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COLLAGEN FIBERS AND PROTEOGLYCANS IN THE GLIAL SCAR IN THE BRAIN AFTER HEMORRHAGIC STROKE AND UNDER CONDITIONS OF MODULATION OF COMPENSATORY-REPAIR PROCESSES

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In the case of a stroke, a glial scar forms around the affected area. According to the hypothesis, scar formation occurs not only by glial cells but also mesenchymal cells, which should be reflected in the cellular composition and extracellular matrix. The aim of this study was to investigate changes in collagen and proteoglycans content in the glial scar in the brain after hemorrhagic stroke and under conditions of modulation of compensatory-repair processes. A local hemorrhagic stroke was modeled in rats, and histochemical methods were used to study changes in collagen and proteoglycans content in the scar formation areas. An increase in collagen accumulation was observed on days 3, 10, and 30 after the stroke, with a tendency to decrease by day 60. The elimination of the hemorrhage was characterized by the accumulation of macrophages with PAS-positive cytoplasm and an increase in proteoglycans content around the hemorrhage. This indicates the involvement of mesenchymal-derived cellular elements in scar formation. In conditions of granulocyte-colony stimulating factor application, collagen accumulation was significantly lower, as it was with dexamethasone treatment, while isolated dexamethasone action led to scar formation characterized by an increase in collagen and macrophage content.

Key words: hemorrhagic stroke, glial scar, collagen, proteoglycans, dexamethasone, granulocyte colony-stimulating factor.

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КОЛАГЕНОВІ ВОЛОКНА ТА ПРОТЕОГЛІКАНИ У СКЛАДІ ГЛІАЛЬНОГО РУБЦЯ У МОЗКУ ПІСЛЯ ГЕМОРАГІЧНОГО ІНСУЛЬТУ ТА ЗА УМОВ МОДУЛЯЦІЇ КОМПЕНСАТОРНО-ВІДНОВЛЮВАЛЬНИХ ПРОЦЕСІВ

За інсульту формується гліальний рубець навколо ділянки ураження. Його формування відбувається не тільки за участі глії, але і за рахунок клітин мезенхімального походження. Це знаходить своє відображення у клітинному складі та стані позаклітинного матриксу. Метою роботи було дослідити зміни кількості колагенових волокон та протеогліканів у гліальному рубці у мозку після геморагічного інсульту та за умов модуляції компенсаторно-відновлювальних процесів. Щурам моделювали локальний геморагічний інсульт і гістохімічними методами досліджували зміни кількості колагену та протеогліканів у ділянках формування рубця. Виявлено збільшення накопичення колагенових волокон через 3, 10 і 30 діб після інсульту і тенденція до його зниження через 60 діб. Елімінація крововиливу характеризувалася накопиченням макрофагів з ШИК-позитивною цитоплазмою і збільшенням вмісту протеогліканів навколо крововиливу. Це могло свідчить про певну участь клітинних елементів мезенхімного походження в утворенні рубця. За умов введення гранулоцитарного колоніестимулюючого фактору накопичення колагену була достовірно меншим, як і у поєднані його дії з дексаметазоном, тоді як дексаметазон сприяв збільшенням вмісту колагену та макрофагів, що фагоцитували протеоглікани.

Ключові слова: геморагічний інсульт, гліальний рубець, колаген, протеоглікани, дексаметазон, гранулоцитарний колоніестимулюючий фактор.

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The glial scar formed after a hemorrhagic stroke is a known phenomenon. However, cellular reactions and their dynamics in this process have numerous unexplained issues. In most studies of the glial scar, attention is focused on the reaction of astrocytes, as the predominant cell elements in it, and, to a lesser extent, on microglia, as mediators of the inflammatory reaction. But scar formation is not limited to

reactive glial cell changes and neuroinflammation. Mesenchymal derivatives, such as blood vessels and connective tissue elements, are also involved in the process of its formation. Moreover, mesenchymal stromal cells are direct participants in cellular reactions that develop in the areas of the brain adjacent to the hemorrhage [3]. The relationship between glial and mesenchymal components can be critical in the process of glial scar formation [12].

