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Immune and Metabolic Biomarkers in a Rodent Model of Spinal Cord Contusion

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Abstract

Study Design: Basic science animal research study.

Objectives: Using T10 spinal contused rats, we sought to identify molecular and circulating, metabolic and immune biomarkers during the subchronic and chronic recovery periods that may inform us concerning neurorehabilitation.

Methods: Gene expression of the cord and ELISA were performed in 28 and 100 days in T10 injured rats and compared to sham-injured rats. Hundred-day injured rats were placed on either a low-fat or high-fat diet following the recovery phase. Linear regression analysis was performed between markers and locomotor score, body weight, body composition, and blood cholesterol and triglycerides.

Results: Gene expression in the thoracic cord for complement marker, C1QC, dendritic cell marker, ITGAX, and cholesterol biosynthesis genes, FDFT1, HMCGR, LDLR, and SREBP1, were significantly associated with BBB score, body weight, composition, and other metabolic parameters. Circulating levels of these proteins, however, did not vary by injury or predict the level of locomotor recovery.

Conclusions: Identification of reliable circulating biomarkers that are durable and based on level of spinal injury are complicated by immune and metabolic comorbidities. Continued work is necessary to identify stable markers of disease progression.

Keywords

spinal cord injury, biomarker, metabolism, obesity, locomotor activity

Introduction

The National Institute of Health has classically defined a biological marker or "biomarker" as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.[1] Spinal cord injury (SCI) biomarkers that are specific, sensitive, and reproducible are greatly sought after in the assessment and care of SCI patients.[2] SCI biomarkers could aid in decision making for patient care and the long-term management of SCI patients, which improve the longevity of the patient, quality of life, and potential restoration of various lost functions.[3] Despite considerable effort, the identification of SCI biomarkers has been challenging.[2] The difficulty in identifying an SCI biomarker lies in the complexity of the mechanisms of injury. SCI may involve various segments (C1-S4) of damage to the spinal cord. The injury may be contusive or a partial or complete transection, which again alters the trajectory of recovery for these individuals. The secondary damage may include autonomic and peripheral nerves, soft tissue, skeletal tissue, and peripheral organs. This plays a large role in the unfolding of the disease for the specific patient and contributes to the burden of care overall.

With improved management of the acute critical care needs of SCI patients, injured individuals can expect to live decades past the primary insult.[4] Medical innovations and specialized

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care for this population have improved in the last decades. As the average age of this injured subpopulation continues to rise, the potential for various comorbidities of metabolic disease increases. SCI persons are at increased risk for dyslipidemia, cardiovascular disease, and glycemic dysregulation, all contributing factors to metabolic dysfunction.[5-7] With this in mind, biomarkers that are useful to predict injury progression, possible regain of lost function, and even metabolic health is important for the long-term care of these vulnerable individuals.

Though there has been an emphasis placed on the need for biomarkers that can identify risk factors for further disease development in the SCI care community,[2,8] the pipeline for this type of identification needs to be primed with preclinical and translational studies. Most of the work accomplished to date have used acute time points in preclinical models of SCI to filter important targets for study.[9-11] More work is required to identify various types of disease progression over the long-term care trajectory of the SCI patient.

Here, we exploit our previously published microarray data[12] to determine whether the immune and metabolic markers we identified in the thoracic cord using a moderate T10 contusion model may become viable biomarkers for the progression of clinical features. We couple quantitative real-time polymerase chain reaction (PCR) of the thoracic region of the cord with blood samples from 2 cohorts of animals. The first cohort was euthanized 4 weeks after injury and remained on chow for the duration. The second cohort was euthanized 16 weeks after injury and was maintained on either low-fat diet (LFD) or high-fat diet (HFD) for the last 12 weeks. We performed a secondary analysis of our gene expression findings to determine the association of these gene markers to locomotor scoring, body weight, lean mass, fat mass, and triglyceride levels. The top identified candidates for biological markers were then screened using terminal plasma.

Methods

Animals Assurance

All procedures for animal use complied with the Guidelines for the Care and Use of Laboratory Animals by the National Research Council and were reviewed and approved by the University of Mississippi Medical Center Institutional Animal Care and Use Committee (IACUC #1469) and the US Army's Animal Care and Use Review Office (ACURO). In conducting research using animals, the investigators adhered to the laws of the United States and regulations of the Department of Agriculture.

Animals

Male, Long Evans rats (250-300 g, Envigo; N = 66) were multiply housed and maintained in a room with a 12/12-hour light/dark cycle at 25 °C and 50% to 60% humidity. Rats were fed an ad libitum access to water and standard chow (#8640, Envigo, 3.0 kcal/g; 17% fat, 54% carbohydrate, 29% protein). Rats were assigned to either sham-laminectomy (Sham; N = 32) or thoracic spinal cord injury (tSCI; N = 34) groups in a counterbalanced fashion based on initial body weight. Following surgery, the rats were single-housed. We chose 2 time frames for assessment following: 4 weeks after injury, which allows for the return of the body weight to preinjury levels 16 weeks, which provides 12 weeks acclimatization to the diets. Animals recovered for 4 weeks following surgery on chow. A subset of Sham and tSCI (N = 8/group) were euthanized 28 days postinjury. The remainder of the rats were switched to either a HFD (#D03082706, Research Diets; 4.54 kcal/g; 40% fat, 46% carbohydrate, 15% protein) or LFD (#D03082705, Research Diets; 3.81 kcal/g, 9% fat, 76% carbohydrate, and 15% protein) until the end of the study totaling 16 weeks. The final N size for each group Sham-LFD (N = 9), Sham-HFD (N = 9), tSCI-LFD (N = 10), and tSCI-HFD (N = 8). The study activities are outlined in Figure 1A and B. Several rats were lost early due to euthanasia due to autophagy produced by neuropathic pain that is more common in Long Evans as previously described.[13]

Surgical Procedures

Injuries were performed on animals that were deeply anesthetized using 5% isoflurane with a gradual decrease to 2.5%. tSCI surgeries were performed as previously described.[13-15] Briefly, following exposure of the thoracic level 10 (T10) spinal cord, a laminectomy was performed while grasping the ventral surface of the lateral spinous processes at vertebral levels T9 and T11. Using an Infinite Horizon Spinal Impactor Device (Precision Systems and Instrumentation, LLC), moderate contusion/compression injuries were delivered to the T10 spinal cord using 150 kdynes of force with a 1-second dwell. The dura mater was not penetrated for any of these injuries. Finally, the overlying muscles were sutured, and the skin was securely closed using stainless steel wound clips.

