Immunohistochemical analysis of TIMP-2 and collagen types I and IV in experimental spinal cord ischemia–reperfusion injury in rats

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Background: Thoracic and thoracoabdominal aortic intervention carries a significant risk of spinal cord ischemia. The pathophysiologic mechanisms that cause hypoxic/ischemic injury to the spinal cord have not been totally explained. In normal spinal cord, neurons and glial cells do not express type IV collagen. Type IV collagen produced by reactive astrocytes is reported to participate in glial scar formation. Tissue inhibitors of metalloproteinases (TIMPs) are endogenous inhibitors that regulate the activity of the matrix metalloproteinases (MMPs). TIMP-2 binds strongly with MMP-2, facilitating activation by membrane-type MMP. Imbalance between TIMPs and MMPs can lead to excessive degradation of matrix components. Type IV collagen involved in the blood–brain barrier disruption and glial scar formation, TIMP-2 influences MMP-2 that controls degradation of collagen I and IV.

Objective: To examine the immunohistochemical analysis of TIMP-2 and collagen types I–IV in experimental spinal cord ischemia–reperfusion in rats.

Methods: Thirty-two male Wistar rats weighing 250–300 g were divided into four groups: group S: sham group ($n = 8$); group 0P: 30-minute occlusion without perfusion ($n = 8$); group 3P: 30-minute occlusion and 3-hour perfusion ($n = 8$); and group 24P: 30-minute occlusion and 24-hour perfusion ($n = 8$). Infrarenal aorta was cross-clamped at two sites by using two aneurysm clips for 30 minutes. Reperfusion was provided after removal of the clips. Lumbar spinal cord segments were removed for immunohistochemical analysis.

Results: TIMP-2 and collagen staining in 3-hour perfused (3P) group were nearly the same with sham group (S). TIMP-2 and collagen staining increased in the 24-hour perfused group.

Conclusion: Alterations in collagen levels may relate to the biphasic breakdown of the blood–brain barrier and collagen staining in new cell types with relation to glial scar formation. Our results demonstrate that 3-hour perfusion after occlusion in hypoxic/ischemic spinal cord injury seems to be the critical reversible period.

Keywords: Spinal cord ischemia, Collagen IV, TIMP-2, Paraplegia, Glial scar formation

Introduction

Spinal cord injury (SCI) has traumatic and non-traumatic origin and often leads to catastrophic dysfunction and disability. The pathophysiologic mechanisms that underlie hypoxic/ischemic injury to the spinal cord have not been totally explained yet.[1–7] Thoracic and thoracoabdominal aortic intervention carries a significant risk of spinal cord ischemia, ranging from 5 to 20%.[8–10] Pouw *et al.*[11] compared the neurological outcome between paraplegia caused by acute spinal cord ischemia syndrome and traumatic SCI, and found that neurological outcome was independent of the diagnosis. In the literature, studies of spinal cord ischemia are based on biochemical markers of oxidative stress[1,2,12,13] and there are various studies of matrix metalloproteinases (MMPs) in traumatic SCI.[14–16] The extracellular matrix (ECM) of the blood–brain barrier (BBB) forms

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a basal lamina, which selectively filters blood elements. Collagen IV is the major structural component of all basement membranes. The response of the adult mammalian central nervous system (CNS) to injury results in a gliosis in the lesion and the formation of a glial scar. Type IV collagen produced by reactive astrocytes is reported to participate in glial scar formation.[17–19] Type IV collagen involved in the BBB and glial scar and high levels of tissue inhibitor of metalloproteinase 2 (TIMP-2) inhibit MMP-2 that degrades collagen I and IV.

In this study, we investigated the levels of TIMP-2 and collagen IV and I that are involved in (non-traumatic) SCI pathogenesis after experimental spinal cord ischemia and reperfusion.

Methods

Thirty-two male Wistar rats weighing 250–300 g were housed in a temperature- and humidity-controlled room (22 ± 3 °C and 67 ± 7%, respectively) in which a 12:12 hours light–dark cycle was maintained. The animals were fed standard rat chow and tap water *ad libitum*. Ethical approval was granted by the University Ethics Committee.