The glial scar is a rather heterogeneous formation consisting of activated astrocytes and extracellular matrix components. The source of the extracellular matrix proteins is still debatable. Reactive astrocytes [7], pericytes and endothelial cells of newly formed vessels, and other non-resident cell populations of mesenchymal origin are considered as it [11]. The population of astrocytes, as it turns out, is also heterogeneous, and their specific number decreases distantly from the zone of brain damage. In this case, astrocytes are usually not observed in the stroke core, while proteoglycans and fibrous proteins, such as collagen, can be found in the lesion site and around the glial scar [5]. Proteoglycans at the site of injury are synthesized by almost all types of scar cells, especially astrocytes [10].

Since the density of the scar cell population changes, scar remodeling occurs, then, in our opinion, it is important to study the dynamics of the accumulation of collagen and proteoglycans in the scar. The reduction of these extracellular matrix components could potentially be seen as a manifestation of matrix degradation and possibly the formation of a favorable microenvironment for repair. No less important is the dynamics of the accumulation of collagen and proteoglycans in the glial scar under the conditions of exposure to drugs that affect the cellular composition of the scar and inflammation.

The purpose of the study was to investigate changes in the amount of collagen and proteoglycans in the glial scar in the brain following hemorrhagic stroke and under conditions of modulation of compensatory and regenerative processes.

Materials and methods. The experiments were conducted on male Wistar rats (mean body weight: 205.6 ± 7.1 g) housed in the vivarium of Bogomolets National Medical University, Ukraine. All procedures were approved by the Bioethics Committee for Human and Animal Research of Bogomolets National Medical University (Protocol No. 160, dated September 22, 2022). 200 rats were randomly divided into five experimental groups (n = 40 per group): sham-operated group (SH) – animals that underwent surgical procedures without stroke induction; hemorrhagic stroke group (HS); HS group with dexamethasone treatment following stroke induction (HS+DEX); HS group with granulocyte colony-stimulating factor treatment (HS+G-CSF); HS group of animals that received both G-CSF and dexamethasone after stroke induction (HS+G-CSF+DEX).

A focal hemorrhagic stroke was induced in the rats by injecting autologous blood into the right cerebral hemisphere. The animals were anesthetized with thiopental sodium (50 mg/kg, intraperitoneally). A volume of 0.02 mL of non-coagulated blood was injected through a fixed 1.0 mL syringe according to stereotaxic coordinates (L = 3.0–4.0; H = 4.0–6.0; AP = -1.0–3.0). The needle remained fixed in the brain, and the same volume of blood was injected again after 10 minutes. Following the second injection, the needle was withdrawn, the wound was sutured with polyamide thread (2 USP), and the surgical site was rinsed with povidone-iodine solution. In the SH group, the needle was inserted without any blood injection. G-CSF (rHuG-CSF, Sanofi) was administered as a single daily subcutaneous injection at a dose of 50 μg/kg. Dexamethasone (Lekhim, Ukraine) was administered at a dose of 10 mg/kg on days 1, 2, and 3 of the study.

Animals were euthanized on days 1, 3, 10, 30, and 60 after stroke induction by administering a lethal dose of thiopental sodium. The intracardiac perfusion was carried out with 200 mL of physiological saline and 200 mL of 4 % formaldehyde prepared in 0.9 % sodium chloride solution (4 °C). The brain was removed and post-fixed in the same fixative (pH 7.4) for 24 hours at 4 °C. Brain samples were dehydrated in isopropanol and embedded in paraffin (Leica Surgipath Paraplast Regular). Frontal brain sections (4 μm thick) were prepared using a rotary microtome. The sections were stained with hematoxylin and eosin (H&E) for general morphological assessment, Sirius Red in picric acid combined with Weigert's hematoxylin for collagen detection, and the PAS (Periodic Acid–Schiff) reaction was used for the identification of proteoglycans. Histological preparations were examined under an Olympus BX51 light microscope, and images were captured using an Olympus C3040ZOOM digital camera with Olympus DP-Soft 3.2 software (Olympus, Japan).