Sham-laminectomy surgery consisted of a laminectomy performed at T10, and then the overlying muscles were sutured, and the skin was securely closed using stainless steel wound clips.

Postoperative Care

As previously described,[13,15] rats received one dose of buprenorphine SR (sustained release; 1.0 to 1.2 mg/kg SQ; ZooPharm) and, 72 hours later, single-dose buprenorphine for postsurgical pain management (0.025 mg/kg, twice daily for a period of 2 days, then as needed). In addition, rats also receive (1) antibiotic, Naxcel (5 mg/kg SQ, Zoetis) once daily for a period of 5 days, and (2) 3 to 5 mL of 0.9% saline, twice daily for a period of 3 days to ensure hydration. Beginning the day of tSCI surgery, each rat's urinary bladder was manually expressed 2 to 3 times daily until the animal recovered the ability to void its bladder. As a rule, bladder care was discontinued for a rat when it exhibited an already voided bladder on 2 consecutive bladder care sessions.

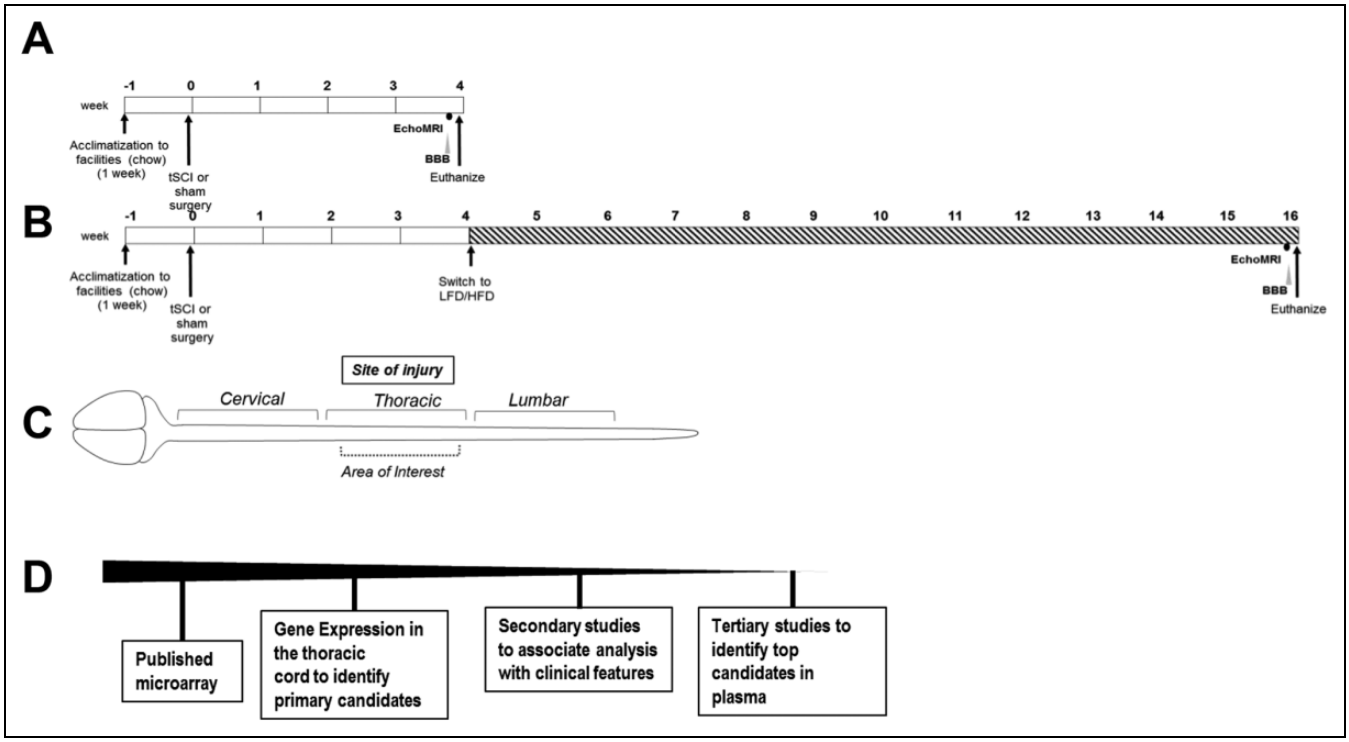

Figure 1. General schema of sham and tSCI animals euthanized at (A) 4 weeks and (B) 16 weeks. (C) Diagram of 1.5 cm area of interest in the thoracic cord (D) pipeline of activities to identify potential biomarkers for SCI disease progression.

Hindlimb Locomotor Function Assessment

Hindlimb locomotor function was assessed using the Basso, Beattie, and Bresnahan (BBB) open-field locomotor scale.[16] BBB scores were initially assessed on days 1, 7, 14, and 28 postinjury. Following diet inductions on day 28, BBB was tested at weeks 8, 12, and 16 on diet. These were performed as previously described.[13,15]

Body Weight and Composition

Following surgery, animals were weighed daily for the first 14 days and then weekly after that. Lean and fat body mass composition was analyzed using Echo Magnetic Resonance Imaging (echoMRI; EchoMedical Systems) before euthanasia for each cohort.

Tissue Harvest

Both at 4 and 16 weeks after injury, rats were euthanized by conscious decapitation starting at 6 hours following the onset of the light cycle. The spinal cord was carefully excised using a Rongeur instrument. The spinal cord was sectioned into 3 pieces measuring approximately 1.5 cm around thoracic enlargements. The T10 lesion site was completely included in the thoracic compartment. Tissue was flash-frozen with methylbutane on dry ice and then stored in −80 °C until further processing.

RNA Processing and Real-Time PCR

RNA was extracted using a QIAGEN miniprep RNA kit (QIAGEN, Inc), and complementary DNA was transcribed using an iScript complementary DNA synthesis kit (Bio-Rad Laboratories). Real-time PCR was performed on a Step-One Plus Real-Time PCR machine coupled with StepOne Software (v2.3; Applied Biosystems) using TaqMan inventoried gene expression assays (Life Technologies) as listed in Tables 3 and 4.