The animals were anesthetized by intramuscular injection of 70 mg/kg ketamine (Ketalar, Parke-Davis, Eczacıbaşı, Istanbul, Turkey) and 5 mg/kg xylazine (Rompun, Bayer, Istanbul, Turkey), and allowed to breath spontaneously. Body temperatures were measured by rectal thermometry and maintained at 37 °C with a heating pad. Arterial pressure and heart rate were monitored continually. Animals were placed in supine position. After sterile preparation, a midline incision was made and the abdominal aorta was exposed through a transperitoneal approach; heparin 150 U/kg was administered for anticoagulation through an intravenous route 5 minutes before clamping. The aorta was cross-clamped at two sites by using two new aneurysm clips of 70 g/0.69 N closing force (Yasargil FE 721, Aesculap, Germany) for each group under surgical microscope. The occlusion sites were just caudal to the left renal artery and above the bifurcation. Cross-clamp time was 30 minutes. At the end of the occlusion period, the clips were removed and restoration of blood flow was visually verified. The incision was closed in layers. Spinal cord segments between L2 and S1 were harvested after total laminectomies from T12 to S1 and cutting the nerve roots. Spinal cord segments between L2 and L5 were removed for immunohistochemical analysis.

Thirty-two rats were divided into four groups:

Group S: Only laparotomy was performed in the sham group ($n = 8$).

Group 0P: Rats were sacrificed after the 30-minute occlusion without perfusion ($n = 8$).

Group 3P: 3-hour perfusion was provided after 30-minute occlusion ($n = 8$).

Group 24P: 24-hour perfusion was provided after 30-minute occlusion ($n = 8$).

Immunohistochemical analysis

After the survival period, all animals were perfused intracardially under deep anesthesia with 4% paraformaldehyde in phosphate buffer solution. After the completion of the perfusion process, all animals were decapitated, and the lumbar section of the spinal cord was removed from the vertebral column, postfixed in the same fixative.

The tissues were embedded in paraffin, coronally sectioned at 5 μm thickness. These sections were placed on positively charged glass slides, heated overnight at 37 °C, deparaffinized in xylene, and rehydrated through a graded series of ethanol. For antigen retrieval treatment, slides were boiled for 20 minutes in a microwave at 100 °C in sodium citrate buffer (10 mM, pH 6). Then the slides were immersed in 3% hydrogen peroxide for 20 minutes at room temperature to block endogenous peroxidase activity. To block non-specific binding sites, the slides were treated with block serum for 10 minutes at room temperature. Thereafter, the slides were incubated with antibodies against collagen I (1:100 dilution, rabbit polyclonal, ab34710), collagen IV (1:250 dilution, rabbit polyclonal, ab6586), and TIMP-2 (1:250 dilution, mouse monoclonal $IgG_{2a,}$ 2Q758, SC-73175) at 4 °C overnight. They were labeled using Universal HRP immunostaining kit (rabbit primer antibodies, KP-50AR). Sections were incubated in biotinylated secondary antibody and streptavidin-HRP for 20 minutes at room temperature. The bound antibodies were visualized using 3-amino-4-ethylcarbazole as chromogen. Finally, the sections were mounted for quantitative analysis. Negative controls consisted of tissue sections incubated without primary antibody.

Evaluation of collagen I and IV and TIMP-2 staining

Images of the immunohistochemically stained sections were captured with a Leica DFC290 HD color digital camera mounted on a Leica DM1000 microscope using a ×20 objective and stored as tagged image file format. Images were recorded in three parts as red, green, and blue (RGB format), each with 640×480-pixel resolution and each pixel having 256 possible gray levels. Images were then analyzed with Image J software. In each image, the parameters measured by the image analysis program were the percentage of antibody-stained area in relation to the whole area.

Statistical analyses

One-way analysis of variance and Tukey honestly significant difference *post hoc* test were applied to analyze the statistical significance of our results. Values were considered to be significant at $P < 0.05$.

Results

Statistical results: Comparison between groups 0P and 24P was not significant for collagen type IV and TIMP-2 ($P > 0.05$). Comparison between groups S and 3P was not significant for collagen types I and IV and TIMP-2 ($P > 0.05$). Comparison between the other groups was significant ($P < 0.05$, $P < 0.0001$). Details of the results with the standard deviations are listed in Table 1.

