Microscopic aspects of macrophage system cells in hemorrhagic stroke in humans

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Abstract
Stroke is an important public health issue because it has high morbidity and mortality rates. In addition, it has one of the highest rates of disability in adults. Recent data show that macrophage system cells, especially microglial cells, are involved both in neuroprotective processes and in the neurotoxicity, depending on the type and extent of the brain damage. In our study, using histology and immunohistochemistry techniques, we evaluated the macrophage-type cell reaction in cerebral hemorrhage. We found that the number of CD68-positive cells increased 7–8 folds per square millimeter of cortical surface in the cerebral parenchyma adjacent to the hemorrhage. We identified a large number of perivascular-activated macrophages, in areas distant to the hemorrhage, showing that individuals with hemorrhagic stroke have profound and extensive alterations of the blood–brain barrier.

Keywords: blood–brain barrier, cerebral hemorrhage, phagocytosis, microglia, CD68-positive cells.

Introduction
Stroke is a severe acute neurological disorder, produced by brain lesions due to ischemic or hemorrhagic vascular events. Worldwide, stroke is one of the leading causes of morbidity and mortality, since each year it “kills” a few million people and causes severe disabilities in another millions.

Depending on the pathophysiological mechanism, there are two different types of stroke:
- Ischemic strokes, also called cerebral infarction or softening of the brain, which occur most often because of a thrombus, embolus or reduction of a lumen of an artery affected by atherosclerosis or other factors;
- Hemorrhagic stroke is due to the leakage of blood into brain tissue. Their cause is the generally high blood pressure or, more rarely, vascular malformations (angioma, aneurysm), coagulopathies or complications of anticoagulant treatment.

Statistics show that 80–85% of ischemic strokes are ischemic and only 15–20% of them are hemorrhagic.

Hemorrhagic stroke or intracerebral hemorrhage, accounts for around 15–20% of all strokes and affects more than two million people worldwide each year [1, 2].

Regarding the age of people with hemorrhagic stroke, statistical studies show that this condition can occur at any age, but that about 75% of cases occur after 65 years of age [3, 4]. Advanced age, linked to other biological deficiencies, make post-stroke recovery of neurological deficiencies to be more difficult in patients with intracerebral hemorrhage [5].

Most of the cases with hemorrhagic stroke are caused by primary arteriolsclerotic hypertension and amyloid angiopathy [6], but vascular malformations, hemorrhaging brain tumors, coagulopathies and the use of thrombolysis in ischemic strokes, are increasingly frequent etiologic factors of intracranial hemorrhage.

Macrophage system cells (i.e., microglia) play an important role in the immune response of the central nervous system. They actively interact with both neurons and the rest of the glial cells. Recent data show that they can exert neuroprotective actions by producing neurotrophic molecules, participating in nervous tissue remodeling after various injuries, but can also cause secondary brain damage that increase neurological deficits by producing proinflammatory factors.

In our study, we aimed to highlight the macrophage system cell reaction in patients who died after hemorrhagic stroke.

Materials and Methods
The biological material in our study was represented by encephalon fragments collected during the necropsy from a total of 24 patients, aged between 68 and 83 years, who died after a hemorrhagic stroke in the Clinical Neurology Hospital of Craiova, between 2006 and 2010. In order to compare the microscopic changes encephalon fragments were collected from the immediate vicinity of the hemorrhagic foci and the contralateral hemisphere, from the same anatomical area. After sampling, the biological material was immediately placed in 4% formaldehyde fixing solution buffered with sodium phosphate for a pH of 7.2–7.4 and processed by the histological technique of paraffin inclusion. Four-µm thick sections were cut using a
microtome (Microm HM350) equipped with a special section transfer system (Section Transfer System, STS, Microm), and collected on special slides covered with a layer of positively charged amino acid residues (poly-L-Lysine) (Sigma), in order to increase adhesion of the sections. After a quick drying period of 5–10 minutes sections were transferred to an incubator at 37°C and kept overnight, during which the biological material adhered perfectly to the surface of the histological slide.

For the histology studies we used two techniques (Hematoxylin-Eosin stain and trichrome Goldner–Szekely stain), and for immunohistochemistry studies we used the anti-CD68 antibody to highlight the macrophage system cells (Table 1).

Table 1 – Antibodies used for the immunohistochemical study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Isotype</th>
<th>Clone</th>
<th>Host/Target/Clonality</th>
<th>Dilution</th>
<th>Antigen retrieval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CD68</td>
<td>AbD Serotec</td>
<td>IgG1</td>
<td>ED1</td>
<td>Ms/Rat/Monoclonal</td>
<td>1:200</td>
<td>Sodium citrate, pH 6</td>
</tr>
</tbody>
</table>

For single immunohistochemistry, after antigen retrieval, sections were allowed to cool down to room temperature and were incubated for 30 minutes in 1% hydrogen peroxide solution. The sections were next washed in PBS, followed by a blocking step of 30 minutes in 1% skim milk. Next, the slides were incubated with the primary antibodies overnight at 4°C, and the next day, the signal was amplified for 30 minutes using a peroxidase polymer-based secondary detection system (EnVision, Dako). Finally the signal was detected with 3,3’-diaminobenzidine (DAB) (Dako) and the slides were coverslipped in DPX (Fluka) after Hematoxylin staining.

