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Clinico-pathological correlations in fatal ischemic stroke. An immunohistochemical study of human brain penumbra

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Abstract

Ischemic stroke is one of the most frequent pathologies with high invalidating potential and a leading cause of death. The brain tissue adjacent to the central necrotic core, defined as penumbra, was extensively characterized mostly by imaging techniques and in animal models. Our goal was to identify a large panel of molecules in this particular area on human brains harvested at autopsy. Twenty-one patients with ischemic stroke and seven control cases were taken into study. We used immunohistochemistry to characterize necrotic lesions. Metalloproteinases, mostly MMP-9, seem to be involved in brain ischemia, but as a protective and not as a deleterious factor. Apoptotic molecules are not increasingly expressed in stroke compared to control cases. Mast cell enzymes chymase and tryptase are described for the first time in neurons and glia, even with unclear significance. Microglia appears active in stroke and stimulating methods directed to it could be useful. Nitric oxide synthases and cyclooxygenase-2 were also involved in stroke cases but not in control ones. Other factors as VEGF and its receptors, PDGF, b-FGF or TNF-alpha showed no significant expression related to ischemic brain injury. Animal study of penumbra and human tissue findings are distinct and research should be focused on the latter approach in order to find valuable and safe therapeutic methods.

Keywords: stroke, penumbra, apoptosis, immunohistochemistry, vascular convolutes, inflammation.

Introduction

Ischemic stroke represent 87% of cerebrovascular disease [1] and third cause of death after diseases of the heart and cancer. Penumbra can be defined as the viable tissue surrounding the irreversibly damaged ischemic core following cerebral ischemia. When studying the process in order to imagine valuable methods for neuro-protection and neurorepair, it must be taken into account which vessel is occluded, the time of evolution of the ischemia, the degree of the ischemia, and the sensitivity to ischemia of the different cells. Most of the techniques used in the treatment of brain infarct use approaches external to the brain itself, i.e. recanalization of the obstructed vessel by the methods of thrombolytic therapy [2]. On the other hand, local characteristics of the nervous tissue injured by ischemia also must be taken into account in order to imagine supplementary therapies directed to local cells – mainly neurons and/or small vessels components. For targeting penumbra in stroke patients, imaging techniques are mandatory for monitoring treatment response as well as for patient screening. The "mismatch" of perfusion-weighted and diffusion-weighted images (PWI–DWI mismatch) is the most commonly used method for imaging penumbra, as well as SPECT and PET techniques [3]. If the central mechanism, which determinates the fate of cell in the penumbra, is its energy state [4], several approaches being designed to improve it [5], other molecular characteristics of the local injured tissue are under discussion. Even though the cellular and molecular changes characteristic for this area were thoroughly studied in animal models in the last years, the current therapies based on these searches proved to be unsuccessful [6–9]. One of the central mechanisms underlying the functional and structural evolution of this region defined as penumbra could be apoptosis. This one appears to be the dominant way of cell death in this area adjacent to the necrotic core of a brain infarct [10, 11]. Detecting the molecular factors involved in apoptotic phenomena occurring after an ischemic injury could permit therapeutic intervention using various anti-apoptotic strategies [11]. Apoptosis is known to be dependent on a family of intracellular cysteine proteases, called caspases (Cysteine Aspartate Specific ProteASEs) [12]. Of these, caspases 2 and 9 are considered as proapoptotic and initiator of apoptosis.

Apoptosis can be induced either from the cell surface, by ligand-dependent triggering of death receptors as CD95/Fas or by the stimulation of intracellular receptor proteins, such as apoptotic protease-activating factor 1 (Apaf-1), which is activated by its ligand cytochrome c once it is released from damaged mitochondria [13]. Except caspases, bcl-2 family members appear to be crucial in regulating the mitochondrial pathway of apoptosis [14] initiated during the ischemia and reperfusion phenomena in brain [15]. Anti- and pro-apoptotic bcl-2 members are thought to play a role in the pathogenesis of several disorders. The expression of Bax was found to be upregulated following ischemia-induced retinal injury in rat [16]. Angiogenesis could also play a role in the penumbra, mostly in a positive way, facilitating the recovery of the ischemic tissue from necrosis and making potential therapeutic intervention feasible [17]. This process involves a series of pro-angiogenic molecules as VEGF and its receptors VEGFR-1 (FLT-1), VEGFR-2 (FLK-1) and VEGFR-3 [18, 19]. Furthermore, PDGF and its receptor PDGFR could be upregulated following cerebral ischemia in experimental studies or in human stroke [20, 21]. Since inflammation is considered as a crucial factor in ischemic brain injury [22], we also tried to asses the presence of various molecules involved in this process, as nitric oxide synthase or cyclooxygenase-2, and also of cellular inflammation represented by polymorphonuclear cells (CD15+), B-lymphocytes (CD20/L26+), T-lymphocytes (UCHL-1+), mast cells (chymase and tryptase+), and macrophages/microglia (CD68+). Mostly mast cells are incriminated to mediate brain injury in ischemia and protective therapies against their degranulation are proposed [23]. In this study, we used immunohistochemical analysis to determine the extent, degree of intensity, and which specific cells expressed the following molecules: bcl-2, Bax, caspase 2, caspase 9, APAF-1, Fas, chymase, tryptase, CD68, VEGF, FLT-1, FLK-1, VEGFR3, PDGFR, MMP-2, MMP-9, smooth muscle actin, u-NOS, COX-2, b-FGF, TNF-alpha, CD15, CD20/L26, UCHL-1, in 21 cases of patients deceased with ischemic stroke and seven control cases, on autopsy harvested material.

