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Cerebral collateral flow defines topography and evolution of molecular penumbra in experimental ischemic stroke

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ABSTRACT

Intracranial collaterals are dynamically recruited after arterial occlusion and are emerging as a strong determinant of tissue outcome in both human and experimental ischemic stroke. The relationship between collateral flow and ischemic penumbra remains largely unexplored in pre-clinical studies. The aim of the present study was to investigate the pattern of collateral flow with regard to penumbral tissue after transient middle cerebral artery (MCA) occlusion in rats. MCA was transiently occluded (90 min) by intraluminal filament in adult male Wistar rats (n = 25). Intracranial collateral flow was studied in terms of perfusion deficit and biosignal fluctuation analyses using multi-site laser Doppler monitoring. Molecular penumbra was defined by topographical mapping and quantitative signal analysis of Heat Shock Protein 70 kDa (HSP70) immunohistochemistry. Functional deficit and infarct volume were assessed 24 h after ischemia induction. The results show that functional performance of intracranial collaterals during MCA occlusion inversely correlated with HSP70 immunoreactive areas in both the cortex and the striatum, as well as with infarct size and functional deficit. Intracranial collateral flow was associated with reduced areas of both molecular penumbra and ischemic core and increased areas of intact tissue in rats subjected to MCA occlusion followed by reperfusion. Our findings prompt the development of collateral therapeutics to provide tissue-saving strategies in the hyper-acute phase of ischemic stroke prior to recanalization therapy.

Introduction

Intracranial collateral circulation represents a multiple-level subsidiary vascular network which is dynamically recruited after occlusion of cerebral arteries to provide a source of residual blood flow (Liebeskind, 2003).

In both humans and rodents, a significant supply of collateral flow after middle cerebral artery (MCA) occlusion is provided by the circle of Willis through the anterior cerebral artery (ACA) and the leptomeningeal anastomoses between the cortical branches of ACA and MCA. However, a significant degree of inter-individual variability exists in the functional performance of intracranial collaterals under ischemic conditions in humans (Qureshi et al., 2008; Liu et al., 2011) and rodents (Armitage et al., 2010; Riva et al., 2012).

Cerebral collateral flow is emerging as a powerful determinant of functional and tissue outcome in unselected ischemic stroke patients (Maas et al., 2009; Menon et al., 2011) and in stroke patients treated with intravenous rtPA (Brunner et al., 2012; Miteff et al., 2009) or endovascular recanalization (Bang et al., 2011; Bang et al., 2008).

For these reasons, an in-depth understanding of the physiology, adaptive response and modulation of intracranial collateral circulation is of foremost importance for acute stroke pathophysiology and therapy.

Ischemic penumbra was originally defined as "tissue at risk", which has been made functionally silent and metabolically metastable by ischemic injury but still has the potential for full recovery if reperfusion is timely achieved (Branston et al., 1974; Hossmann, 1994; Baron, 1999). The concept of ischemic penumbra is gradually evolving, after pre-clinical and clinical studies showed a heterogeneous and variable pattern of perfusion deficit, molecular response, topographical

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distribution and evolution of the penumbra in relation to the ischemic core (Del Zoppo et al., 2011; Manning et al., 2014).

HSP70 is the major inducible heat shock protein (Sharp et al., 2000), whose expression after focal cerebral ischemia reflects an endogenous cell response to injury. The neuronal expression of HSP70 is considered as a molecularly defined penumbra, where injured neurons have activated endogenous pathways for protein renaturation and protection against further ischemia.

In the present study, we investigated the relationship between intracranial collateral flow during transient MCA occlusion and the development of molecular penumbra and ischemic infarct after 24 h. The hemodynamic monitoring of central and peripheral MCA territories was analyzed in terms of perfusion deficit and biosignal fluctuation. Hemodynamic data were correlated with the corresponding areas of molecular penumbra, infarct volume and functional deficit.

Material and methods

Study design

The experimental protocol was approved by the Committee on Animal Care of the University of Milano Bicocca, in accordance with the national guidelines on the use of laboratory animals (D.L. 116/1992) and the European Union Directive for animal experiments (2010/63/EU), under project license from the Italian Ministry of Health.

A group of consecutive animals undergoing successful MCA occlusion (see below) were used to explore the effect of cerebral collateral flow on molecular penumbra. Cerebral collateral flow (defined as perfusion deficit in the MCA–ACA borderzone territory) was considered as a continuous independent variable. Primary outcome was defined as the quantification of molecular penumbra (HSP70 positive areas). Secondary outcomes were infarct volume and neurobehavior. Outcome assessment was blinded to hemodynamic data on cerebral collateral flow.

Animals and surgery

Animals were housed in single cages, exposed to 12/12 hour light/dark cycle, at controlled room temperature, with free access to food and water, in a specific pathogen free (SPF) facility.