The specific density of collagen in glial scars was quantified using the ImageJ 1.46 image analysis system (64-bit Java 1.8.0_172; Wayne Rasband, NIH, USA) based on microphotographs captured at ×200 magnification (2272 × 1704 pixels, RGB, brightfield mode, standardized exposure settings). In areas with positive staining for Sirius Red, color segmentation was performed using the ROI to isolate color-specific regions. The RGB images were split into individual color channels and converted to 8-bit grayscale. A

threshold was set within each ROI to identify positively stained areas, which were expressed as a percentage of the total image area. The relative number of macrophages with PAS-positive cytoplasm was counted manually in microphotographs taken at ×200 magnification (2272 × 1704 pixels).

Statistical analysis was performed using StatPlus software (version 7.0; AnalystSoft Inc.). One-way analysis of variance (ANOVA) followed by Bonferroni post hoc test and the Mann–Whitney U test were used for data analysis where appropriate. Differences between groups were considered statistically significant at $p < 0.05$.

Results of the study and their discussion. The conducted observations demonstrated that reactive changes developed around the site of cerebral hemorrhage, leading to the formation of a glial scar that confined the area of tissue destruction. In the SH group only a moderate increase in cell number was observed along the needle track. In the HS group, as the hematoma was gradually resorbed, the surrounding brain tissue exhibited an increase in cell density, the appearance of newly formed blood vessels and fibrous elements. Sirius Red staining enabled the visualization of collagen fibers and the assessment of the dynamics of their deposition (Fig. 1). Mature collagen was detected around pre-existing blood vessels in the perihematomal area as early as days 1 and 3 after stroke induction. Between days 3 and 30, thin Sirius Red-positive fibrous structures were detected within in the developing glial scar, typically located adjacent to newly formed blood vessels. The amount of these fibers progressively increased, peaking by day 30, and then slightly decreased (Fig. 2).