Materials and Methods

Autopsy material

We studied the brains of 21 patients deceased after suffering an ischemic stroke (11 males, 10 females, aged between 18 and 86 years; mean = 73.9 years; SD = 14.36). We took into account only the strokes caused by large vessel thrombosis and cardioembolic mechanism, not including the lacunar type or those with undetermined cause. Patients with large infarcts, with destruction of parenchyma and no detectable viable adjacent tissue, presumably to be considered as penumbra, were also excluded from the study. As control we used seven patients (three males, four females, 46–86-year-old, mean 65.14 years, SD = 17.07), deceased with either hemorrhagic stroke or other than brain vascular conditions (ischemic heart disease, viral encephalitis, septic shock, myocardial infarct, renal failure, bronchopneumonia). The interval between death and autopsy varied from 6 hours to 280 hours, but was cvasisimilar for the two series, with a mean of 35.4 hours in the stroke series and 40.7 hours in the control series. The time from admission to death was 11 to 413 hours (mean 190 hours) for the stroke patients and 16 to 334 hours (mean 67 hours) for the control series.

All patients have been admitted to the National Institute of Neurology and Neurovascular Diseases in Bucharest, in a two-year period. Patient clinical information was retrieved retrospectively at the end of the study period in a blinded fashion. The study was approved by the Ethical Committee of the Institute and informed consent was obtained from the relatives in each case.

Neuropathology

Samples were obtained from a region located at the margins of the necrotic area and comprising also a rim of the necrotic core. Corresponding area from the contralateral hemisphere were also sampled. In control cases, two standard regions were harvested: a frontal sample from Brodmann's area 8 (an association area) and the hippocampus. These latter cases had no imagistic and did not exhibit macroscopic signs of brain necrosis, either at examination or palpation of tissue.

The whole brains were first fixed in buffered-saline formalin for at least two weeks prior to sectioning. The section of brains was performed in clinical-pathological staff with the neurology clinic members in which clinical-pathological diagnostic correspondence was evaluated. Sectioning was performed in coronal plane, beginning with the mammillary bodies and continuing toward the frontal and occipital poles, using a guiding metal frame, at 1 cm width. The presence of hemorrhagic transformation of infarct was noted.

The samples comprised, usually, a fragment of both grey and white matter and were routinely embedded in paraffin and stained with Hematoxylin & Eosin. The neuropathological examination was performed blinded to the clinical data or the localization of the sample. The neuronal morphology (normal, shrunken), degree of edema, aspect of large, medium and small-size vessels in both parenchyma and leptomeninges) were studied.

Immunohistochemistry

The paraffin blocks were sent to the National Institute of Pathology for immunohistochemical assessment. Five-micrometer sections from the blocks were cut. Immunohistochemistry was performed on the paraffin-embedded material using the EnVision+ Dual Link System Peroxidase kit (Dako, Carpinteria, CA, USA), according to manufacturer's instructions. The slides were stained with the following antibodies: Bax (1:50), chymase (1:1000), tryptase (1:1000), FLT-1 (1:50), FLK-1 (1:50), u-NOS (1:50) , MMP-2 (1:50), MMP-9 (1:50) (Thermo Fisher Scientific Inc., Fremont, CA, USA), bcl-2 (1:50), CD68 (1:50), smooth muscle actin (1:50), CD31 (1:50), CD34 (1:50), FVIII (factor VIII associated-antigen) (1:50), COX-2 (1:100), CD15

(1:50), UCHL-1 (1:50) (DakoCytomation, Glostrup, Denmark,), caspase 2 (1:20), caspase 9 (1:20), Fas (1:50), L26/CD20 (1:50), VEGFR-3 (1:50) (Novocastra, Newcastle upon Tyne, UK), APAF-1 (1:100), VEGF (1:50), PDGFR (1:200), b-FGF (1:50), TNF-alpha (1:50) (Santa Cruz Biotechnology, CA, USA). Antigen retrieval was performed for each antibody as indicated by the manufacturer. Finally, the slides were counter-stained with Mayer's hemalum.

We assessed the cell types potentially expressing each antibody: neurons, astrocytes, oligodendrocytes, vascular wall cells (i.e. endothelium, smooth muscle cells, pericytes, and fibroblasts). The positivist of cells was assessed semi quantitatively using a modified Quick score, which take into account the intensity and distribution of positivity [24]. This classification scores 0 – negative, 1 – only visible at high magnification, 2 – readily visible at low magnification, and 3 – strongly visible art low magnification. Therefore, we classified the intensity of staining in our cases as weak (1), medium (2) or strong (3), the cases lacking any positive cell or structure being considered as negative (0).

Statistical analysis

Statistical analyses were performed using the Excel software for Windows. The logistic regression was used to assess the association between variables. $P<0.05$ was considered as significant.

Results

Our series, of 21 patients, was composed of 11 cases with infarcts in the territory of middle cerebral artery, three cases in the anterior cerebral artery, one case with internal carotid origin, one with mixed localization (middle and posterior cerebral arteries) and five cases within the basilary territory. The necrotic tissue extension was variable in the samples but always comprised a large zone of peri-ischemic tissue. All cases presented various degrees of edema. This was distinct from a case to another, being present adjacent to the infarct zone but also in the contralateral hemisphere, or in only one of these areas. Furthermore, the degree of edema was very different even in the same area, being present in a zone and totally absent adjacent to it, regardless the distance from the ischemic core. Neurons showed signs of ischemic injury but no gliosis or inflammatory infiltrates were visible. Microscopic hemorrhagic foci of the infarcted parenchyma were seen in 10 of the stroke cases and absent in the control ones. No special changes in the small vessels were detected, as microatheroma, thrombosis, fibrinoid necrosis. Fibrosis and hyaline changes were present in only rare, scattered microvascular profiles, not related to the presence or absence of ischemic changes or to the hemorrhagic transformation of the infarct in stroke cases.