The sections were imaged with a Nikon Eclipse 55i microscope (Nikon, Apidrag, Romania) equipped with a 5-megapixel cooled CCD camera. Images were captured and archived using a Nikon frame grabber and the Image ProPlus 7 AMS software (Media Cybernetics Inc, Buckinghamshire, UK).

Results

In hemorrhagic stroke, extravasation of plasma proteins and cellular elements from the blood vessels into the nervous tissue represents the triggering factor for the mobilization and activation of the macrophage system cells. In other words, intracerebral hemorrhage can cause primary brain lesions through the formation of the hematoma and a resulting increase in intracranial pressure, as well as secondary lesions through the local inflammatory reaction. Inflammation is characterized by activation and accumulation of inflammatory cells and secretion of specific mediators in the hemorrhagic brain.

Microscopic aspects of nervous tissue in stroke cases examined by us varied depending on the severity and time period elapsed from the onset, but also on the state of cerebral vessels. In relatively recent hemorrhages, in which the death occurred within five days after the onset of the brain hemorrhage, at the periphery of the hematoma, there were numerous red blood cells diffusely infiltrating the cerebral parenchyma, associated with rare round mononuclear cells showing a diffuse pattern around them (Figure 1). Histological examination at high magnification allowed us to notice that these mononuclear cells had an average size of around 18–25 μm, large nuclei with heterogeneous chromatin and abundant cytoplasm, similar to circulating monocytes. Therefore, we considered that these cells belong to the macrophage system.

Unfortunately, specific identification of these cells could only be performed by immunohistochemistry techniques. The use of anti-CD68 antibody allowed the specific labeling of macrophage-type cells and quantification of their response in cerebral hemorrhages, since CD68 is a transmembrane glycoprotein with a molecular weight of 110 kD present in the macrophage system cells, especially in lysosomes and endosomes.

For a proper evaluation of macrophage-type cell reaction in the area around the hemorrhage, we sought to identify and quantify macrophages in the contralateral hemisphere, which is not directly affected by the cerebral hemorrhage. As can be seen (Figure 2), in the central nervous system macrophages are relatively rare and small, with long and thin branches. They are represented by microglial cells in physiological condition and appear to be more numerous around the blood vessels. In our study, we identified a number of 72–85 microglia/mm² of cortical surface.

In contrast, in recent hemorrhages (within 5–10 days after stroke), the number of macrophage-type cells at the periphery of the hemorrhage was relatively high (Figure 3). The quantification of macrophage system cells in recent hemorrhages allowed us to identify an average of 460–580 macrophages/mm² of cerebral substance. Regarding the origin of macrophages around the hemorrhage core, we believe that some of them result from local microglial activation and conversion into macrophages, but a large number of macrophages come from the blood flow, by activation of circulating monocytes.

A similar high density of CD68-positive cells was also identified in the white matter, sometimes at quite large distances from the hemorrhage core (Figure 4). While macrophages in the gray matter showed a homogeneous cytoplasm, those in the white matter had a heterogenous stippled cytoplasm. Uneven immunolabeling is because macrophages in the white matter internalized increased amounts of myelin that accumulated within the phagolysosomes, because myelin is much harder to digest by lysosomal or endosomal enzymes of macrophages.

In older hemorrhage cores, also macrophage-type cell counts remained high, even where collagen fibrillogenesis occurred (Figure 5), which shows that cerebral macrophages can be seen for a long time, even months after a hemorrhagic stroke.

In our study, we also found numerous perivascular reactive macrophages, even at large distances from the hemorrhage core in both recent and older hemorrhages (Figures 6 and 7). This suggests that the alteration of the blood–brain barrier occurs long before the onset of the hemorrhagic stroke due to hemodynamic stress to which...
Meningo-cerebral vessels are exposed in hypertension or other diseases. It is also possible that small discontinuities occur in the cerebral microcirculation, particularly in the arterioles, capillaries and venules, even after an acute stroke that stimulates the development of perivascular macrophage-type cells. In our study, we frequently noted, using conventional techniques, the presence of small red blood cell suffusions in the Virchow–Robin space (Figure 8), which makes us believe that the development of perivascular macrophage-type cells is due to blood–brain barrier disruptions arising from various causes.
Discussion

For an optimal function, neurons, glial cells and blood–brain barrier (BBB) require a very precise balance of several variable factors such as temperature, local pH, the amount of glucose and oxygen [7].