Immunohistochemical results: Immunohistochemical staining was strong in groups 0P and 24P for types IV and I collagen and TIMP-2. Weak vascular staining was observed in groups 3P and S for types IV and I collagen and TIMP-2 (Figs 1–3).

Discussion

The ECM of the BBB forms a basal lamina, which surrounds and anchors endothelial cells and astrocytes, which, in turn, provide a structural barrier that selectively filters blood elements. Major components of the cerebral microvascular basal lamina include collagen IV, laminin, and fibronectin. Collagen IV is the major structural component of all basement membranes. It provides the scaffolding onto which the rest of the basement membrane is built.[20–26] In normal spinal cord, neurons and glial cells do not express type IV collagen. Type IV collagen produced by reactive astrocytes is reported to participate in glial scar formation.[17–19]

Relationship of TIMP-2 and collagen I and IV

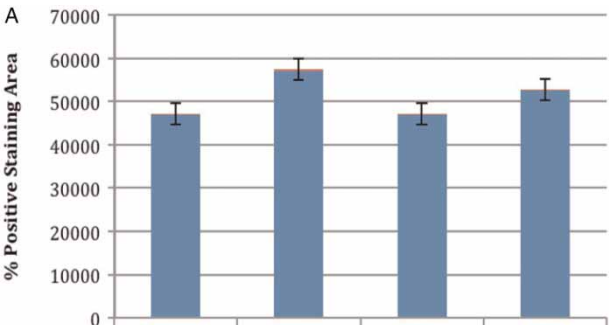
Brain vascular tissue is an important source of TIMP-2, which is highly produced in brain microvessels. TIMPs are endogenous inhibitors that regulate the activity of the MMPs. MMP-2 degrades types IV and I collagen, and thus has the potential to modify constituents of basal laminae. TIMP-2 reduces the tracer uptake. Metalloproteinase inhibitors reduce ECM proteolysis and protect the BBB.[15,27–33]

There are four TIMPs, differentially expressed by cells in various tissues. TIMP-1, TIMP-2, and TIMP-4 are secreted, whereas TIMP-3 is bound to the ECM and TIMP-4 is mainly found in vascular tissue.[32–35]

An important feature of the MMPs is their latency. Secreted in a proform, they require activation by a variety of mechanisms before they can act. Matrix prometalloproteinase-2 (ProMMP-2) is activated by the membrane-type MMP (MT-MMP).[36,37] TIMP-2 binds with MT-MMP and proMMP-2 to facilitate activation of MMP-2. MMP-2 degrades collagen IV and I. Low concentrations of TIMP-2 facilitate activation, while

Table 1 Statistical results with standard deviations of collagen types I and IV and TIMP-2 in groups S, 0P, 3P, and 24P

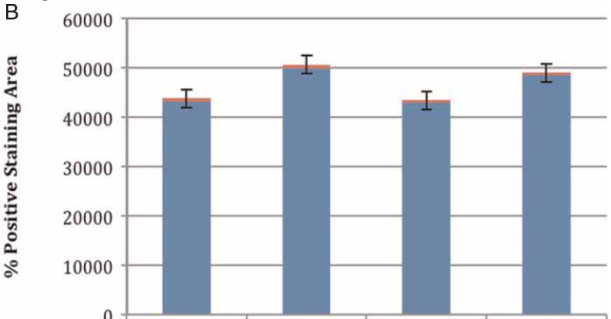
A



	S Group	0P Group	3P Group	24P Group
Std Error	365.97836	546.37314	338.78019	299.94141
Collagen I	46832.533	56925.267	46812.533	52515.733

(A) Collagen I: comparison between groups S and 3P was not significant and comparison between all other groups was significant ($P < 0.005$, $P < 0.0001$)

