is increased in subjects with SCI, independently of age, blood

pressure, smoking, diabetes mellitus and metabolic variables, supporting the notion that traditional cardiovascular risk factors might not influence the development of early atherosclerosis in such individuals.³

Inflammation is thought to have a major function in cardiovascular diseases, and elevated levels of systemic inflammatory markers have been associated with worse cardiovascular outcomes and increased carotid IMT.⁴ In this context, subjects with SCI are reported to be exposed to higher circulating levels of inflammatory markers in comparison to healthy ones,^{5–7} suggesting that inflammatory mechanisms might be potential candidates to explain the increased atherosclerotic burden in these individuals.

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Received 12 November 2009; revised 12 January 2010; accepted 15 January 2010; published online 16 February 2010

Therefore, the aim of this study was to evaluate carotid surrogates of cardiovascular risk in SCI and able-bodied subjects and investigate the impact of inflammatory markers in this regard.

Materials and methods

A total of 65 nondiabetic, nonhypertensive, sedentary men (34 with SCI and 31 healthy subjects) were cross-sectionally evaluated. Subjects with SCI were enrolled from a university hospital outpatient clinic, whereas healthy subjects were recruited among the employees of the hospital. Further exclusion criteria for both groups included current or past smoking, known coronary artery, cardiac or pulmonary disease, cancer, regular medical therapy and clinical evidence of active infection. The SCI group comprised 18 tetraplegic and 16 paraplegic individuals. SCI level ranged from C4 to T12 and the American Spinal Injury Association (ASIA) Impairment Scale was defined as complete (ASIA A) for all patients with SCI, except for 2 paraplegics classified as incomplete (ASIA B).

Clinical data included information on the participants' ages and, when applicable, injury duration. Body mass index was calculated as body weight divided by height squared and body surface area was evaluated by the Dubois formula. Blood pressure was measured using a validated digital oscillometric device (Omron HEM-705CP; Omron Corp., Kyoto, Japan).

Blood samples were obtained on the morning after 12 h of fasting for analysis of glucose, lipid fractions and inflammatory markers.⁸ Inflammatory markers in sera (tumor necrosis factor (TNF) receptor-I, TNF receptor-II, interleukin-6, e-selectin, intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and transforming growth factor- β) were determined by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA). C-reactive protein in sera was measured by latex-enhanced immunonephelometric assays (Dade Behring, Newark, NJ, USA).

In vitro stimulation of mononuclear cells was performed as previously described,⁸ with minor modifications. Briefly, peripheral blood mononuclear cells were isolated by density gradient centrifugation over Histopaque 1077 and washed twice with phosphate-buffered saline. Cells were resuspended at 2×10^6 cells per ml and incubated in 1 ml of RPMI in 24-well tissue culture plates. Monocytes were isolated by adherence for 2 h in a humidified 5% CO₂ incubator at 37 °C; nonadherent cells were removed by changing the medium. Monocytes were then stimulated for 24 h with 10 $\mu\text{g ml}^{-1}$ lipopolysaccharide or phytohemagglutinin 10 $\mu\text{g ml}^{-1}$. Interleukin-17 and interferon- γ released in the supernatants of phytohemagglutinin-stimulated cells and interleukin-6 and TNF produced by lipopolysaccharide-stimulated monocytes were determined by enzyme-linked immunosorbent assay (R&D Systems).

Carotid studies were performed by a skilled physician on each subject in the sitting position with a Vivid 3 Pro apparatus (General Electric, Milwaukee, WI, USA) equipped with 10 MHz transducers, as previously described.⁴ To

measure carotid IMT, we identified a region 2 cm proximal to the carotid bifurcation, and evaluated the IMT of the far wall as the distance between the lumen-intima interface and the media-adventitia interface. The average from both right and left common carotid artery measurements was used for analyses. All measurements were made using an automatic border recognizer (Vivid 3 Pro IMT software analyzer) on still images obtained during the sonographic scanning. No carotid plaques were visualized while measuring IMT. The resistive index was calculated as follows: $1 - (\text{minimum diastolic velocity}/\text{maximum systolic velocity})$.⁹ Intraobserver and interobserver common carotid IMT variabilities were <4 and <5%, respectively, whereas intraobserver and interobserver variabilities of internal carotid resistive index measurements were <4%.