Fas was positive in four stroke cases (19%) and negative in the control series. The staining was weak (1) in the endothelial lining of small intraparenchymal vessels (Figure 1), but also in neurons (Figure 2). Fas was statistically found to increase in parallel with the presence of hemorrhagic transformation of the infarct in the penumbra (p=0.015), with the accumulation of VEGF in small intraparenchymal vessels (p=0.04), and to decrease in a significant manner with longer times from death to the moment of the autopsy (p=0.02).

Bcl-2 was positive in two cases from the stroke series (9.52%) and negative in all control cases. The mean intensity of staining in the positive cases was 1.5. Expression of bcl-2 was found in the brain microvessels (Figure 3) and negative in the rest of cerebral parenchyma. Unlike Fas, however, bcl-2 was more widely distributed within the vascular walls, being both expressed in the endothelial layer and also in intermediate cells (smooth muscle cells or pericytes). The large leptomeningeal arteries also expressed bcl-2 mostly within the media, in the smooth muscle cells. Bax was positive in five cases of the stroke series (23.8%) and negative in all control patients. The mean intensity of staining was 1.6. It was mostly expressed by small intraparenchymal vessels and only very rarely by neurons or astrocytes (Figure 4). Caspase 2 showed positivity in 18 of 21 cases with stroke (85.71%) and also in five of seven control cases (71.42%). Its expression was strong; the mean intensity grade of staining was 2.16 in the stroke series. In the control cases, the value was 1.25 for the frontal lobe samples, and 1.66 for the hippocampal fragments. In stroke cases it was positive mostly in the cells of the necrotic core, in almost all cells in the penumbra (neurons and astrocytes) (Figure 5), absent in the endothelial and other vascular cells, but the same cell localization and intensity were also present in the contralateral non-ischemic hemisphere. Caspase 2 was found at significantly higher levels in cases with arterial hypertension in the stroke series (p=0.003) and in those also featuring ischemic heart disease (p=0.006).

Caspase 9 was found positive in five of 21 cases with stroke (23.8%) and in three of seven control cases (42.85%). The intensity of staining was weak, with a mean of 1.2 in stroke cases and one in both frontal and hippocampal samples in the control cases. Caspase 9 was expressed mostly by neurons and less by astrocytes and not by vascular structures (Figure 6).

APAF-1 was positive in five of the stroke cases (23.8%) and in one control case (14.28%), regardless of the associated pathology and with no statistically significant correlations with the other studied molecules. APAF-1 was also expressed in neurons and astrocytes (Figure 7). The overall positivity for apoptotic molecules had a maximum of intensity for caspase 2 in the stroke series, followed by the same molecule in the hippocampal region of control cases. Neurons and glial cells seemed equally positive, but not endothelial cells. A lesser degree of positivity was found for Bax and bcl-2 in the stroke patients, followed by caspase 2 in the frontal region of control cases and caspase 9 in the stroke series. Lowest values were seen for caspase 9 and APAF-1 in both stroke and control cases.

Except for the separate control cases, in the series of patients with stroke we also studied in parallel the zone adjacent to the infarct (penumbra) and the contralateral, symmetric one and/or a "neutral" region, imagistic and macroscopically non-affected by ischemia, usually the hippocampus. When considering the presence of our target molecules from this point of view, several patterns

were seen. The most frequent was a similarity in the expression of molecules between the two regions (penumbra and control area). Other cases showed positivity for either caspase 2 or 9 in the penumbra and its absence in the contralateral area, but also the opposite situation (i.e. presence of one of two caspases in the control area and its absence in the penumbra). The same was also found for bcl-2, which was intensely positive in one case in the control area and negative in the infarcted tissue.

An interesting situation was obvious by comparing the infarct localization. In the middle cerebral artery strokes, not a single patient exhibited positivity for caspase 9. On the other hand, all patients with infarcts within the territory of the anterior cerebral artery (even though only three cases) showed positivity for caspase 9, although at low degrees (grade 1 in all cases). Nine of 11 patients (81.81%) had positive caspase 2, all patients (100%) with ACA infarcts were positive for the same antibody, approximately the same situation as that seen in cases with brain stem infarcts, where four of five patients (80%) were positive.

The inflammatory cells were practically absent from both the stroke penumbra and contralateral hemisphere and from the control cases. CD15, L26/CD20, UCHL1, chymase and tryptase did not disclose inflammatory cells in all the specimens, with the exception of one or two isolated cells in the blood vessels or the blood extravasate in cases with hemorrhagic transformation of infarcts. However, a special situation was seen regarding the mast cell markers chymase and tryptase, which proved to be positive in almost all neurons and a high number of glial cells (Figure 8). They were strongly expressed in stroke cases (mean = 2) and only at lower levels in control cases (mean = 1), even though all patients (stroke and control series) exhibited positivity for both markers at least in a region (penumbra or contralateral control area in stroke cases and one of the two studied regions in control series). In control patients the frontal lobe area consistently lacked positivity for chymase or tryptase, whereas the hippocampus in the same cases showed a strong positivity. In stroke cases, the number of cells and the degree of positivity were similar in both penumbra and control areas of the same case and paralleled the age in a statistically significant manner (p=0.015). We also found a parallel increase of both enzymes with the accumulation of neuronal VEGF (p=0.02).