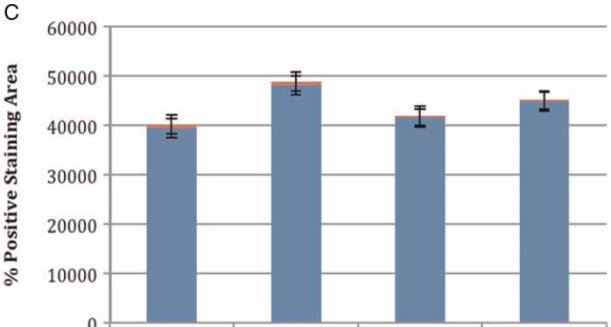
B



	S Group	0P Group	3P Group	24P Group
Std Error	592.97522	825.26078	590.16611	664.56824
Collagen IV	43166.4	49829.267	42823.333	48372

(B) Collagen IV: comparison between groups 0P and 24P, and comparison between groups S and 3P was not significant; comparison between all other groups was significant ($P < 0.005$, $P < 0.0001$)

C



	S Group	0P Group	3P Group	24P Group
Std Error	764.5631	844.59321	546.53643	312.91833
TIMP-2	39469.6	48060.467	41465.2	44802.267

(C) TIMP-2: Comparisons between groups 0P and 24P and between groups S and 3P were not significant; comparison between all other groups was significant ($P < 0.005$, $P < 0.0001$)

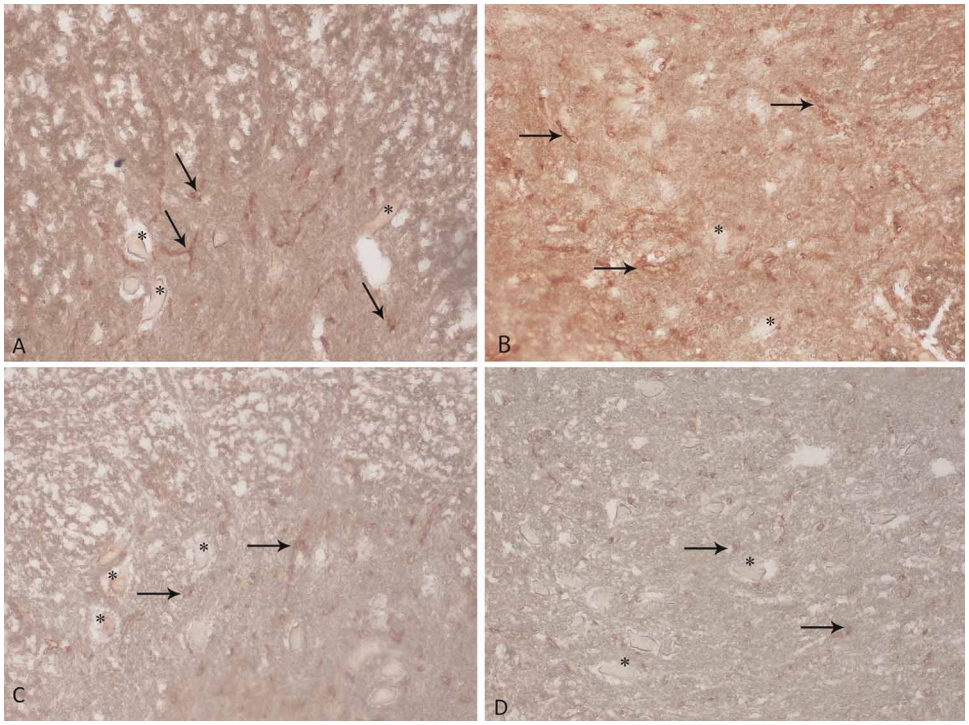


Figure 1 The anterior horn of spinal cord was positive for type IV collagen (A–D). Immunohistochemical staining for type IV collagen in 0P group (A). Strong vascular basement membrane staining of type IV collagen. Immunohistochemical staining for type IV collagen in 24P group (B). Weak vascular staining of type IV collagen in spinal cord in 3P (C) and S group (D). Asterisk (*) indicates neurons and arrows indicate type IV collagen positive stained blood vessels. Magnification ×20 in all panels.