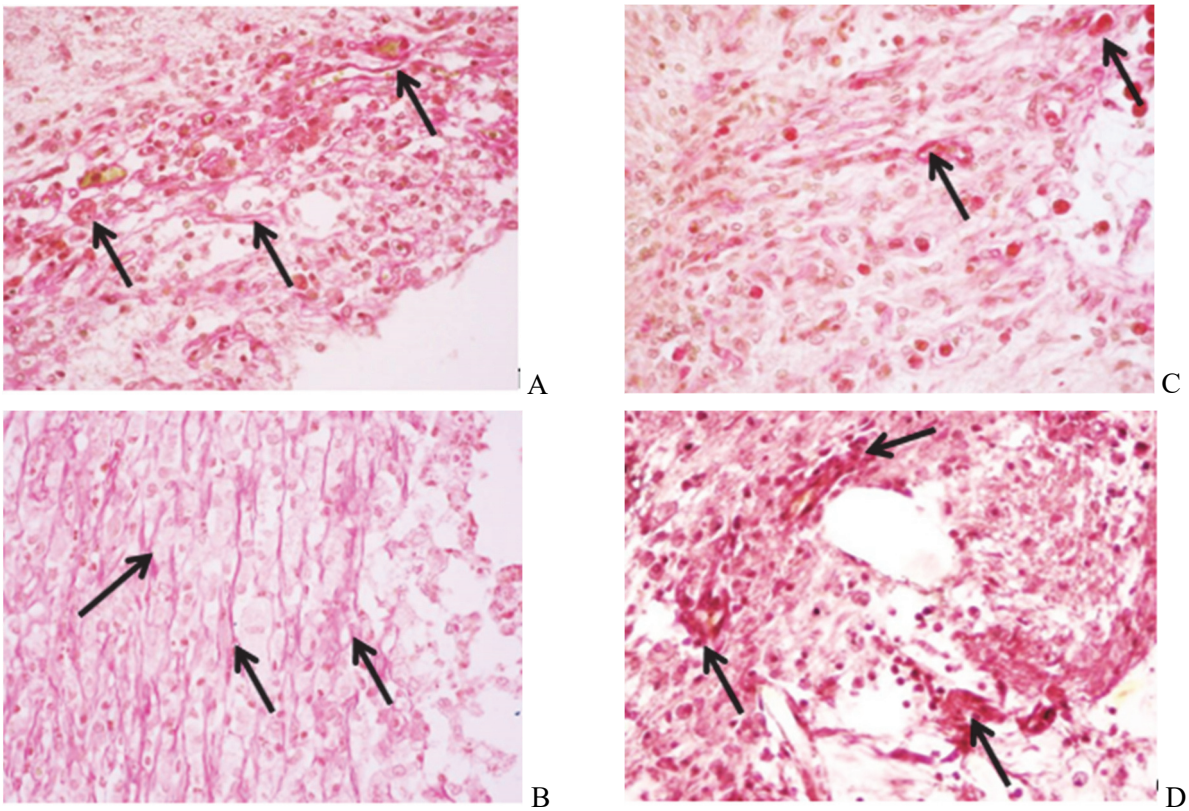

Fig. 1. Histochemical detection of collagen in the forming scar 30 days after hemorrhagic stroke induction: a – HS; b – HS+DEX; c – HS+G-CSF; d – HS+G-CSF+DEX. Areas of positive collagen staining are indicated by arrows. Sirius Red staining with Weigert's hematoxylin counterstain. Magnification ×400.

In the HS+DEX group, the specific density of collagen in brain regions adjacent to the hematoma was significantly lower than in the HS group on day 3 after stroke induction ($p < 0.05$). However, this parameter subsequently increased, surpassing the HS group by day 30, with a further upward trend observed by day 60 ($p = 0.06$). In the HS+G-CSF group, significantly fewer Sirius Red-positive fibrous structures were detected in the perilesional area on day 3 compared to the HS group ($p < 0.05$). This difference persisted throughout the observation period, but by day 60 it was no longer statistically significant. In the HS+G-CSF+DEX group, collagen density remained significantly lower than in the HS group from day 3 ($p < 0.05$). Starting from day 10, the amount of Sirius Red-positive fibrous structures showed a slight increase, reaching a plateau that persisted until the end of the experiment. A significant difference was observed between the two DEX-treated groups (HS+DEX vs. HS+G-CSF+DEX) on days 30 and 60 ($p < 0.05$), with lower collagen content in the HS+G-CSF+DEX group. No statistically significant differences were found between the two G-CSF-treated groups.

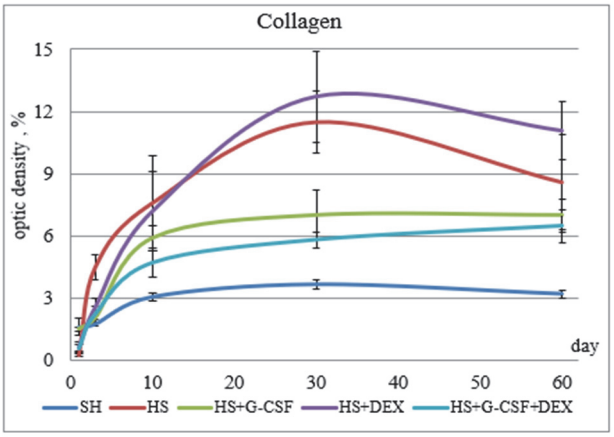

Fig. 2. Quantitative dynamics of collagen accumulation in the glial scar area at different time points following hemorrhagic stroke induction.