Systolic volume was generated from Doppler interrogation of transaortic flow at the aortic annular level and aortic cross-sectional area using a Vivid 3 Pro apparatus equipped with a 2.5 MHz transducer. Cardiac output was calculated as systolic volume \times cardiac frequency, whereas peripheral vascular resistance was obtained by the formula, mean blood pressure/cardiac output.

Continuous parametric and nonparametric variables are presented as means \pm standard errors and medians (interquartile ranges), respectively. The Kolmogorov-Smirnov test was used to test for normal distribution of the variables. χ^2 -Test was used to compare categorical variables whereas unpaired *t*-test and Mann-Whitney test compared parametric and nonparametric continuous variables, respectively. Pearson's or Spearman's methods were used to assess univariate correlations between carotid variables and inflammatory parameters. General linear model analyses were used to assess intergroup differences in carotid parameters after adjustment for relevant covariates. A *P*-value of <0.05 was considered significant.

The study was approved by the ethics committee of our institution and informed consent was obtained from all participants. We certify that all applicable institutional and governmental regulations concerning the ethical use of human volunteers were followed during the course of this research.

Results

Clinical and hemodynamic features of SCI and healthy subjects are presented in Table 1. Mean age, body size, diastolic blood pressure, heart rate, cardiac output, peripheral vascular resistance, serum lipid fractions and glucose levels were similar between the studied groups, whereas systolic blood pressure was significantly lower in individuals with SCI.

Table 2 presents the inflammatory parameters of the studied subjects. Higher serum C-reactive protein, TNF receptor-I, TNF receptor-II as well as increased leukocyte-derived interleukin-6 production were detected in individuals with SCI, in comparison to able-bodied ones. Conversely, no differences in serum interleukin-6, e-selectin, intercellular adhesion molecule-1, vascular cell adhesion

molecule-1 and transforming growth factor- β levels as well as in interleukin-17, interleukin-10 and interferon- γ released by stimulated mononuclear cells were found between the groups.

Carotid parameters are shown in Table 3. Common carotid IMT was significantly higher in SCI than in healthy individuals, even after adjusting for C-reactive protein and TNF receptor-II levels. Conversely, common carotid lumen diameter and common carotid and internal carotid resistive indexes were comparable in both groups (Table 3). In addition, results of univariate correlation analysis showed no significant relationship between inflammatory markers and carotid parameters in the whole sample as well as in injured subjects (data not shown).

Features of subjects with SCI were then assessed according to injury level (Table 4). Tetraplegic individuals displayed

Table 1 Clinical and hemodynamic features

Variable	SCI (n = 34)	Healthy subjects (n = 31)
Age (years)	31.9 \pm 1.3	31.1 \pm 1.3
Time of injury (years)	6.7 \pm 0.8	—
Body mass index (kg m ⁻²)	23.6 \pm 0.7	24.5 \pm 0.7
Systolic blood pressure (mm Hg)	108.5 \pm 3.4	119.9 \pm 1.9*
Diastolic blood pressure (mm Hg)	71.1 \pm 2.3	71.9 \pm 1.9
Heart rate (b.p.m.)	73.1 \pm 2.1	71.2 \pm 1.5
Cardiac output (ml min ⁻¹)	4.7 \pm 0.2	4.9 \pm 0.2
Peripheral vascular resistance (dynes s cm ⁻⁵)	1459 \pm 56	1486 \pm 68
Glucose (mg per 100 ml)	81.6 \pm 2.4	76.5 \pm 3.0
LDL-cholesterol (mg per 100 ml)	108.5 \pm 6.6	99.8 \pm 7.0
HDL-cholesterol (mg per 100 ml)	42.2 \pm 1.8	44.2 \pm 2.0
Triglycerides (mg per 100 ml)	110.2 \pm 10.6	92.4 \pm 9.2

Abbreviations: HDL, high-density lipoprotein; LDL, low-density lipoprotein; SCI, spinal cord injury.