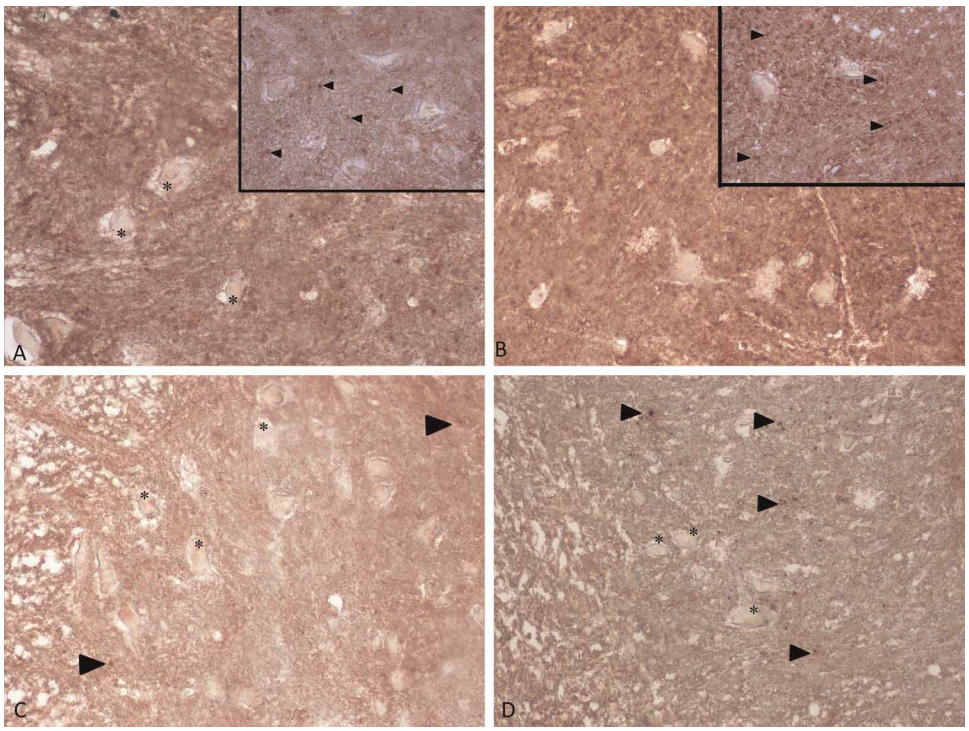


Figure 2 Immunohistochemical staining for type I collagen. Insets show a close-up of the spinal cord. Strong staining of type I collagen in 0P and 24P groups (A, B). Weak staining of type I collagen in spinal cord in 3P and S groups (C, D). Type I collagen staining is indicated by arrowheads. Asterisk indicates neurons. Magnification ×20 in all panels and ×40 in insets.