Plasma Determination by ELISA

Terminal plasma for 28-day and 100-day rats was probed for the following proteins using commercially available ELISAs: ApoE, FDFT1, Lcat, LDLR, and PLTP. The following kits were used: APOE (#MBS2512633), FDFT1 (#MBS9331891), LCAT (#MBS2881993), LDLR (#MBS260387), and PLTP (#MBS036126; MyBioSource). All plasma samples were used undiluted except when probing for LDLR in which we used a 1:10 dilution with provided sample diluent.

Statistical Analyses

All statistical analyses were performed using GraphPad Prism version 7.02 (GraphPad Software). Differences between 2 groups were assessed by using unpaired Student's *t* test and 2-tailed distribution. To observe timewise differences, 2-way ANOVA (variables: injury group/diet and time) with the Bonferroni post hoc test was used. All results are given as means ±

Table 1. Body parameters and blood lipids for Sham and SCI rats four weeks postinjury.

	4 Weeks postinjury[a]	
	Sham	SCI
Body weight (g)	395.9 ± 13.3	377.3 ± 12.4
Fat mass (g)	27.6 ± 2.0	22.7 ± 0.8*
Lean mass (g)	340.6 ± 10.1	316.7 ± 12.0
BBB score	21.0 ± 0.0	11.3 ± 0.9*
Plasma triglycerides (mg/dL)	131.5 ± 14.9	146.1 ± 7.2
Plasma cholesterol (mg/dL)	103.7 ± 2.2	88.6 ± 3.2*

Abbreviations: SCI, spinal cord injury; BBB, Basso, Beattie, and Bresnahan scale.
[a]Data is presented as mean ± SEM.
*$P < .05$ (Student's t test).

SEM. Results were considered statistically significant when $P < .05$.

Results

Paradigm Used for the Study

Two cohorts of male rats were obtained for this study. Male Long Evans rats were acclimatized to the vivarium for 1 week and then were scheduled to receive either a sham-laminectomy (Sham) or tSCI in a counter-balanced fashion by body weight (Figure 1A and B). Rats received palliative care for several weeks and were placed on standard chow and euthanized 4 weeks after injury (Figure 1A) or were placed on either LFD or HFD for an additional 12 weeks and were euthanized 16 weeks postinjury (Figure 1B).

Thoracic-level tissues were dissected 1.5 cm around the thoracic enlargement as depicted (Figure 1C) and used for primary gene expression analyses that were prioritized based on immune and lipid metabolism functions as described previously.[12] Our generalized scheme was to further distill previous microarray gene targets, validate their expression at 2 time points, perform secondary linear regression with key clinical features, and measure top candidates in circulation at 4 and 16 weeks postinjury (Figure 1D).

General Characterization of tSCI Cohorts

No difference in body weight was observed in rats in the 4-week cohort (Table 1). tSCI animals had reduced fat mass in comparison to Sham rats ($P < .05$) following 4 weeks on chow (Table 1). No statistical difference between Sham and tSCI lean body mass by EchoMRI (Table 1). Sham animals received a BBB locomotor rating of 21 after 4 weeks of injury, while tSCI rats received a score of 11.3 ± 0.9 (Table 1). There were no differences in fasting triglycerides, but tSCI animals had reduced cholesterol in comparison to Sham, $P < .05$ (Table 1).

After 12 weeks on either LFD or HFD, there was a significant main effect of injury ($P < .01$) and diet ($P < .05$) on body weight, such that the HFD-fed animals were heavier than the LFD-fed animals and tSCI animals weighed less than the Sham animals (Table 2). No statistical differences were measured concerning fat mass by diet or injury (Table 2). tSCI animals had a reduced lean body mass in comparison to Sham animals 16 weeks after injury (main effect of injury, $P < .001$; Table 2). tSCI-LFD rats had an average score of 10.6 ± 1.0, and tSCI-HFD animals had a score of 11.9 ± 0.9 (Table 2). HFD-fed animals had both reduced triglycerides and cholesterol in comparison to Sham (main effect of diet, $P < .05$; Table 2).

Immune Gene Expression in Thoracic Cord

Microglial marker IBA1 (AIF1) was significantly elevated in tSCI animals at 4 weeks ($P < .01$) in comparison to Sham (Figure 2A) and trended to be higher in tSCI animals in comparison to Sham 16 weeks postinjury (Figure 2A). Macrophage marker CD68 was significantly elevated in tSCI cord in comparison to Sham ($P < .001$; Figure 2B). CD68 continued to be elevated 16 weeks postinjury (main effect of injury, $P < .0001$) with no effect of diet (Figure 2B). Complement C1q C Chain (C1QC) was significantly elevated in tSCI cord in comparison to Sham ($P < .001$; Figure 2C). C1QC continued to be increased 16 weeks postinjury (main effect of injury, $P < .05$) with no effect of diet (Figure 2C). Glial fibrillary acid protein (GFAP) mRNA was significantly increased in tSCI animals in comparison to Sham ($P < .05$; Figure 2D). GFAP elevation continued at 16 weeks postinjury in tSCI animals (main effect of injury, $P < .05$) with no effect of diet (Figure 2D). Galectin 3 (LGALS3), a carbohydrate-binding lectin whose expression is associated with inflammatory cells including macrophages, neutrophils, and mast cells, was significantly elevated in tSCI animals in comparison to Sham ($P < .0001$) and continued to be elevated at 16 weeks postinjury (main effect of injury, $P < .001$; Figure 2E). Integrin subunit alpha X (ITGAX) (dendritic cell marker) mRNA was significantly elevated in tSCI animals in comparison to Sham ($P < .0001$; Figure 2F). ITGAX elevations continued at 16 weeks postinjury in tSCI animals (main effect of injury, $P < .0001$) with no effect of diet (Figure 2F).