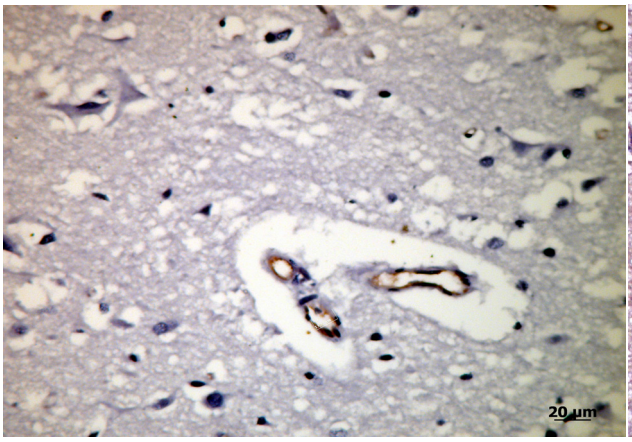

Figure 1 – ***Fas is conspicuously expressed in the endothelium of a 13.31 µm diameter arteriole within the parenchyma, in a zone adjacent to the infarcted area (Immunohistochemistry, ob. 40×).***

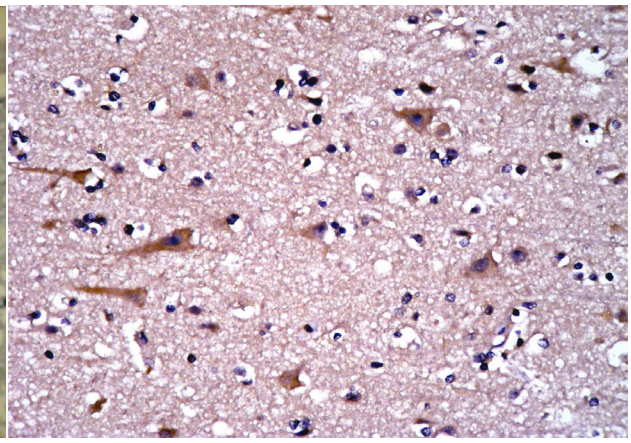

Figure 2 – ***Fas positivity in numerous neurons in the penumbra of a hemorrhagic middle cerebral infarct (Immunohistochemistry, ob. 40×).***

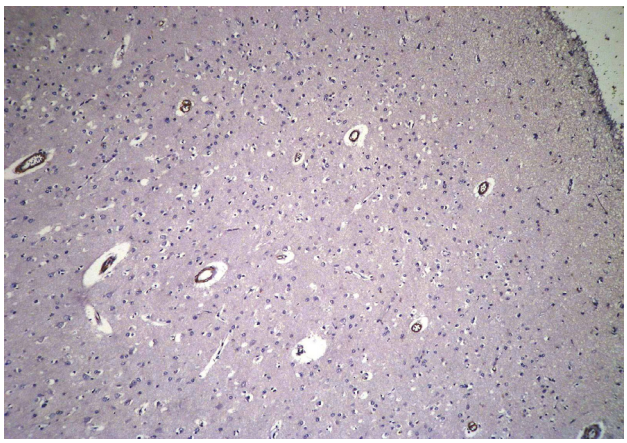

Figure 3 – ***Bcl-2 appears strongly expressed (grade 3) in all medium-sized cortical vessels. The neurons and astrocytes are completely negative (Immunohistochemistry, ob. 10×).***

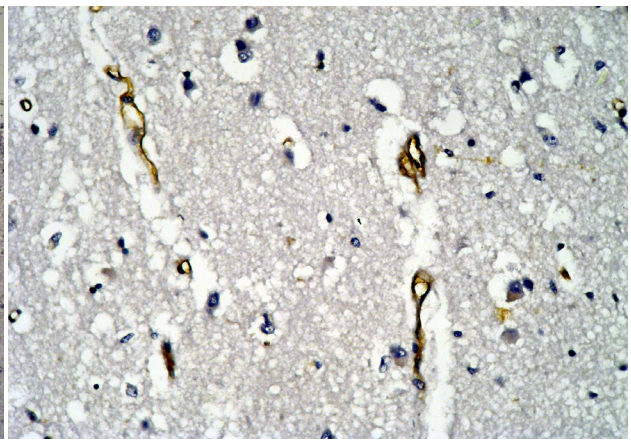

Figure 4 – ***Some small intraparenchymal vessels around 10 µm in diameter show a diffuse positivity of the wall for Bax. A strong degree of edema gives the "clear" aspect for spaces around cells and vessels (Immunohistochemistry, ob. 40×).***

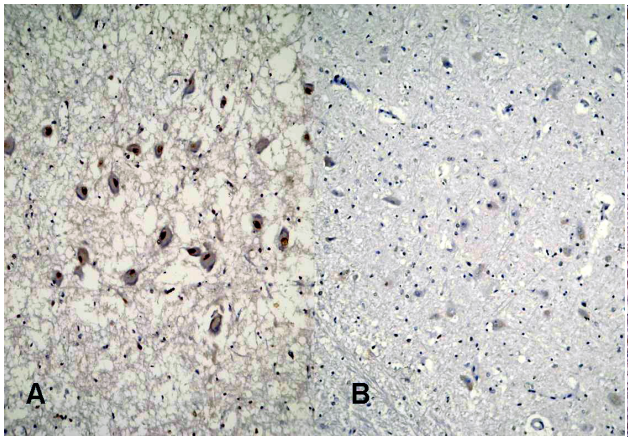

Figure 5 – ***A stroke case with mesencephalic infarct. Caspase 2 is strongly (grade 3) expressed in the nuclei of neurons in the core of the infarct (panel A). In the adjacent zone (penumbra), the marker is almost negative (panel B) (Immunohistochemistry, ob. 20×).***

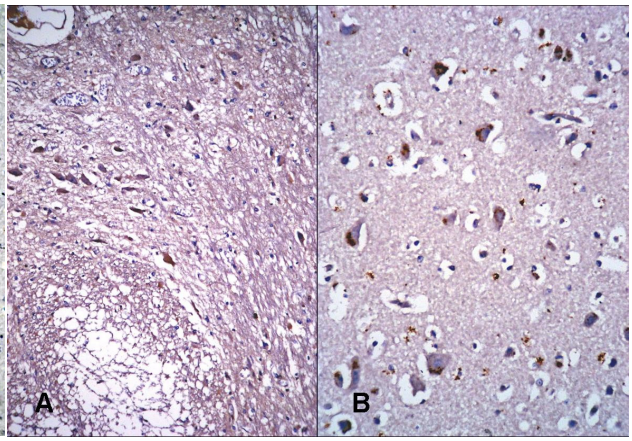

Figure 6 – ***Caspase 9 is expressed in both stroke and control cases. In panel A, neurons are strongly positive for caspase 9 adjacent to an infarct area (Immunohistochemistry, ob. 20×). In a control case (panel B) neurons are also the cells expressing caspase 9, in a conspicuously cytoplasmic, mitochondrial manner (Immunohistochemistry, ob. 40×).***