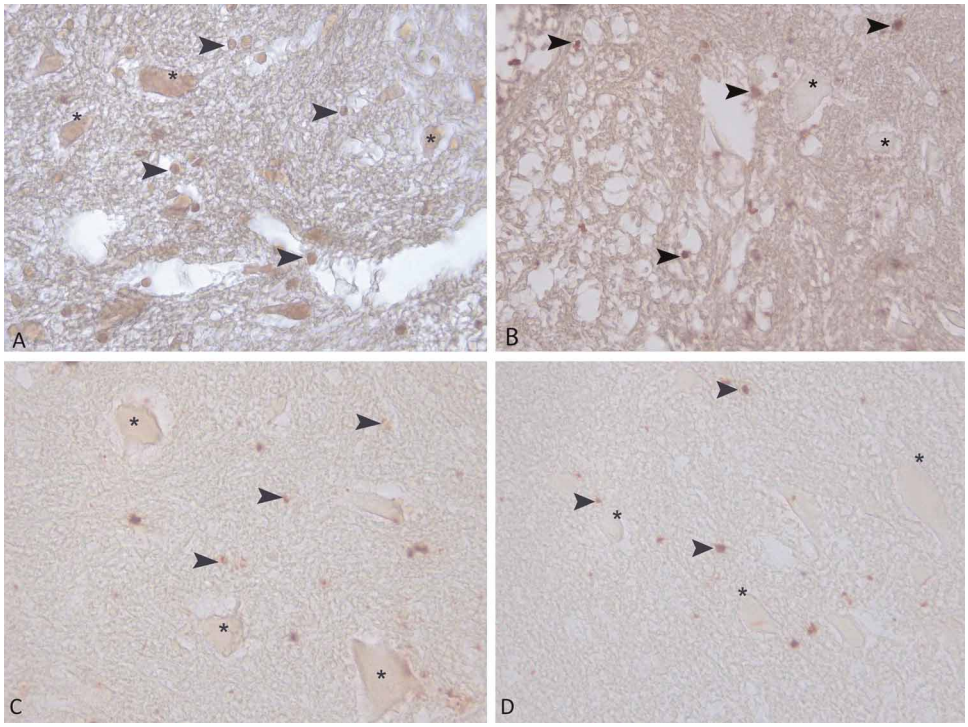


Figure 3 The anterior horn of spinal cord was positive for TIMP-2. Immunohistochemical staining for type TIMP-2 in 0P group (A). Strong positive staining of TIMP-2 (A). Immunohistochemical staining for TIMP-2 in 24P group (B). Weak vascular staining of TIMP-2 in spinal cord in 3P (C) and S groups (D). Asterisk (*) indicates neurons and arrowheads indicate TIMP-2 positive stained glia cells. Magnification ×40 in all panels.

higher concentrations inhibit the processing of proMMP-2.[36,38]

In our study, TIMP-2 and collagen staining in 3-hour perfused group were nearly the same as in sham group, suggesting that 3 hours of perfusion is not sufficient to activate the process. However, as the perfusion continues, TIMP-2 and collagen staining increased in 24-hour perfused group due to an increase of TIMP-2. Elevated TIMP-2 levels inhibited MMPs (MMP-2 and MMP-9) and thus collagen staining (I and IV) increased.

We demonstrated that high percentage of antibody-stained area in relation to the whole area and staining density of TIMP-2 provided the increased levels of collagens, probably inhibiting the activation of MMPS as mentioned in the literature.[36,38]

Relationship of TIMP-2 and collagen I and IV to glial scar and breakdown of BBB

The response of the adult mammalian CNS to injury results in a gliosis in the lesion and the formation of a glial scar. The formation of the glial scar is a complex process, involving astrocytes, oligodendrocyte precursors, and meningeal cells. One of the most remarkable characteristics of astrocytes is their powerful response to various neurologic stimuli. Such activated astrocytes are called reactive astrocytes and are thought to be directly responsible for the glial scar formation. Thus, its final form is a structure composed of crowded, process-bearing reactive astrocytes and surrounded by ECM.[39–42]

TIMP-2 is a 21 kDa molecule that binds most strongly with MMP-2, facilitating activation by MT-MMP. MMP activity is required for the inflammatory cell infiltration that occurs following SCI and most likely contributes to early barrier disruption. The early inflammatory response involves an initial wave of infiltrating neutrophils, followed by migration of monocytes and macrophages into injured segment. Each of these inflammatory cells expresses MMPs, including MMP-2 (gelatinase A), MMP-8 (neutrophil collagenase), MMP-9 (gelatinase B), MMP-11 (stromelysin-3), and MMP-12 (metalloelastase). Imbalance between TIMPs and MMPs can lead to excessive degradation of matrix components.[32] When the proteolytic activity is greater than the inhibition by the TIMPs, ECM breakdown occurs. Conversely, when the inhibitors are excessively expressed, and proteolysis is restricted, glial fibrosis occurs.[36]

Alteration of TIMP-2 during the hypoxia and reperfusion

The alternation of TIMP-2 expression in an acute stage of ischemia has not been identified *in vivo*. There are

several mechanisms defining the initial expression of TIMP-2 in ischemia. TIMP-2 may influence BBB permeability independent of MMPs. TIMP expressions in the CNS can also be upregulated in response to MMPs under various pathologic conditions.[43–45] However, the function of TIMPs in acute cerebral ischemia, especially with respect to their influence on MMP levels and BBB disruption, is less well understood. It is also defined that ECM breakdown products are additional regulators of MMPs and TIMPs.[46,47] Pagenstecher *et al.*[43] noted that some cytokines released during the ECM breakdown induce an increase of TIMP gene expression.