Upregulated Lipid Metabolism Gene Expression in the Thoracic Cord

Gene transcripts involved in cholesterol and lipid metabolism were chosen from a previously published data set, and the effect of time and diet were considered. Gene expression ATP-binding cassette transporter 1 (ABCA1, cholesterol efflux regulatory protein; Figure 3A), apolipoprotein E (APOE; Figure 3B), lecithin-cholesterol acyltransferase (LCAT; Figure 3C), phospholipid transfer protein (PLTP; Figure 3F) sterol regulatory element-binding protein 1 (SREBP1; Figure 3G) mRNA were all significantly elevated in tSCI cord in comparison to Sham. Each gene target was also significantly elevated in tSCI animals 16 weeks after diet in comparison to Sham (Figure 3A-C, F-G). No changes in mRNA expression were measured in peroxisome proliferator-activated receptor alpha (PPARα) at either time point (Figure 3D). Concerning PPARγ, no difference was measured at the early time point, but

Table 2. Body parameters and blood lipids for Sham and SCI rats 16 weeks postinjury.

	16 Weeks postinjury[a]			
	Sham-LFD	SCI-LFD	Sham-HFD	SCI-HFD
Body weight (g)	583.8 ± 27.2	515.1 ± 27.9	640.1 ± 34.0	551.3 ± 27.5
Fat mass (g)	106.9 ± 14.1	87.9 ± 18.1	130.0 ± 20.5	122.7 ± 15.2
Lean mass (g)	444.8 ± 15.3	396.4 ± 6.4	454.7 ± 14.9	397.3 ± 13.3
BBB score	21.0 ± 0.0	10.6 ± 1.0	21.0 ± 0.0	11.9 ± 0.9
Plasma triglycerides (mg/dL)	305.9 ± 67.1	217.9 ± 47.2	430.1 ± 68.0	233.0 ± 51.7
Plasma cholesterol (mg/dL)	135.1 ± 11.7	110.8 ± 12.6	130.5 ± 15.3	92.8 ± 11.3

Abbreviations: LFD, low-fat diet; SCI, spinal cord injury; HFD, high-fat diet; BBB, Basso, Beattie, and Bresnahan scale.
[a]Data is presented as mean ± SEM. Two-way ANOVA by injury and diet.

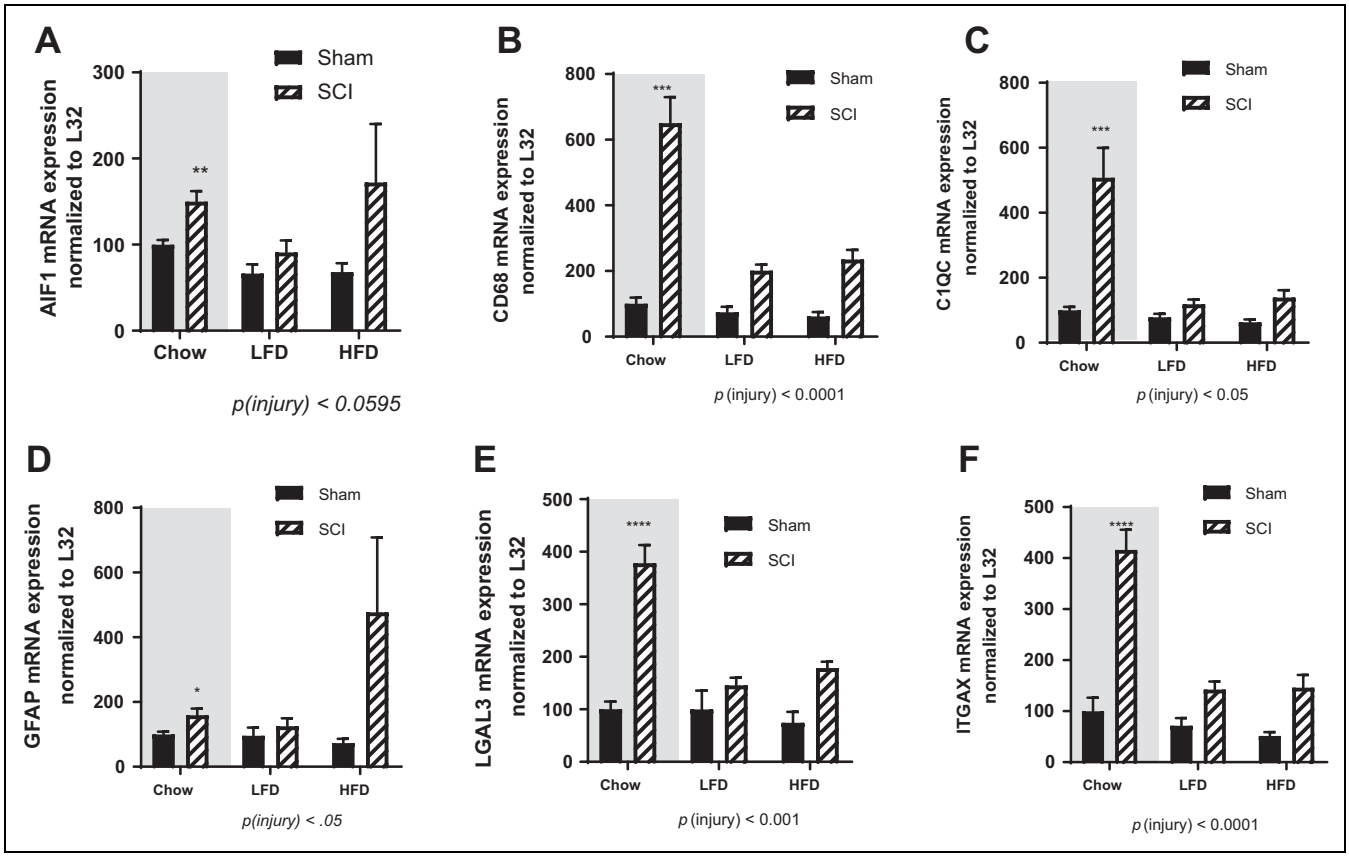

Figure 2. Upregulated immune-related transcripts in thoracic spinal cord region from animals injured 4 and 16 weeks prior. (A) AIF1, (B) CD68, (C) C1QC, (D) GFAP, (E) LGAL3, (F) ITGAX. Data is presented as mean ± SEM. Four-week injured animals were chow-fed and comparisons performed using Student's *t* test. Sixteen-week animals were fed either LFD or HFD and comparisons were performed using 2-way ANOVA. N = 8 to 10/group with main effects listed. *$P < .05$, **$P < .01$, ***$P < .001$.

we measured elevations in PPARγ mRNA in tSCI animals 16 weeks postinjury (Figure 3E).