Stroke, irrespective of its clinical form or etiopathogenesis, results in a drastic reduction or total interruption of cerebral blood flow in an anatomical area, more or less extensive, depriving brain cells of glucose and oxygen supply needed to function normally. In these circumstances cellular necrosis develops, which triggers a prompt response from the immune system. Thus, within and around the cerebral necrosis core there are many granulocytes, lymphocytes and macrophages that ensure local immune protection, but also remove cellular and tissue debris.

The reaction of the macrophage system cells is very prompt. Thus, Ekdahl CT et al. (2009) reported an increased number of activated microglial cells, from two to 16 weeks after middle cerebral artery occlusion in rats [8].

In non-pathological conditions, microglial cells have a small body, with numerous long and thin branches, and are evenly distributed throughout the brain. They are considered the primary effector immune cells present in the brain, often referred to as cerebral macrophages. It seems that they play a critical role as immunocompetent phagocytic cells in the central nervous system.

In normal brain, microglial cells are account for approximately 5–20% of the total glial population and, although they are not derived from the neural tube or neural crest, they are perfectly integrated into the cerebral parenchyma. Thus, several studies have noted that microglial extensions and protrusions dynamically interact with surrounding neurons, astrocytes and blood vessels [9]. In response to various types of brain injury, microglial cells become activated (or reactive) and undergo morphological and functional changes. More specifically, the cell body becomes larger with thickened or shortened processes and produce pro-inflammatory proteins. Subsequently they become migratory cells moving through the cerebral parenchyma and acquire phagocytic properties. Microglial cells are considered the first non-neuronal cells that react to various brain injuries, acting as guardians for neuronal survival in various pathological conditions [10].

In our study, we observed a significant increase in macrophage system cells in the perilesional cerebral parenchyma after hemorrhagic stroke. Thus, their number increased 7–8 folds per square millimeter of cortical surface compared to areas of normal cerebral parenchyma. Unfortunately, we cannot say by histology and immunohistochemistry techniques which of these cells are microglia and which are blood-derived monocytes. We believe that the appearance of perilesional CD68-positive cells is due mostly to activation of blood monocytes and their transformation into macrophages, and to a lesser extent to microglial migration. Also, evidence of cellular or myelin debris in the cytoplasm of these cells makes us believe that their main role is the phagocytosis of cellular debris and altered cells (extravasated erythrocytes) from and around the hemorrhagic area.

Some authors [11, 12] indicate that under pathological conditions, microglial cells, in addition to their phagocytic activity, synthesize and release cytokines, chemokines and cytotoxin. Of these, the most frequently mentioned ones are the pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6. Also, microglial cells can exert neuroprotective effects by producing neurotrophic molecules such as the brain-derived neurotrophic factor (BDNF), insulin-like growth factor I (IGF-I) or other growth factors still unknown.

There is a considerable body of evidence suggesting that in addition to the beneficial effects of the pro-inflammatory activity, microglial cells may have detrimental effects on the brain. Thus, activated microglias have the potential to release some potentially cytotoxic molecules, including nitric oxide (NO), oxygen free radicals and prostanoids [13]. It is believed that the activated microglia has both neuroprotective and neurotoxic properties, depending on the severity of pathological conditions and brain injuries.

The persistence of macrophage-type cells in old
hemorrhagic cores observed by us may be explained by the fact that overall, the blood–brain barrier is compromised and that certain molecules and RBCs can penetrate into the cerebral parenchyma. Other authors [14] consider that the inflammatory cells are involved in the remodeling of the nervous tissue long after the various injuries.

The presence of perivascular CD68-positive cells, distant from the hemorrhagic core in both recent hemorrhages and old lesions, can be explained by the impairment of the blood–brain barrier by other factors, mainly metalloproteinases. It is a known fact that matrix-metalloproteinases (MMPs) are a family of proteolytic enzymes responsible for extracellular matrix remodeling, which can degrade all its constituents. MMPs expression in adult brain is very low to undetectable, but many MMPs are overexpressed in the brain in response to injury [15]. Neurons, astrocytes, microglial cells and endothelial cells synthesize and express MMPs after an injury. Numerous experimental studies have shown that blood–brain barrier is compromised by the activation of MMPs [16], leading to vasogenic edema and local microhemorrhages.

The present study allowed us to see that all individuals who died from hemorrhagic stroke showed significant changes in intraparenchymal vessels that may explain neuronal injury. The fact is that in the elderly, the vascular system undergoes atherosclerotic changes, cerebral amyloid angiopathy and small vessel disease, which can lead to cerebral hemorrhage or indirectly cause damage to both gray and white matter.

Conclusions

The number of macrophage-type cells increased 7–8 folds in the cerebral parenchyma around the hemorrhage core both in recent and old injuries. Numerous perivascular-activated macrophages were identified as evidence of blood–brain barrier disruption. We believe that the main role of these cells is the phagocytosis of cellular and fibrillar debris resulting from stroke.

Acknowledgments

The study is part of the explorative research project IDEI/2009, code ID_2188, financed by CNCSIS.

References


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Received: October 3rd, 2011

Accepted: December 16th, 2011