TIMP-2 levels may be increased due to both breakdown of BBB and release of cytokines or as a counter-regulator function of increased MMPs, consequently preventing the activation of proMMP-2 following the occlusion.

Cavdar *et al.*[48] investigated the effects of hypoxia and hypoxia/reoxygenation on MMP-2 and MMP-9, and their inhibitor (TIMP-2) and activator (MT1-MMP), in human umbilical vein endothelial cells. They demonstrated a significant increase in both MMP-2 activity and TIMP-2 protein levels by reoxygenation for 24 hours following a short-term hypoxia. They defined that the increase in MMP-2 secretion caused by reoxygenation may be stimulated by the oxidative stress that occurs following reoxygenation and the increase in TIMP-2 protein level with the duration of reoxygenation may be interpreted as a compensation mechanism related to increased proMMP-2.

In studies of both Pagenstecher *et al.*[43] and Cavdar *et al.*[48] and according to our results, there is an induction of TIMP-2 levels during the 24-hour perfusion. Prolonged perfusion pressure may have adverse effects on the vascular structures, and inter-correlated multifactorial mechanisms (oxidative stress, breakdown of BBB, and release of cytokines) lead to an increase of TIMP-2 levels due to increased MMPs to prevent the activation of proMMP-2 following the occlusion. Thus, during the 24-hour perfusion, increased TIMP-2 levels cause blockage of MMP-2 activation, and this results in elevation of the collagen IV and I levels. Further studies are needed to define the mechanism of the increase of TIMP-2 during reoxygenation.

Biphasic pattern of BBB

Rosenberg demonstrated the biphasic BBB pattern of brain injury. The first phase includes the initial injury, which is thought to be related to the activation of the constitutively expressed MMP-2 by MT-MMP in the 3–6-hour period. Rosenberg defined that the early opening of the BBB secondary to the activation of MMP-2 can be blocked by treatment with an MMP inhibitor but, in the absence of treatment, the permeability reverts to normal in several hours. After 24–48 hours, there is a second, more severe disruption of the BBB.[36]

In another study, Rosenberg demonstrated that middle cerebral artery occlusion for 90 minutes with reperfusion caused a biphasic opening of the BBB with a transient opening at 3 hours and a second more severe injury at 48 hours. The early opening at 3 hours correlated with an increase in MMP-2.[31]

In our study, we investigated TIMP-2, which binds most strongly with MMP-2, controlling the activation by MT-MMP and collagen IV and I levels that participate in glial scar formation.

We observed that critical reversible period in hypoxic/ischemic spinal cord injury is 3 hours after the perfusion provided, which is similar to Rosenberg's results of hypoxic brain studies. TIMP-2 levels activate proMMP-2; thus, the collagens will not increase or stabilize in this 3-hour period. This period seems to represent the first phase of the process. Thus, possible ECM of spinal cord was recovered during the 3 hours of reperfusion. However, after the 24-hour perfusion, MMP-2 was probably increased by reactive astrocytes during the process as mentioned in the literature.[17] This elevation induced TIMP-2 activation due to increased proMMP-2. Increased TIMP-2 provided the blockage of MMP-2 and increased collagens to the levels of the non-perfused group. This period may represent the beginning of the irreversible second phase causing the glial scar formation due to increased collagens. Further studies including measurement of MMPs by immunoassay and various groups researched for long time processes are needed to provide more clear results.

Encouraging results were provided with the use of MMP inhibitors in the studies including intracerebral hemorrhage, experimental allergic neuritis, delayed hypersensitivity, and bacterial meningitis.[27,36,49–54]

Ischemic injuries that are complicated by hypoxia induce cell pathways that lead to cell death, making ischemic injury more challenging to treat. The therapeutic goal in the ischemic lesion is to reverse the process at an early stage blocking the factors that lead to the production of MMPs.[36]

Conclusion

Biphasic opening of BBB plays an important role in ischemic spinal cord injury. Since TIMP-2 blocked the MMP-2-induced BBB opening in MMP-induced injury in the brain,[36,55] synthetic MMP-2 inhibitors

may help to recover BBB opening in spinal cord ischemic injury in the early stages.

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