Downregulated Lipid Metabolism Gene Expression in Thoracic Cord

A certain portion of cholesterol metabolism genes was downregulated after injury and never appeared to recover substantially. Gene expression of farnesyl-diphosphate farnesyltransferase 1 (FDFT1; Figure 4A), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMCGR; Figure 4B), 17-beta-hydroxysteroid dehydrogenase enzyme (HSD17B7; Figure 4C), low-density lipoprotein receptor-related protein 1 (LRP1; Figure 4D), and low-density lipoprotein receptor (LDLR; Figure 4E) were all significantly reduced in tSCI animals in comparison to Sham after 4 weeks postinjury. The only transcript to recover expression was LRP1, in which there was no significant differences among the animals at 16 weeks. All the remaining transcripts exhibited suppressed mRNA expression at 16 weeks and were not affected by diet.

Table 3. Linear Regression for Relationship Between Immune-Related mRNA Transcript Levels and Either BBB (Basso, Beattie, and Bresnahan) Locomotor Score, BW (Body Weight), LM (Lean Mass), FM (Fat Mass), CHOL (Cholesterol Levels), or TRIGS (Triglyceride Levels) for 4- and 16-Week SCI[a].

Gene	Name		BBB	BW	LM	FM	CHOL	TRIGS
AIF1	Allograft inflammatory factor 1	*P*	.58	**.01**	.11	**.02**	.38	.06
		F	0.31	**8.49**	2.70	**6.38**	0.82	3.87
		R^2	0.01	**0.27**	0.11	**0.22**	0.03	0.14
C1QC	Complement component 1, q subcomponent, C chain	*P*	**.00**	**.00**	**.00**	**.00**	.28	.09
		F	**10.51**	**11.52**	**18.26**	**9.85**	1.21	3.18
		R^2	**0.31**	**0.33**	**0.44**	**0.30**	0.05	0.12
CD68	Cluster of differentiation 68	*P*	**.00**	.06	**.00**	.09	.74	.85
		F	**14.87**	3.99	**15.40**	3.17	0.11	0.04
		R^2	**0.39**	0.15	**0.40**	0.12	0.00	0.00
GFAP	Glial fibrillary acidic protein	*P*	.15	.27	.62	.67	.99	.85
		F	2.21	1.29	0.25	0.19	0.00	0.04
		R^2	0.09	0.05	0.01	0.01	0.00	0.00
ITGAX	Integrin, alpha X, (CD11c)	*P*	**.00**	**.00**	**.00**	**.00**	.28	.04
		F	**13.14**	**28.91**	**47.89**	**20.77**	1.23	5.01
		R^2	**0.36**	**0.56**	**0.68**	**0.47**	0.05	0.18
LGALS3	Galectin-3	*P*	**.00**	**.00**	**.00**	**.00**	.16	.10
		F	**12.79**	**14.65**	**30.48**	**12.88**	2.10	2.85
		R^2	**0.36**	**0.39**	**0.57**	**0.36**	0.08	0.11

[a]*F* statistic and R^2 reported. N = 8 to 10/group. Data is presented as mean ± EM. Boldfaced parameters denote statistically significant differences. $P < 0.05$.

Relationship Between the Injured Thoracic Cord Immune-Related Transcripts and Outcomes

To determine which transcripts in the cord may inform us concerning the outcome of each rat, we performed linear regression using only the transcript information of the SCI animals and important endpoints. We compared BBB score, terminal body weight, lean mass, fat mass, and fasting plasma cholesterol and triglycerides. We report *P* value, *F* statistics, and R^2 for each transcript reported. Of the immune targets, AIF1, LGALS3, and C1QC are most informative in their association with BBB score, terminal body weight, lean mass, and fat mass (Table 3). CD68 levels in the cord are associated significantly with BBB and lean body mass (Table 3). Surprisingly, ABCA1, GFAP, and AIF1 have diminished value to inform concerning the output measures in question (Table 3).

Relationship Between the Injured Thoracic Cord Metabolism-Related Transcripts and Outcomes

Similarly, linear regression was performed in the metabolically relevant genes. The relationship between gene expression for FDFT1, HMCGR, LDLR, PLTP, and SREBF1 was significantly associated with the outcomes outlined for this study (Table 4). HSD17B7, LRP1, and PPARG were less informative in our analysis (Table 4).

Circulating Markers Following SCI

Using conventional ELISAs we measured APOE, LCAAT, PLTP, LDLR, and FDFT1 in circulation at both 4 and 16 weeks after injury. Despite local gene expression differences reported, we were not able to measure differences by time or injury or diet in the cord for these analytes (Table 5).

Discussion

In the current study, we probed the relationship of important immune and metabolic gene expression within the injured cord in the subacute and chronic phases of cord injury with important measures of locomotor function and metabolic health. We find close relationships between the cord expression and BBB, body weight, lean mass, fat mass, cholesterol, and triglyceride levels. The goal of this work was to determine if any circulating factor could inform us of disease progress in the rat SCI contusion model.

Immune-Related Gene Targets

Our distilled list of genes suggests elevations in integral cellular immune responses in the area of injury at both 4 weeks and 16 weeks. It is not surprising that we observed significant elevations in markers of microglia (AIF), macrophages (CD68), complement cascade factors (C1QC), astrocytes (GFAP), dendritic cells (ITGAX), and pan lectin (LGALS3). Integrin alpha X (ITGAX also known as CD11c) is highly expressed in immune tissues and adipose tissue.[17,18] These all remained elevated 16 weeks after injury and, in most cases, were not impacted by the type of diet consumed by the animals in this study. However, in the case of the gene markers for microglia (IBA1) and astrocytes (GFAP), HFD does, in fact, appear to exacerbate the expression of the markers.

Temporal expression analysis of macrophage markers has previously been reported in SCI.[19] The work reported was

Table 4. Linear Regression for Relationships Between Lipid-Related mRNA Transcript Levels and Either BBB (Basso, Beattie, and Bresnahan) Locomotor Score, BW (Body Weight), LM (Lean Mass), FM (Fat Mass), CHOL (Cholesterol Levels), or TRIGS (Triglyceride Levels) for 4- and 16-Week SCI[a].