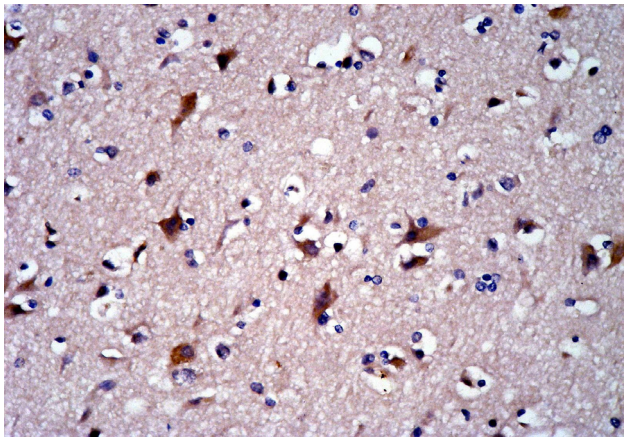

Figure 7 – ***APAF-1 expression is intense in the neurons and rare astrocytes of the penumbra zone (Immunohistochemistry, ob. 40×).***

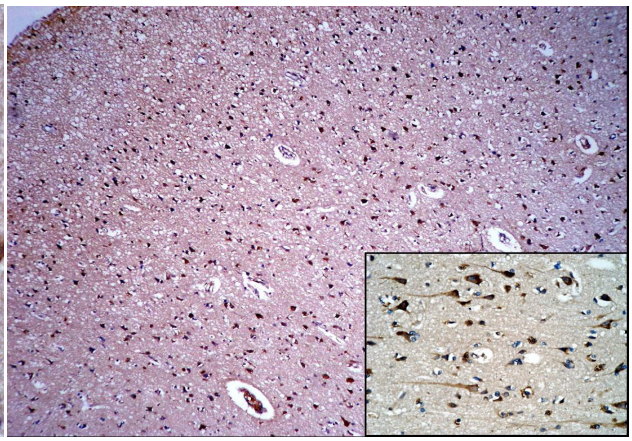

Figure 8 – ***Chymase expression is especially strong (grade 3) in all the cortical area from a penumbra. Inset: neurons as well as astrocytes are strongly positive (Immunohistochemistry, ob. 10×; inset: ob. 40×).***

CD68-positive activated microglial cells (Figure 9) were present in both series, in all cases; however, they were positive at a much higher intensity and in greater number in the stroke cases than in the control cases (mean 2.6 *vs.* only 0.8). This finding did not take into account the macrophage present in large number in the necrotic areas or immediately adjacent to these in long standing stroke cases, which were present in great number. In stroke patients, the degree of positivity was also constantly higher in the penumbra than in the contralateral symmetric or remote, unaffected areas. The pro-angiogenic molecules showed low levels of significance in stroke. VEGF was expressed mostly in neurons (Figure 10), in the majority of cases (n=17; 80.98%) but also in all control cases (100%) in both regions we studied. It was also positive in the wall of small intraparenchymal vessels (not shown). Neuronal VEGF paralleled an increase with chymase (as reported above) and microvascular VEGF with Fas (as mentioned above), but also with the interval between patient admission to death (p=0.02). VEGF receptors (FLT-1, FLK-1, and VEGFR3) were negative in both series of patients. PDGF was positive in six cases of the stroke series (28.57%), but in a diffuse, interstitial manner, without certain cell localization. Its positivity however increased with that of MMP-2 (p=0.02) and was also much lower in the patients with myocardial infarct (p=0.04).

Endothelial markers CD31, CD34, and FVIII labeled small intraparenchymal vessels and the endothelial lining of larger arachnoidal arteries and veins. Smooth muscle actin was positive in the small vessel walls in the parenchyma and leptomeningeal larger arteries. However, it revealed the presence of vascular convolutes, described by [37] in seven stroke cases (33.33%) and in only two control cases (28.57%) (Figure 11). In stroke cases they were conspicuous in all areas of brain, either adjacent to the ischemic core or the contralateral unaffected hemisphere. In the control cases, they were present in only one of the two tested areas (hippocampus or frontal lobe). Vascular convolutes showed a significant inverse relationship with the hemorrhagic

transformation of infarct (p=0.04), a tendency toward higher number in the presence of metalloproteinase MMP-2 (p=0.055) and also a tendency to increase (even though not statistically significant) with the presence of HTA (p=0.06). MMP-2 was expressed in small vessels within the parenchyma in only four of the stroke series (19%), but also in four control patients (57.14%) (Figure 12). MMP-9 appeared also in four cases of the stroke series (19%) but in six cases of the control patients (85.71%) (Figure 13). Except the already mentioned relationship of metalloproteinase 2 with the vascular convolutes, MMP-9 presence was correlated with NOS at microvascular level (p=0.02). nNOS was present in 13 of the stroke cases (61.9%) and was totally negative in the control series (Figure 14).