*P = 0.009 in comparison to SCI.

Table 2 Inflammatory parameters

Variable	SCI (n = 34)	Healthy subjects (n = 31)	P
<i>Serum markers</i>			
TNF-receptor I (ng ml ⁻¹)	1.81 \pm 0.12	1.48 \pm 0.13	0.043
TNF-receptor II (ng ml ⁻¹)	3.14 \pm 0.05	2.93 \pm 0.07	0.024
Interleukin-6 (ng ml ⁻¹)	7.3 \pm 1.7	7.6 \pm 2.9	0.702
E-selectin (ng ml ⁻¹)	16.2 \pm 2.5	10.7 \pm 1.7	0.172
ICAM-1 (ng ml ⁻¹)	91.1 \pm 7.8	78.2 \pm 9.6	0.395
VCAM-1 (ng ml ⁻¹)	402 \pm 35	438 \pm 42	0.681
C-reactive protein (ng ml ⁻¹)	2.22 (6.63)	0.55 (1.0)	0.001
TGF- β (ng ml ⁻¹)	30.3 \pm 2.9	31.2 \pm 5.1	0.617
<i>Monocyte-stimulated markers</i>			
Interleukin-17 (pg ml ⁻¹)	213 \pm 40	163 \pm 48	0.322
Interferon- γ (pg ml ⁻¹)	51.9 (69.7)	41.5 (70.4)	0.551
Interleukin-6 (ng ml ⁻¹)	112.7 (172.7)	36.3 (53.9)	0.042
Interleukin-10 (pg ml ⁻¹)	2915 \pm 393	2413 \pm 451	0.316

Abbreviations: ICAM-1, intercellular adhesion molecule-1; SCI, spinal cord injury; TGF- β , transforming growth factor- β ; TNF, tumor necrosis factor; VCAM-1, vascular cell adhesion molecule-1.

Table 3 Carotid parameters

Variable	SCI (n = 34)	Healthy subjects (n = 31)	P	P*
CCA intima-media thickness (mm)	0.74 \pm 0.02	0.52 \pm 0.02	<0.0001	<0.0001
CCA lumen diameter (mm)	5.29 \pm 0.10	5.42 \pm 0.10	0.643	0.675
CCA resistive index	0.82 \pm 0.01	0.79 \pm 0.02	0.246	0.312
ICA resistive index	0.71 \pm 0.01	0.70 \pm 0.01	0.901	0.893

Abbreviations: CCA, common carotid artery; ICA, internal carotid artery; SCI, spinal cord injury.

*Adjusted for C-reactive protein and tumor necrosis factor receptor-II levels.

Table 4 Clinical and carotid features of SCI subjects according to injury level

Variable	Tetraplegic (n = 18)	Paraplegic (n = 16)	P	P*
<i>Clinical parameters</i>				
Age (years)	32.9 \pm 1.8	30.8 \pm 1.9	0.313	
Time of injury (years)	7.0 \pm 1.0	6.4 \pm 1.3	0.682	
Body mass index (kg m ⁻²)	22.0 \pm 0.8	25.4 \pm 0.9	0.016	
Systolic blood pressure (mm Hg)	98.1 \pm 3.8	120.2 \pm 4.4	<0.001	
Diastolic blood pressure (mm Hg)	65.5 \pm 3.0	77.5 \pm 2.8	0.007	
Heart rate (b.p.m.)	68.1 \pm 2.4	78.8 \pm 2.9	0.007	
Cardiac output (ml min ⁻¹)	4.6 \pm 0.3	4.9 \pm 0.2	0.725	
Peripheral vascular resistance (dynes s cm ⁻⁵)	1382 \pm 86	1547 \pm 67	0.538	
Glucose (mg per 100 ml)	82.4 \pm 2.1	80.8 \pm 4.7	0.782	
LDL-cholesterol (mg per 100 ml)	101.9 \pm 9.0	117.2 \pm 9.7	0.433	
HDL-cholesterol (mg per 100 ml)	39.9 \pm 2.0	45.2 \pm 3.0	0.081	
Triglycerides (mg per 100 ml)	115.0 \pm 16.7	104.7 \pm 12.9	0.462	
<i>Carotid parameters</i>				
CCA intima-media thickness (mm)	0.77 \pm 0.02	0.70 \pm 0.02	0.046	0.002
CCA lumen diameter (mm)	5.34 \pm 0.15	5.23 \pm 0.15	0.712	0.692
CCA resistive index	0.82 \pm 0.01	0.81 \pm 0.01	0.471	0.390
ICA resistive index	0.76 \pm 0.02	0.67 \pm 0.02	0.008	0.151