Gene	Name		BBB	BW	LM	FM	CHOL	TRIGS
APOE	Apolipoprotein E	*P*	0.09	**0.01**	**0.01**	**0.00**	0.33	0.09
		F	3.04	**7.11**	**9.37**	**9.92**	0.99	3.22
		R^2	0.12	**0.24**	**0.29**	**0.30**	0.04	0.12
FDFT1	Farnesyl-diphosphate farnesyltransferase 1	*P*	**.02**	**.00**	**.00**	**.00**	**.05**	.28
		F	**6.33**	**13.29**	**13.57**	**10.36**	**4.33**	1.21
		R^2	**0.22**	**0.37**	**0.37**	**0.31**	**0.16**	0.05
HMCGR	HMG-CoA reductase	*P*	**.02**	**.04**	**.02**	**.05**	.57	.93
		F	**6.58**	**4.96**	**6.43**	**4.10**	0.33	0.01
		R^2	**0.22**	**0.18**	**0.22**	**0.15**	0.01	0.00
HSD17B7	17-beta hydroxysteroid dehydrogenase type 7	*P*	.10	**.04**	.06	.06	.41	.84
		F	2.89	**4.56**	4.07	3.92	0.70	0.04
		R^2	0.11	**0.17**	0.15	0.15	0.03	0.00
LCAT	Lecithin–cholesterol acyltransferase	*P*	0.47	1.00	0.63	0.90	0.99	0.41
		F	0.53	0.00	0.23	0.01	0.00	0.71
		R^2	0.02	0.00	0.01	0.00	0.00	0.03
LDLR	Low-density lipoprotein receptor	*P*	.07	**.02**	**.03**	**.04**	.09	.68
		F	3.50	**5.92**	**5.06**	**4.59**	3.09	0.18
		R^2	0.13	**0.20**	**0.18**	**0.17**	0.12	0.01
LRP1	Low density lipoprotein receptor-related protein 1	*P*	**.01**	.13	**.03**	.19	.99	.86
		F	**8.00**	2.44	**5.06**	1.81	0.00	0.03
		R^2	**0.26**	0.10	**0.18**	0.07	0.00	0.00
PLTP	Phospholipid transfer protein	*P*	.40	**.01**	**.01**	**.01**	.18	**.02**
		F	0.73	**7.95**	**8.43**	**7.99**	1.96	**6.35**
		R^2	0.03	**0.26**	**0.27**	**0.26**	0.08	**0.22**
PPARA	Peroxisome proliferator-activated receptor alpha	*P*	.41	.20	.13	.33	.64	.32
		F	0.70	1.72	2.53	1.00	0.23	1.01
		R^2	0.03	0.07	0.10	0.04	0.01	0.04
PPARG	Peroxisome proliferator-activated receptor gamma	*P*	**.04**	.13	**.04**	.17	.39	.92
		F	**4.73**	2.46	**4.79**	1.97	0.78	0.01
		R^2	**0.17**	0.10	**0.17**	0.08	0.03	0.00
SREBF1	Sterol regulatory element-binding transcription factor 1	*P*	.56	**.03**	**.04**	**.02**	.29	**.04**
		F	0.35	**5.30**	**4.82**	**6.32**	1.18	**4.93**
		R^2	0.02	**0.19**	**0.17**	**0.22**	0.05	**0.18**

[a] *F* statistic and R^2 reported. N = 8 to 10/group. Boldfaced parameters denote statistically significant differences. $P < 0.05$.

specifically done to determine the temporal pattern of M1 and M2 subtype macrophages following a contusive injury to the cord at the mid-thoracic region from 1 to 28 days postinjury.[19] Workers showed that though the M1 polarized macrophages were elevated throughout the time investigated, M2 spiked 7 days postinjury and was back to normal within 7 days.[19] Transplantation of rat-derived microglia into the area of SCI does appear to improve various measures of locomotor activity in a rat model of contusion and suggests a role for microglia in the recovery of locomotor function.[20] Peripheral blood mononuclear cell secretome infused into the area of cord injury increased recruitment of CD68-positive macrophages resulting in reduced oxidative stress via nitric oxide and increased angiogenesis in the site of the lesion.[21] Treatment of the spinal cord with a nano-carrier fused to minocycline (microglial inhibitor) was successful in reducing the level of expression of pro-inflammatory mediators, IL-6, and reduce the expression of CD68 in area of trauma.[22] Thus, inhibiting microglial propagation in the area of the lesion may improve long-term outcomes.

LGALS3 is a galactose-specific lectin that mediates IgE binding. In our studies, LGALS3 is 3-fold elevated and remains significantly increased at 16 weeks. Lectins have a wide range of roles; they are known to specifically mediate cell adhesion and to be important in the migration of astrocytes. SCI performed in GAL3 knockout mice has reduced recovery of motor function in comparison to wild-type mice, suggestive that LGALS3 presence exacerbates neuroinflammation.[23] The continued elevation of LGALS3 is not surprising considering the high level of infiltration of many cell types that perform immune response, phagocytosis, and debris clearing in the area of injury.

Lipid-Related Transcripts That Were Upregulated

Among the differentially regulated genes identified,[12] there was a robust representation of lipid/cholesterol-related genes. We focused on some targets which were promising candidates. For example, ABCA1, a cholesterol and phospholipid efflux