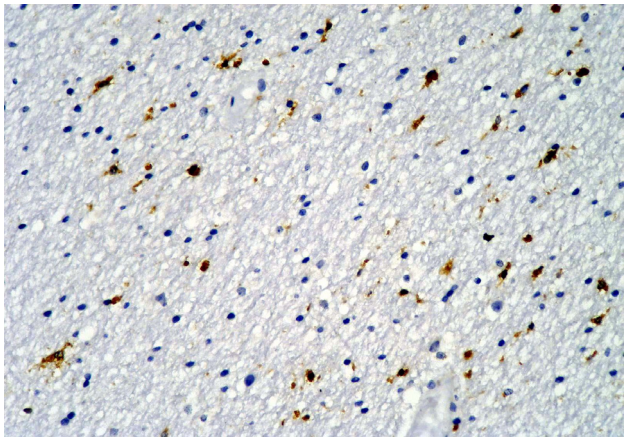

Figure 9 – ***CD68 is expressed in frequent microglial cells in the white matter adjacent to a middle cerebral artery infarct (Immunohistochemistry, ob. 40×).***

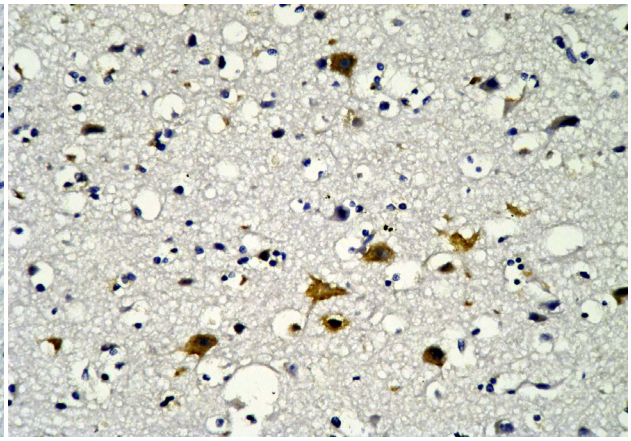

Figure 10 – ***VEGF appear positive in neurons in the penumbra of a stroke case (Immunohistochemistry, ob. 40×).***

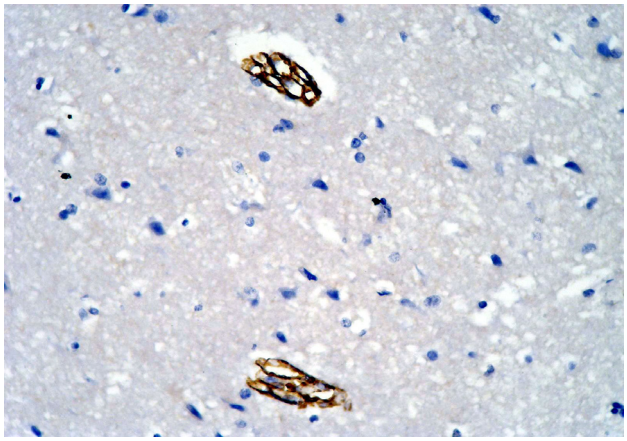

Figure 11 – ***Vascular convolutes are conspicuous in a stroke case, remote from the infarct area (Immunohistochemistry for smooth muscle actin, ob. 40×).***

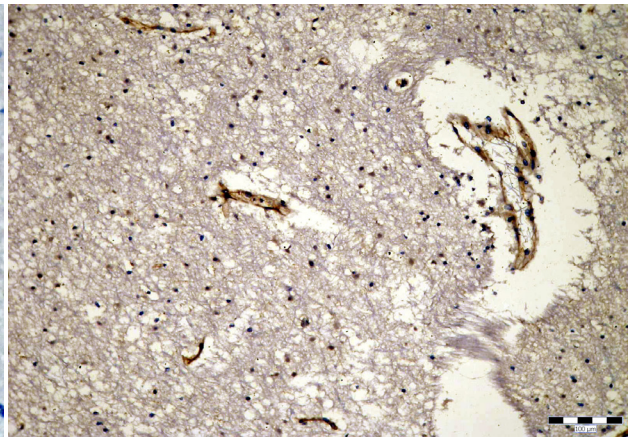

Figure 12 – ***The expression of MMP-2 is restricted to small intraparenchymal vessels. Note the intense degree of edema surrounding the vessels (Immunohistochemistry, ob. 20×).***

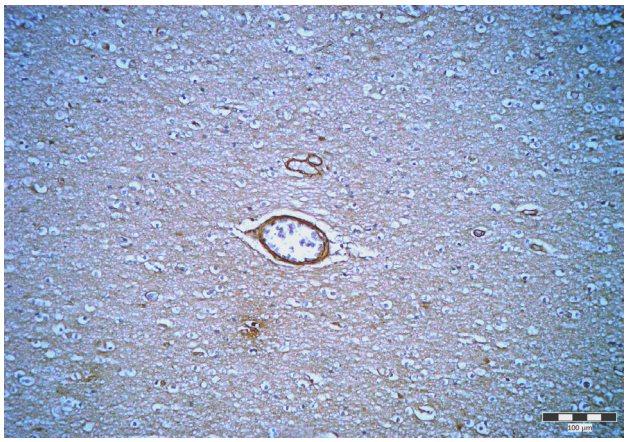

Figure 13 – ***MMP-9 is expressed in small vessels in an area remote from the necrotic core (Immunohistochemistry, ob. 20×).***

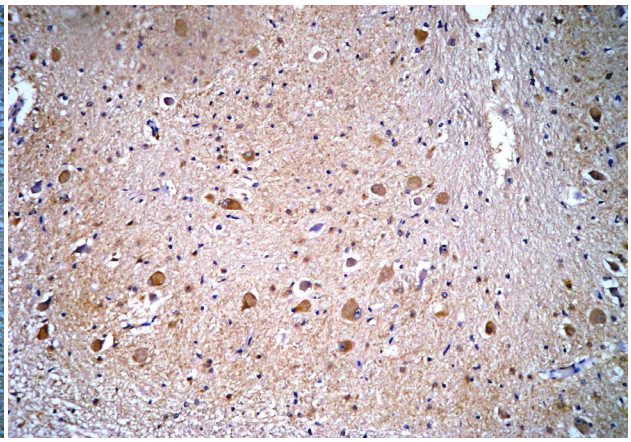

Figure 14 – ***nNOS appears intensely expressed in neurons in penumbra in a brainstem infarct (Immunohistochemistry, ob. 20×).***