Abbreviations: CCA, common carotid artery; HDL, high-density lipoprotein; ICA, internal carotid artery; LDL, low-density lipoprotein; SCI, spinal cord injury.

*Adjusted for systolic blood pressure and body mass index.

lower systolic blood pressure, diastolic blood pressure, heart rate and body mass index in comparison to paraplegic ones. In contrast, similar values of serum and leukocyte-derived inflammatory markers were found in the injured subgroups (data not shown). Higher common carotid IMT and internal carotid-resistive indices were detected in tetraplegic subjects; however, the difference in internal carotid resistive index between the injured subgroups was no longer observed when the analysis was adjusted for systolic blood pressure and body mass index, whereas adjustment for these confounders increased the statistical significance of the difference in carotid IMT between tetraplegic and paraplegic subjects (Table 4).

Discussion

In this report, the evaluation of a sample of young, nonhypertensive, nondiabetic, nonsmoker, sedentary SCI and healthy men with average normal metabolic profile revealed that (1) subjects with SCI exhibited higher carotid IMT and increased systemic inflammatory markers in comparison to able-bodied ones, (2) mononuclear cells were more activated in individuals with SCI, as detected by lipopolysaccharide-induced IL-6 production, (3) the difference in carotid IMT between SCI and healthy subjects persisted after adjusting for serum inflammatory markers, (4) tetraplegic subjects presented a higher carotid IMT, but similar inflammatory profile in comparison to paraplegic patients. Overall, these findings show that individuals with SCI exhibit a clustering of vascular and inflammatory surrogates of cardiovascular risk. However, the higher carotid IMT seen in individuals with SCI compared to healthy subjects was not explained by discrepancies in inflammatory profile.

Our group recently showed that carotid IMT is increased in subjects with SCI, independently of age, blood pressure levels, body mass index, lipid profile and glucose levels.³ Given that individuals with SCI are reported to be exposed to increased circulating levels of inflammatory markers,⁵⁻⁷ this study investigated whether inflammatory parameters were associated with carotid IMT in this population. Initially, our results confirmed data from another source,^{6,7} showing that C-reactive protein is elevated in the sera of injured individuals. As such, TNF receptors were also found to be higher in subjects with SCI. Cell-surface TNF receptors shed in the serum compartment may behave as modulators of TNF biological activity and are thought to behave as markers of inflammatory activity.¹⁰ Moreover, novel evidence is provided showing that lipopolysaccharide-induced interleukin-6 secretion by peripheral blood mononuclear cells was higher in the group with SCI, indicating that inflammatory cells might be more activated in injured patients. Nevertheless, results of general linear model analysis revealed no significant influence of inflammatory parameters on carotid IMT difference between SCI and able-bodied subjects. Although these latter results suggest that inflammatory mediators exerted no major impact on carotid remodeling in injured subjects, an additive effect of inflammatory

markers on atherosclerosis development cannot be ruled out in such individuals.