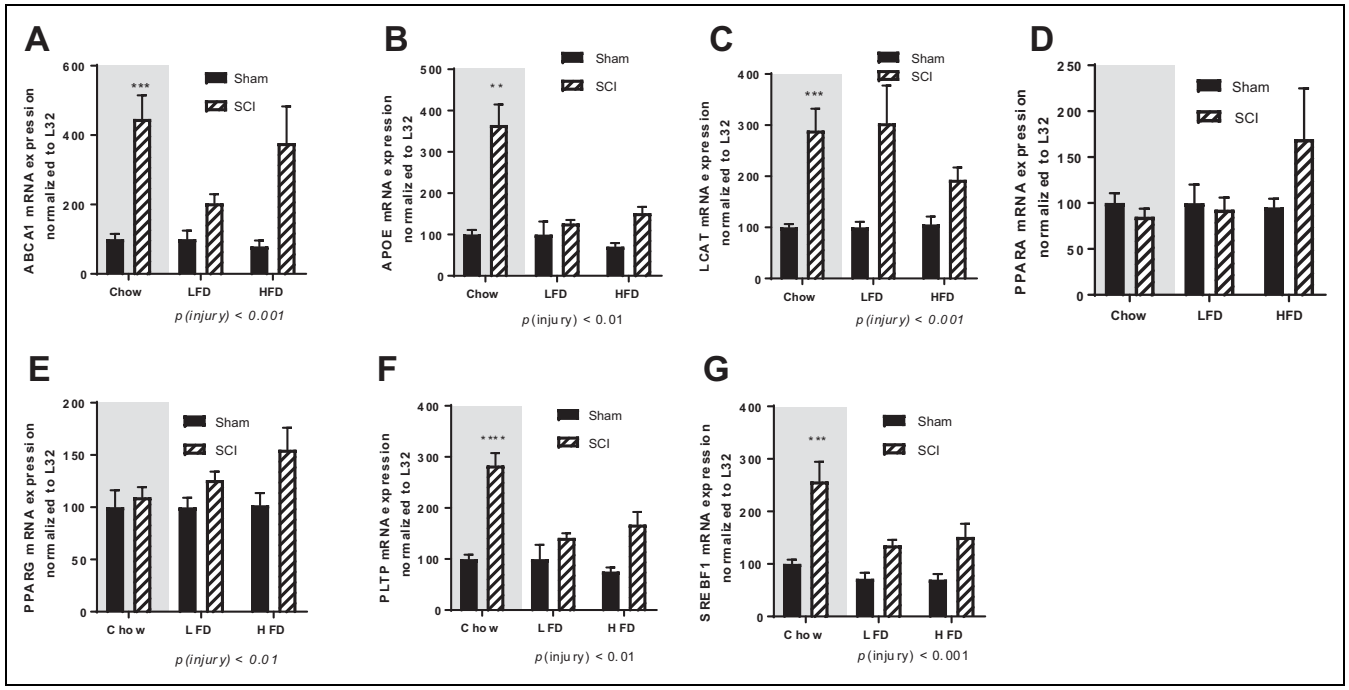

Figure 3. Upregulated lipid-related transcripts in thoracic spinal cord region from animals injured 4 and 16 week prior. (A) ABCA1, (B) APOE, (C) LCAT, (D) PPARA, (E) PPARG, (F) PLTP, (G) SREBP1. Data is presented as mean ± SEM. Four-week injured animals were chow-fed and comparisons performed using Student's *t* test. Sixteen-week animals were fed either LFD or HFD and comparisons were performed using 2-way ANOVA. N = 8 to 10/group with main effects listed. **P* < .05, ***P* < .01, ****P* < .001.

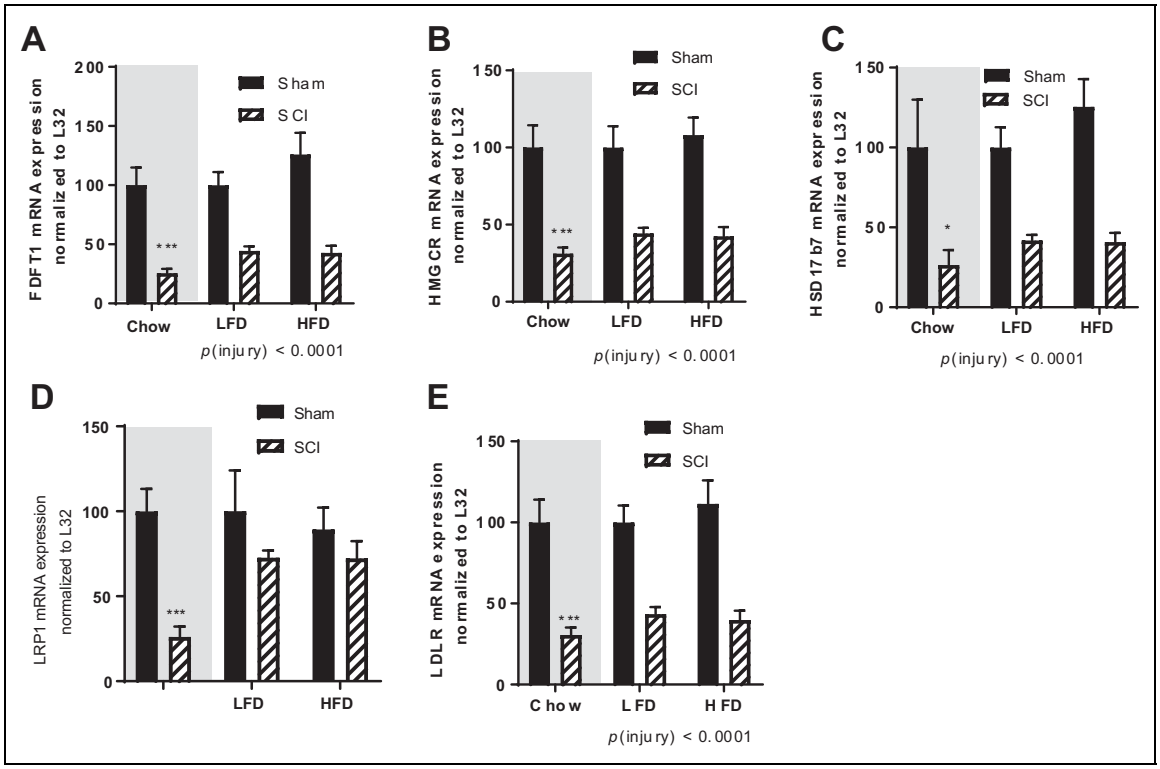

Figure 4. Downregulated lipid-related transcripts in thoracic spinal cord region from animals injured 4 and 16 week prior. (A) FDFT1, (B) HMCGR, (C) HSD17b7, (D) LRP1, (E) LDLR. Data is presented as mean ± SEM. Four-week injured animals were chow-fed and comparisons performed using student's *t* test. Sixteen-week animals were fed either LFD or HFD and comparisons were performed using 2-way ANOVA. N = 8 to 10/group with main effects listed. **P* < .05, ***P* < .01, ****P* < .001.

Table 5. Plasma Biomarkers in Circulation (A) 4 Weeks Postinjury and (B) 16 Weeks Postinjury[a].