Regarding the comparison between the penumbra and the contralateral symmetric area, it was found either to be expressed in both areas or only in the non-ischemic territory, sparing the peri-infarct tissue and small intraparenchymal vessels of the penumbra. eNOS was expressed in only four of the stroke cases (19%) and was also negative in the control series (Figure 15). The degree of expression for nNOS was strongly related to the time from death to autopsy (p=0.02) and had a much lower degree of expression in patients with atrial fibrillation, when com-pared with those lacking this condition (p=0.0007). COX-2 was expressed by neurons (Figure 16) in nine of 21 control cases (42.85%) and showed a similar expression between penumbra and the contralateral control area. In the control series, it was negative. No statistic-ally significant correlations were seen with other molecules or pathological associated disease, and no vascular or glial expression was obvious. Its distribution within the same patient was identical in the ischemic penumbra and contralateral control area. In our study, bFGF was expressed in all cases, in both the stroke and control series, in vessels and neurons (Figure 17). Its mean expression degree was 1.6 in both series. TNF-alpha was negative in all cases, in both series.

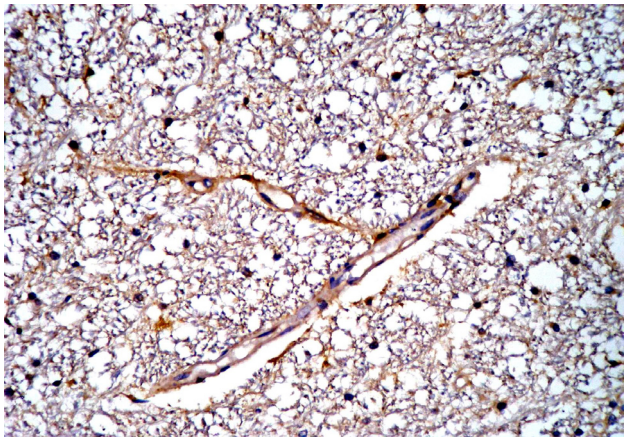

Figure 15 – ***eNOS appears positive in small intraparenchymal vessels in the penumbra (Immunohistochemistry, ob. 40×).***

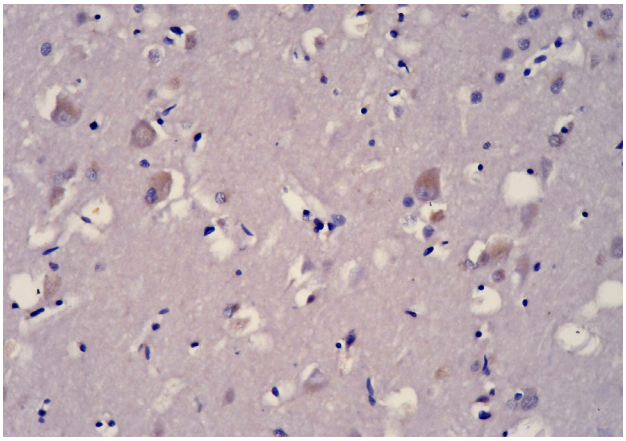

Figure 16 – ***COX-2 is expressed in a medium grade in neurons in the penumbra of a stroke case (Immunohistochemistry, ob. 40×).***

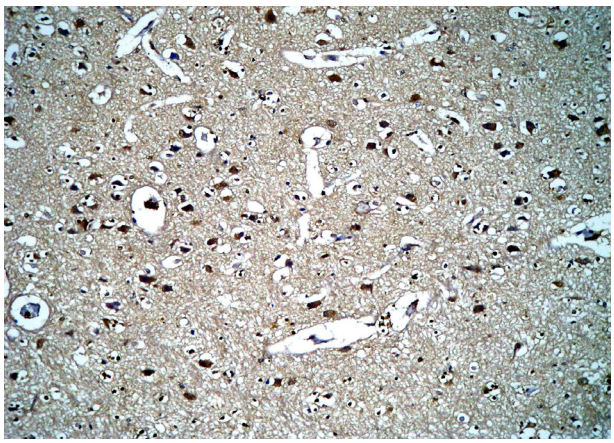

Figure 17 – ***bFGF appears intensely expressed by all neurons in the penumbra (Immunohistochemistry, ob. 20×).***

Discussion

In ischemic stroke, apoptosis appear as a phenomenon related to endoplasmic reticulum stress and presumably acts as a protective element against necrosis [25]. As stated by other authors, multiple pathways for apoptosis are functioning in the ischemic penumbra, eliciting multiple therapeutic agents for neuroprotection and neurorecovery [26]. In our series, however, comprising only human subjects, the molecules under study did not show statistically significant differences between peri-infarct area, contralateral non-ischemic tissue or control subjects.

This could be mostly due to a massive degree of edema in both zones, which could obviously trigger the apoptotic cascade, regardless the distance from the necrotic core. This could also explain the difference between human stroke and animal models, where no study on the contralateral hemisphere has focused on the degree of edema and related to apoptotic cell death. In animal studies, bcl-2 and Bax were found to be increasingly expressed in the penumbra [27], fact which was not present in our cases. The presence of caspase 2 in both stroke and control series at higher levels and with no significant differences could be related first to its better detection, since its expression is nuclear and not cytoplasmic, unlike the other ones.

Inflammation, even though stated as having a certain role in cerebral ischemia [28]. Cellular response in our cases was practically negative, no lymphocytes, polymorphonuclear cells or mast cells accumulated adjacent to the infracted area or remote to it. COX-2 was present in a significant number of cases, however without being related to the location, duration or other factors associated to ischemia. However, since no control cases exhibited COX-2 positivity, its relationship with ischemic phenomena could be stated. Even though mast cells are found to accumulate and degranulate in stroke in animal models [29], we did not find a similar situation in human stroke cases. On the other hand, ***we describe for the first time the presence of chymase and tryptase***, two enzymes considered to be almost specific to mast cells, ***in the neurons and glial cells*** of our patients.