The lack of relationship between inflammatory markers and carotid IMT in subjects with SCI deserve further comments. First, some markers could be not sensitive to evaluate the presumed chronic inflammatory state of afebrile persons with SCI. Second, it is possible that atherosclerosis might be more related to high-grade rather than low-grade inflammatory status¹¹ in injured individuals. Third, recurrent infections from pressure ulcers and in the urinary tract might have contributed to alter inflammatory markers in patients with SCI independent of mechanisms related to atherosclerosis.^{7,12} Nevertheless, the fact that none of the enrolled subjects exhibited clinical signs of acute infection turns this hypothesis less probable. At last, it is possible that increased IMT in injured individuals might not reflect carotid atherosclerotic burden, but instead, media layer growth. This hypothesis is based on the observation that carotid IMT is usually predicted by age and blood pressure and, thus, may also represent hypertensive medial hypertrophy.¹³ In contrast to this assumption, subjects with SCI displayed lower systolic blood pressure and similar age in comparison to able-bodied individuals. In addition, tetraplegic subjects exhibited increased IMT in comparison to paraplegic ones, in agreement with previous reports showing higher prevalence of cardiovascular disease in patients with a more rostral level of injury¹⁴⁻¹⁶ and strengthening the idea that carotid IMT is indeed a marker of early atherosclerosis in subjects with SCI.

In the light of the present data, it can be speculated that aspects other than inflammatory, metabolic and hemodynamic risk factors might have a function in carotid remodeling in individuals with SCI. In this regard, recent evidence from another source has shown that paraplegic athletes present similar IMT values in comparison to recreationally active able-bodied subjects.¹⁷ Likewise, tetraplegic subjects, who are subjected to extreme inactivity, were found herein to exhibit higher IMT than paraplegic individuals. This body of evidence points toward physical inactivity and diminished muscle mass as determinants of increased IMT in individuals with SCI and further suggests that exercise might be a potentially useful strategy to prevent atherosclerosis in these subjects. Physical inactivity has been classically assigned to influence atherogenesis by leading to a worse glycemic and lipidic profile as well as favoring obesity, increased blood pressure levels and higher inflammatory status.¹⁸ Interestingly, the results shown herein showed that none of these aforementioned factors explained the increased IMT in injured patients. Therefore, further studies are necessary to unveil whether physical inactivity-related mechanisms might influence atherosclerosis development in subjects with SCI.

Internal carotid resistive index has been considered a predictor of cardiovascular risk in subjects with cardiovascular risk factors, at least comparable to the well-established IMT.^{9,19} This latter parameter is assigned to identify early vascular alterations that may not be detectable by sonographic measurements of the vessel wall.⁹ Notably, no substantial differences in internal carotid resistive index

measurements were found between SCI and healthy individuals. Similarly, general linear model analysis adjusted for blood pressure and body mass index revealed comparable internal carotid resistive index among tetraplegic and paraplegic patients. Altogether, these results showed that changes in common carotid IMT were not paralleled by variations in internal carotid resistive index in the SCI population. In addition, they suggest that internal carotid resistive index may not be equivalent to carotid IMT, as a surrogate of cardiovascular risk in injured subjects. Nevertheless prospective studies are necessary to confirm this assumption.

One potential limitation of this study was that serum markers were measured at a single time point for each subject. Therefore, variability in the levels of these markers with time cannot be excluded. However, a previous study showed that in about 90% of cases, two independent C-reactive protein measurements taken 3 months apart were within one quartile of each other.²⁰ In addition, the inclusion of only male patients means that the results cannot yet be applied to female patients.

In summary, this report showed that the increased common carotid IMT seen in individuals with SCI compared to healthy subjects was not explained by variation in inflammatory profile. In addition, tetraplegic subjects presented a higher common carotid IMT than paraplegic ones, suggesting that diminished muscle mass is involved in this process. Nevertheless, further studies are necessary to unveil the exact mechanisms by which increased atherosclerotic burden is detected in individuals with SCI.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by grants from FAPESP (Proc. 05/56986-5 and 07/55148-1) and CNPq (Proc. 304329/06-1 and 474206/07-6), Brazil. We thank Dr Nicola Conran for English language editing.

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