The analysis revealed a slight increase in PAS positivity of the extracellular matrix within the developing glial scar surrounding the hemorrhage. One day after stroke induction, PAS-positive homogeneous material was detected around the hematoma, distributed along its periphery. By day 3, the PAS-positive matrix became localized in areas of increased cell density near the hematoma. On day 10, the homogeneous material was largely absent; instead, fibrous matrix elements within the scar exhibited a more intense PAS-positive reaction. By days 30 and 60, the morphological pattern remained similar, with the scar matrix containing a higher amount of proteoglycans compared to the surrounding neuropil, particularly around newly formed blood vessels. At the same time, during hematoma resorption, the appearance of macrophages phagocytosing cellular debris was observed in adjacent brain regions. Among these phagocytic cells, macrophages with PAS-positive cytoplasmic inclusions were identified (Fig. 3). On day 3, such cells were mainly localized within the hematoma. From day 10, they were also observed in the perihematomal areas. A peak in the number of PAS-positive macrophages was recorded on day 10, followed by a decrease by day 30, which mirrored the reduction in other phagocytic cells with PAS-negative cytoplasm.

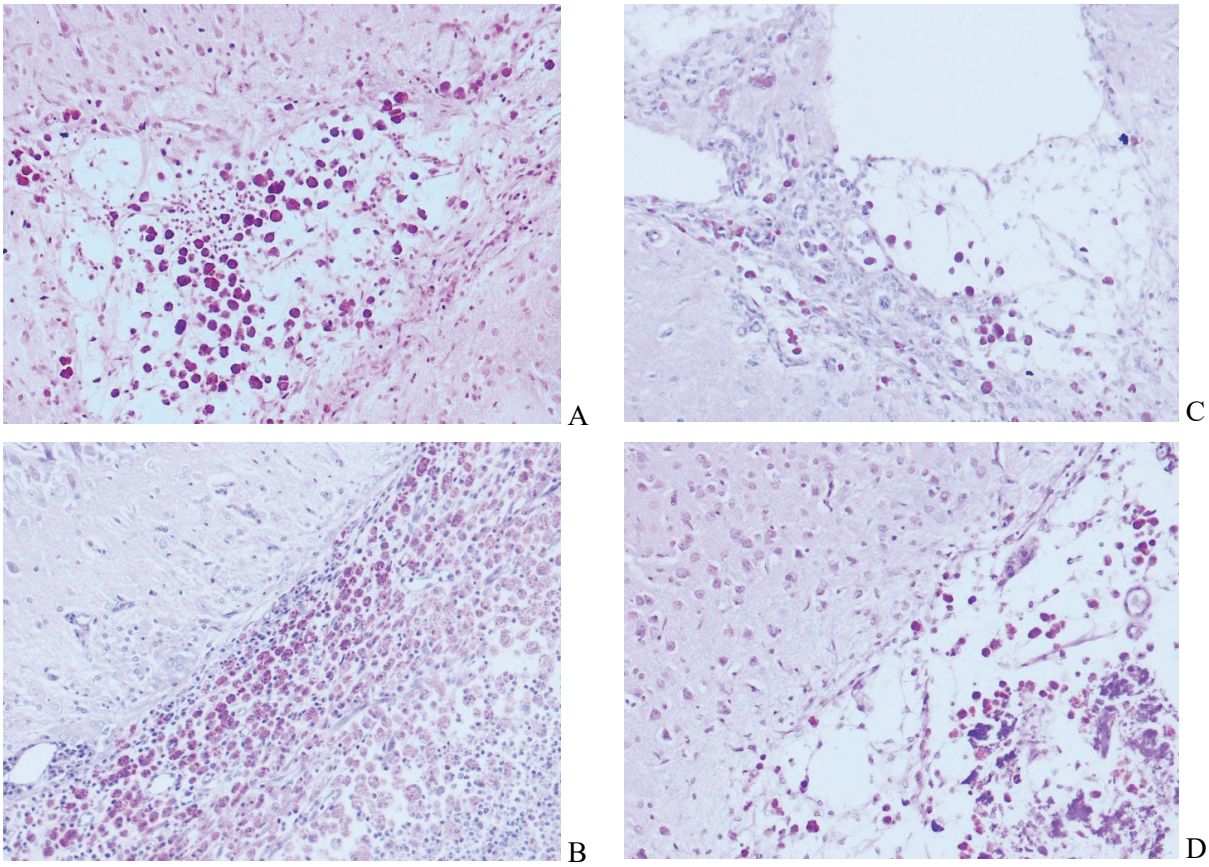

Fig. 3. Macrophages with PAS-positive cytoplasm in the forming glial scar 30 days after hemorrhagic stroke induction: a – HS; b – HS+DEX; c – HS+G-CSF; d – HS+G-CSF+DEX. PAS staining, ×200 magnification.

The dynamics of macrophage accumulation with PAS-positive cytoplasm in the perihematomal area generally mirrored the pattern of collagen deposition (Fig. 4). On day 3 following hemorrhagic stroke induction, no significant differences were observed among the stroke-model groups. However, by day 10, the specific number of PAS-positive macrophages differed markedly across groups. In the HS group, day 10 represented the peak of activated macrophage accumulation, followed by a statistically significant decrease in their density within the forming glial scar ($p < 0.05$). In contrast, in the HS+DEX group, the specific number of macrophages on day 10 did not differ from the HS group. Their number continued to