Adult male Wistar rats (280 ± 5% g; n = 28) were anesthetized with 3% isoflurane in O_2/N_2O (1:3), and maintained with 1.5% isoflurane. Occlusion of the origin of the right MCA was induced transiently for 90 min with a reperfusion period of 24 h. Briefly, a silicone-coated filament (diameter 0.39 ± 0.02 mm, Doccol Corporation, Redlands, CA, USA), was introduced in the right external carotid artery and pushed through the right internal carotid artery to occlude the origin of the right MCA. The common carotid artery was transiently occluded immediately before the insertion of the filament and subsequently re-opened during ischemia period and reperfusion. The pterygopalatine artery was transiently occluded during both the surgical insertion of the filament and ischemia, then re-opened during reperfusion.

During surgery, the core temperature of 37 °C was controlled by a rectal thermometer connected to a feedback-controlled heating pad. In a subset of animals (n = 12) cardio-respiratory parameters (arterial pressure, heart rate, respiratory rate) were continuously monitored using a Samba Preclin 420 transducer (Samba Sensors, Harvard Apparatus, UK) inserted in the right femoral artery before ischemia induction.

After reperfusion, rats were allowed to recover and had free access to food and water. After 24 h from the onset of ischemia, animals were assessed for neurobehavioral score (see below), then euthanized using CO_2 inhalation and the brains were collected for fixation.

Three rats were excluded from the experimental series for early death occurred before successful MCA occlusion (one rat died for anesthesiological complications, two rats died for subarachnoid hemorrhage). All successfully occluded rats (n = 25) survived the 24 hour reperfusion time and were used for analysis.

Laser Doppler multi-site perfusion monitoring

The induction of focal cerebral ischemia was assessed using laser Doppler (LD) perfusion monitoring (dual channel moorVMS-LDF™, Moor, Axminster, UK) using two blunt needle probes (VP12). Probes were positioned in a custom made silicon holder attached with surgical glue over the intact skull and further secured in place using sutures to surrounding soft tissues (Beretta et al., 2013). The first probe (Probe 1) was attached to the skull 1 mm posterior to the bregma and 5 mm lateral to the midline (lateral probe, corresponding to the central MCA territory). The second probe (Probe 2) was attached to the skull 2 mm anterior to the bregma and 2 mm lateral to the midline (medial probe, corresponding to the borderzone territory between ACA and MCA territories). Cerebral perfusion monitoring was performed continuously during the entire period of anesthesia (approximately 140 min), including reperfusion. The mean duration of cerebral perfusion monitoring after reperfusion was 7.8 (4–15) min.

Three hemodynamic parameters were considered for signal analysis of each probe: i) drop in cerebral perfusion (expressed as % of baseline) during MCA occlusion following successful filament insertion; ii) biosignal fluctuation analysis during the pre-ischemic period and during MCA occlusion (see below); and iii) cerebral reperfusion (expressed as % of baseline), from the removal of the filament until the end of anesthesia.

Hemodynamic assessment was blinded to outcome data (neurobehavior, infarct volume, HSP70 staining).

Biosignal fluctuation analysis of cerebral perfusion

Fluctuation analysis of blood microcirculation from LD signals or alternative laser speckle imaging signals has been undertaken with various measures (see Humeau-Heurtier et al., 2013 for a recent review). In this study, auto-correlation $R(k)$ analysis was based on linear second order statistics (Humeau et al., 2008), see Supplementary methods for details.

The auto-correlation $R(k)$ measures the self-memory of the fluctuations around the average value and constitutes an analysis different from the record of the drop of average value between the two probes. As shown in Fig. 2, the two autocorrelation functions estimated for the two probes display a decrease but with a distinct characteristic time decay. To quantify this observation, we compute the time in seconds that autocorrelation $R(k)$ takes to drop from 1 to 0.9 for each probe.

Neurobehavioral assessment

Rats were assessed 24 h after transient MCA occlusion with a functional neuroscore for neurological outcome on a scale from 3 (most severe) to 18 (no deficit) (Garcia et al., 1995). Behavioral assessment was blinded to hemodynamic data and consisted of scoring the spontaneous movement, sensory function and motor function.

Histology and infarct volume determination

Brains were fixed in ice-cold 10% neutral buffer formalin and coronal sections (50 μm) were stained using Cresyl Violet 0.1% (Bioptica, Milano, Italy). Infarct areas were measured in 19 consecutive sections with 250 μm interval (bregma +2.5 mm to −3.0 mm).

Infarct volume was calculated using ImageJ image processing software (National Institute of Health, Bethesda, MD, USA), corrected for inter-hemispheric asymmetries due to cerebral edema, and expressed in mm^3. Infarct volume calculation was blinded to hemodynamic data.

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