(A) Postinjury (4 weeks)	Sham	tSCI		
APOE (mg/mL)	224.2 ± 9.13	266.3 ± 9.11		
LCAT (ng/mL)	2.20 ± 0.08	2.00 ± 0.08		
PLTP (ng/mL)	2.90 ± 0.06	3.08 ± 0.06		
LDLR (ng/mL)	6.40 ± 0.83	8.44 ± 1.71		
FDFT1 (ng/mL)	1.96 ± 0.13	2.19 ± 0.24		
(B) Postinjury (16 weeks)	Sham-LFD	tSCI-LFD	Sham-HFD	tSCI-HFD
APOE (mg/mL)	259.5 ± 9.74	259.2 ± 14.84	270.8 ± 9.30	237.3 ± 16.34
LCAT (ng/mL)	2.27 ± 0.11	2.28 ± 0.13	2.37 ± 0.06	2.27 ± 0.08
PLTP (ng/mL)	2.96 ± 0.09	3.00 ± 0.08	2.93 ± 0.06	2.91 ± 0.06
LDLR (ng/mL)	12.61 ± 1.37	11.65 ± 1.11	13.90 ± 1.15	11.67 ± 1.02
FDFT1 (ng/mL)	2.22 ± 0.17	2.14 ± 0.13	2.08 ± 0.14	2.02 ± 0.10

Abbreviations: tSCI, thoracic spinal cord injury; LFD, low-fat diet; HFD, high-fat diet.
[a]Plasma samples were used to determine circulating levels of proteins for APOE, LCAT, PLTP, LDLR, and FDFT1 in 4- and 16-week injured rat plasma. Data is presented as mean ± SEM.

transporter, is elevated both subacutely and chronically in the tSCI model. ABCA1 is commonly upregulated in pro-inflammatory environments and is associated with enhanced neurotoxicity and impaired wound healing.[24] ABCA1 responds to elevations in myelin debris, and continued myelin debris results in foamy macrophages being overwhelmed by the level of injury resulting in reduced capacity to continue phagocytosis in the area of the injury.[24] In our HFD-fed animals a trend to higher ABCA1 gene levels suggests that the addition of saturated fats to the diet drive gene expression to greater levels.

APOE, a plasma protein responsible for lipid/cholesterol transport, is very significantly elevated 4 weeks after injury and then dissipates 16 weeks after injury, though still clearly elevated in tSCI animals. Previous reports suggested that it reached maximal levels 2 weeks postinjury.[25] However, we report a 4-fold elevation at 4 weeks. Using an APOE-mimetic, short-term recovery of motor function was possible in a T8 contusion rat model suggestive that APOE may be neuroprotective and reduces microglial activation in the lesion site.[26]

LCAT (lecithin–cholesterol acyltransferase) is an enzyme that converts free cholesterol into cholesteryl ester that is then transferred into a lipoprotein particle core. In our studies, LCAT was elevated 3-fold 4 weeks after injury but still remained 2- to 3-fold elevated 16 weeks after injury with no clear impact of diet. No work has been done identifying whether targeting LCAT could improve spinal cord related measures. Based on its function, its elevation may be linked to continued levels of myelin debris and the inability to clear the area of injury.

Lipid-Related Transcripts That Were Suppressed Chronically

Some lipid-related genes were chronically reduced following SCI and whose expression did not recover even after 16 weeks. Among these, FDFT1 and HMCGR were downregulated in SCI in the cord. FDFT1 is a membrane-associated enzyme important in cholesterol biosynthesis, whereas HMCGR is the rate-limiting enzyme for cholesterol synthesis. Though the expression of cord FDFT1 and HMCGR varied significantly with BBB, body weight, lean mass, and fat mass, we failed to identify variations by ELISA in plasma. This was similarly true for LDLR, which encodes for the receptor that carries cholesterol in the blood and APOE, which encodes for apolipoprotein E. Our attempt to determine circulating markers by using this method did not prove fruitful.

The Intersection of Metabolic and Immune Health After SCI

Many facets of metabolic health are altered after SCI. SCI is accompanied by a loss in lean mass,[27] an increased ratio of fat mass to lean[28] and altered metabolic rate.[13,29] These metabolic parameters are worsened with consumption of HFD,[13] and saturated fat consumption is further damaging to the immune system after SCI.[15] We have previously reported the chronic impact SCI has on circulating peripheral leukocytes and immune organs such as the spleen and thymus.[15] The complex way in which metabolic health and inflammation are interrelated suggest that improving metabolic health would have direct and indirect positive influence on immune health and vice versa. Obtaining a clear view of the markers that could inform us concerning their temporal progression could refine holistic health management of the SCI individual and provide new avenues of intervention for long-term care.

Significance, Limitations, and Future Directions

In the current study, we were able to specifically identify immune and lipid markers in the cord at a subacute and chronic time frame that had enduring expression level changes. Given the importance of the immune response to neurotrauma and metabolic requirements for mending and regrowing

connectivity, the enduring differential expression may provide opportunities for enhanced metabolic "feeding" of the lesion site with a cocktail of substrates that may improve and accelerate the rate of recovery.

We were limited by access to reagents that were validated to appropriately assay the protein products of the mRNAs we reported. We concede that western blot analysis of the various proteins of interest would have had greater specificity and would be important for validation for further follow-up of these gene targets. We regret not having the resources of reagents and tissues to formally perform these. Future work to identify high-fidelity biomarkers would be to use a proteomics approach to validate the transcripts of future work to identify high-fidelity biomarkers would be to use a proteomics approach to validate the transcripts of interest reported here. We were also limited by the time frames we were able to test. Acute injury was not the focus of this study, and the addition of this time point may indeed have brought to light a different outlook on the trajectory of cord neurorecovery. The timeframe was chosen to allow for weight stabilization to occur after injury, which in our hands occurs between weeks 3 and 4. The latter time frame was chosen because various studies suggest that obesity-related parameters have onset between 8 and 16 weeks after expose to diet. Given the large number of animals we handled in this study, we choose the intermediate time point of 12 weeks on diet for our study. Shorter and longer time frames may have produced diverse changes in the trajectory of some of the genes of interest.

Taken together, because of the complex interaction of neural, metabolic, hormonal, and immune aspects of SCI, biomarker identification continues to be challenging in the realm of cord injury and recovery.

Declaration of Conflicting Interests

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