rise by day 30, with only a slight decline by day 60, indicating prolonged macrophage persistence likely associated with delayed hematoma resorption.

In the HS+G-CSF group, the specific number of detected macrophages was significantly lower than in the HS group on day 10 ($p < 0.05$) and continued to show a downward trend at later time points. In the HS+G-CSF+DEX group, the dynamics of macrophage presence were similar to those in the HS+G-CSF group, though slightly higher overall. However, the differences between these two groups were not statistically significant.

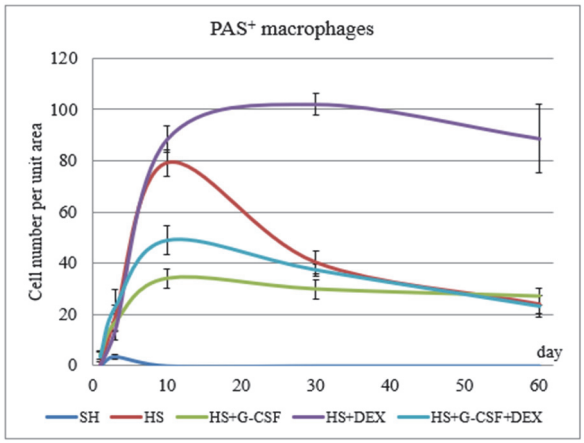

Fig. 4. Dynamics of accumulation of macrophages with PAS-positive cytoplasm in the areas of glial scar formation.

The study results indicate that the glial scar following stroke is not exclusively glial in nature; elements of non-glial (mesenchymal) origin also participate in its formation. It is well established that astrocyte activation occurs in the rat brain hemorrhagic stroke model, with astrocytes representing the dominant cellular population within the scar. However, the scar also contains microglia and cells that are hypothesized to be of mesenchymal origin. There is a functional interdependence between reactive astrocytes, microglia, and phagocytes of monocytic origin. For example, a reduction in microglial presence is associated with a decrease in the number of GFAP-positive astrocytes [14]. Both phagocytic and astrocytic populations within the injured brain areas are heterogeneous. While the functional role of phagocytes is more evident – mainly related to the elimination of cellular debris – the exact role of astrocyte proliferation and their functional differentiation into neurotoxic A1 and neuroprotective A2 astrocyte subtypes remains incompletely understood.