Even though their presence could not be related to a specific ischemic condition or to any other systemic disease present in our patients, the significant increase in the grade and the higher extent of positivity at older age could suggest their putative role in the apparition of pathologic changes at brain level and further studies are needed to elucidate this occurrence.

VEGF is normally expressed by the brain in astrocytes and neurons [30]. Ischemia is reported to induce the VEGF expression in neurons [31, 32] but also in microvessels within the penumbra [33, 34]. All these studies were however performed in animal models. A study in the human tissue also found an upregulation of VEGF levels in the penumbra, with neurons, endothelial cells and astrocytes as cells expressing the marker [35]. The increase in VEGF expression in the infarcted hemisphere was described to appear at three hours after the ischemic insult and to progress for several days [36]. In our series, VEGF did not appear to be overexpressed in the ischemic cases. Moreover, its neuronal expression was much intense in the control series (2.5 *vs.* 1.8 intensity degrees), while its microvascular expression was equal between stroke and control cases.

The demonstration of vascular convolutes, reported by us previously [37] showed no major differences between stroke and control series. The fact that smooth mucle actin was much more strongly expressed in these unusual structures than endothelial markers as CD31, CD34, and FVIII suggests that their wall is mostly composed of pericytes and/or smooth muscle cells, possibly intervening in blood flow regulation in a positive or negative way (favorizing or decreasing local blood flow velocity). Their inverse relationship with the hemorrhagic transformation of infarcts could suggest a protective role against this phenomenon. Preconditioning stimuli as associated arterial hypertension or higher levels for MMP-2 expression have a tendency to be present when convolutes appear and could be presumed as factors involved in their generation, even though without definite statistical value.

The presence of metalloproteinases did not show particular significance in stroke patients, since both MMP-2 and MMP-9 were almost equally expressed in this series and in control cases. Therefore, at least in humans, potential therapeutic strategies destined to lower their levels appear as unsuitable.

Demonstration of a much higher number of activated microglial cells in our stroke cases confirms their putative role as neuroprotectors and potential target for stimulation as suggested by other studies [38].

Nitric oxide synthases (NOS) are known to be expressed in blood vessels (endothelial nitric oxide synthase – eNOS) and to be increasingly found following ischemia [39]. Since our antibody detects both neuronal (nNOS) and endothelial nitric oxide synthases, we were able to study simultaneously their expression in the brain tissue. Our study confirmed these findings, more than half of stroke patients exhibiting positivity for nNOS and almost 20% for eNOS, as compared to the control series, which was negative.

Fibroblastic growth factors (FGFs) stimulate the growth of endothelial cells [40]. The studies by Issa R *et al.* [41] showed basic fibroblastic growth factor (bFGF) to be increasingly expressed in the penumbra. It seemingly has a protective effect, preventing down-regulation of bcl-2 protein [42]. In our study, bFGF was equally expressed by both series of patients; therefore, a difference regarding the pathogenesis of ischemic injury or the clinical outcome related to it could not be confirmed.

Several studies [43–45] demonstrated that TNF-alpha could be a mediator of focal ischemic brain injury. However, we did not find any positive case for it, in both the stroke series and control cases; therefore, its role in the ischemic brain injury was not seen in our cases.

The role of cyclooxygenase-2 (COX-2) as a deleterious factor in cerebral ischemia is debated [28]. We also found it to be significantly associated with cerebrovascular injury.

On the other way, in the postmortem evaluation of a certain tissue, and mostly of the brain, a factor of paramount importance is the pre-stroke condition of patient and possible pathological changes of local existing microvessels resulting from previous pathologies which could have been recognized during the patient's life or not. These have most probably already induced subtle or more conspicuous molecular and cellular transformations due to chronic hemodynamic aggression and metabolic stress during the patient's life, before the present acute event occur. The presence of arterial hypertension in almost all our patients, even at high values and during a long period of their life had certainly deleterious effects on the status of their small intraparenchymal vessels, with plasma leakage, interstitial edema and subsequent triggering of apoptotic pathways. Diabetes mellitus could play similar roles, aside modifying the individual susceptibility of some cells, mostly neurons, to these aggressive factors. Many patients of both our series suffered and even died of a myocardial infarct, a condition associated with a marked lowering in arterial pressure, which could also enhance the neuronal vulnerability and initiate the apoptotic process at a moment difficult to be assessed in the evolution of a particular patient.

Some weaknesses of this study could arise from the relatively small samples of patients taken into study. Another problem is the interval from death to autopsy, which could modify in an unpredictable manner the tissue reactivity for various molecules. Overall, we assessed with enough accuracy the expression of various molecules possibly involved in the fate of brain tissue in ischemic injury and their association to infarct localization, duration, or associated diseases.

Conclusions

The presence of metalloproteinases seems to have a protective role against ischemia, since they were practically absent from the stroke series but expressed in the majority of the control cases. Therefore, therapies should be imagined to enhance their presence in the

brain of stroke patients appear as appropriate. Vascular convolutes appear to be generated by arterial hypertension as a preconditioning state of the brain and their presence is associated with a lower degree of hemorrhagic transformation of brain infarcts. We found systemic inflammatory markers as NOS and COX-2 to be associated with ischemic cerebrovascular disease but no inflammatory cells were involved. We also describe for the first time the presence of mast cell and basophilic polynuclear cell enzymes in neurons and astrocytes. Their significance, even though not related to a specific condition, must be further investigated. Microglia appears as being activated in stroke and methods directed toward its stimulation could be imagined in order to enhance neuroprotection and neurorecovery. Our findings underscore notable differences between animal model studies and human pathological tissue. Attention should be focused on the latter by an increase in the autopsy rate worldwide.

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