The dynamics of glial scar formation and its density are heterogeneous. At the border with the brain injury site, a relatively dense network of astrocytes forms, resembling a compact barrier whose density significantly decreases with distance. Interestingly, astrocytes during scar formation are found predominantly in the perihematomal region, while they are absent within the core of the lesion [6]. In contrast, other non-glial cells are present both within the scar and in the lesion foci. The immunophenotypes of these cells require further detailed investigation; however, it is already known that they express CD44, CD68, and CD146 markers [3].

Reactive gliosis involves not only the accumulation of astrocytes but also the deposition of extracellular matrix (ECM) proteins. Accumulation of proteoglycans has been described in cortical peri-infarct regions of the human brain after ischemic stroke, as well as in rodent models of brain injury [13]. Proteoglycans have been considered components of the glial scar; however, their specific cellular source remains unclear. It is likely that all scar-forming cells, including astrocytes, secrete these ECM components [5]. In our study, we only noted the presence of macrophages phagocytosing proteoglycans. In connective tissues, perivascular fibroblasts, pericytes, and endothelial cells synthesize protein complexes and macromolecules and serve as key mediators of fibrotic scar formation in injured tissues [1]. The role of collagen in glial scar formation remains controversial. Some studies suggest that type I collagen is not involved in the scar [14], whereas others report collagen deposition following spinal cord injury [4]. A consensus among researchers is that the scar inhibits or even prevents axonal regeneration and reparative migration of progenitor cells [15]. Therefore, further investigation into the regulation of scar formation and degradation is crucial.

The potential benefit of such research lies in uncovering both direct and indirect mechanisms by which pharmacological agents can modulate glial scar formation. It is now widely accepted that cells with a pro-inflammatory phenotype, such as neutrophils, macrophages, and microglia, produce matrix metalloproteinases (MMPs), which contribute to disorganization of the glial scar and likely reduce its density [8]. Scar degradation has been associated with MMP-9 overexpression within the scar tissue itself [2]. Moreover, MMP-9 facilitates the migration of progenitor cells from the subventricular zone into the injured brain area [9]. Thus, modulation of inflammation and accumulation of progenitor cells may represent a viable approach for regulating scar formation.

In our previous studies, we described the dynamics of astrocyte accumulation around the hemorrhagic lesion and demonstrated that dexamethasone enhances astrogliosis while delaying hematoma elimination [3]. In the present work, we observed that scar regions contain a certain amount of collagen

fibers. Their amount increases under the influence of dexamethasone, whereas granulocyte colony-stimulating factor – either alone or in combination with dexamethasone – prevents excessive collagen deposition. Although we did not investigate the molecular mechanisms underlying the effects of these drugs, our findings clearly indicate that pharmacological treatment alters the dynamics of scar formation.

Conclusions

1. The glial scar that forms in the brain following hemorrhagic stroke comprises not only reactive astrocytes but also fibrous structures including collagenous and proteoglycans. This indicates the active involvement of cells related to mesenchymal stem cells in scar formation.

2. Activation of the mesenchymal stem cell pool by granulocyte colony-stimulating factor leads to a reduction in both collagen fiber content and the number of macrophages with PAS-positive inclusions in the glial scar, whereas dexamethasone treatment induces predominantly opposite effects.

3. The observed increase in collagen content in the glial scar by day 30 after stroke modeling reflects the prolonged activity of cellular responses around the lesion, including reactive astrocytes, macrophages, and potentially diverse immunophenotypic populations of mesenchymal stem cells. The cessation of collagen accumulation by this time point under G-CSF treatment may suggest that this growth factor promotes earlier scar maturation.

Prospects for further research. The study of glial scar formation expands our understanding of tissue responses in the injured brain, including the roles of reactive astrocytes and mesenchymal stem cells. These findings lay the groundwork for developing strategies to optimize regenerative processes in the brain following injury.

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