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Short Communication

Workflow for automated quantification of cerebromicrovascular gelatinase activity

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ABSTRACT

The gelatinase enzymes, matrix metalloproteinases -2 and -9, are central mediators of blood–brain barrier disruption, actively studied in experimental models of neurological disease. Staining with in situ zymography (ISZ) allows visualization of gelatinase activity directly in brain tissue sections. However, quantifying microvascular gelatinase activity from ISZ-images is challenging and time consuming, as surrounding cell types often show significant confounding activity. We describe validation and performance of a workflow for automated image analysis of cerebromicrovascular gelatinase activity, now released for open-access use. In comparison to manual analysis, the automated workflow showed superior accuracy, was faster to execute and allows for more detailed analysis of heterogeneity in the microvasculature. We further suggest recommendations for quantifying and reporting this type of activity in experimental studies, focusing on ischemic stroke.

Introduction

Matrix metalloproteinase enzymes (MMPs) are zinc-dependent endopeptidases with central physiological proteolytic functions (Rosenberg, 2009). The gelatinase enzymes, MMPs -2 and -9, are capable of degrading vascular basal lamina, and participate in microvascular events such as angiogenesis, leukocyte infiltration and ischemia/reperfusion injury. In the cerebral microvasculature, gelatinase enzymes are central mediators of blood–brain barrier disruption in pathologies such as ischemic stroke, cerebral hemorrhage and infections, and remain a focus of active investigation (Rosenberg, 2009).

Enzymatic activity of gelatinases is regulated at several levels, including expression, secretion, activation, and inhibition (by Tissue inhibitors of metalloproteinases, TIMPs) (Ra and Parks, 2007). Because of this, traditional methods like western blot and immunohistochemistry are largely insufficient in determining the genuine local activity of gelatinases. Likewise, gel zymography does not give spatial information, and may not always represent true gelatinase activity, as natural inhibitors are dissociated from gelatinases by SDS-treatment (Vandooren et al., 2013). These limitations were addressed by in situ zymography (ISZ), in which fluorescently overlabeled gelatin is applied directly on tissue sections. Active gelatinases fragment gelatin, leading to dequenching of fluorescence in gelatin fragments, and a local fluorescent signal that correlates with in situ gelatinase activity (Vandooren et al., 2013).

Previously, we set out to determine differences in cerebromicrovascular gelatinase activity between treatment groups in an experimental stroke study (Mattila et al., 2011). Using ISZ, we quickly found that quantification of microvascular gelatinase activity using traditional manual image-analysis was time-consuming, and that in brain tissue, other active cellular structures confounded precise selection of active microvascular structures, especially perpendicularly cut microvessels. Here, we describe the validation and performance of our workflow for quantification of microvascular gelatinase activity, including staining and visualization of ISZ, and novel software for automated image-analysis (which we have now made freely available for download at: http://anduril.org/pub/anima/ISZ_activity/). Our main finding is that automated image analysis has superior accuracy and speed compared to manual analysis in this specific setting of cerebromicrovascular research, and allows for more detailed investigation of microvascular gelatinase activity. We further suggest methods to unify quantification and reporting of changes in microvascular gelatinase activity in experimental studies. Most importantly, automated analysis is preferable, and selection of "gelatinase active" microvessels should be based on defined levels of ISZ brightness.

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Methods

Animal model of focal cerebral ischemia

The experimental procedure has been described previously (Strbian et al., 2006). Briefly, adult male Wistar rats (n = 5), weighing 290–340 g, were anesthetized with ketamine (i.p., 50 mg/kg, Ketalar, Parke-Davis, Detroit, MI, USA) and medetomidine (s.c., 0.5 mg/kg, Domitor, Orion, Espoo, Finland). Focal ipsilateral cerebral ischemia was induced with the suture MCAO model. After 1 h of ischemia and 3 h of reperfusion animals were sacrificed with pentobarbital (60 mg/kg, Mebunat, Orion) and transcardially perfused with ice-cold saline. Infarction was confirmed with 2,3,5 triphenyltetrazolium chloride staining as described previously (Strbian et al., 2006). A representative 1 mm coronal brain section was embedded in Tissue-tek (Sakura Finetek Inc., Tokyo, Japan), snap-frozen with liquid nitrogen and stored at −80 °C. 8 μm thick sections were prepared for staining. All experiments were approved by local authorities (ELLA animal experiment board, Finland), and conducted in accordance with The Finnish Act on Animal Experimentation (62/2006).

In situ zymography and immunohistochemistry

For ISZ the brain sections were air-dried for 5 min at room temperature and washed with phosphate-buffered saline (PBS) for 5 min. DQ-gelatin (0.1 mg/ml, EnzChek® gelatinase/collagenase assay kit, Invitrogen, Carlsbad, CA, USA) was applied on the sections and incubated in a humid chamber for 2 h at 37 °C. Sections were then rinsed in PBS and dH_2O. Control sections were incubated with gelatinase inhibitors Ilomastate (500 mM, GM6001, Millipore) or TIMP-1 (500 nM, R&D Biosystems) for 1 h before and during DQ-gelatin incubation, and reduced ISZ-activity was seen.

After ISZ, sections were stained for detection of neurons (Neuronal Nucleus, NeuN), astrocytes (Glial Fibrillary Acidic Protein, GFAP) or endothelial cells (von Willebrand Factor, vWF). Primary antibodies used were: NeuN (A60 Chemicon, 0.5 μg/mL, 1 h), GFAP (G-A-5 Sigma-Aldrich, 1/1000, 1 h) and vWF (rabbit polyclonal Abcam, 19.5 μg/mL, o/n). Alexafluor 594 secondary antibodies were used (anti-mouse 10 μg/mL or anti-rabbit 5 μg/mL, 30 min, Invitrogen). Control sections incubated with nonspecific mouse IgG1 (Dako) or rabbit IgG (Vector labs) in equivalent concentrations showed no specific immunostaining. We mounted sections with Prolong Gold (Invitrogen).

Digital imaging

Imaging was performed using an Axioplan 2 epifluorescent microscope (Carl Zeiss, Hallbergmoos, Germany) with a 20×-objective. Five region of interest (ROI) images were acquired from predefined sites (three cortical and two subcortical) from both hemispheres with an AxioCam camera, (1300 × 1030 pixels) and Axiovision software (v3.0.6, Carl Zeiss). Image sets were acquired using constant exposure times for all samples, and using the Zeiss Vision Image (ZVI) file format. A sample image is shown in Fig. 1. For a schematic procedure of sample preparation and imaging see Fig. 2a.

Automated high-throughput image analysis

We created a novel automated analysis workflow (Fig. 2b) for analyzing microvascular gelatinase activity, which was implemented using the Anduril workflow framework (Ovaska et al., 2010). The pipeline utilizes Mathworks MATLAB and CRAN R and can be installed on any modern Ubuntu operating system, and is now available for free download in the public domain (http://anduril.org/pub/anima/ISZ_activity/).

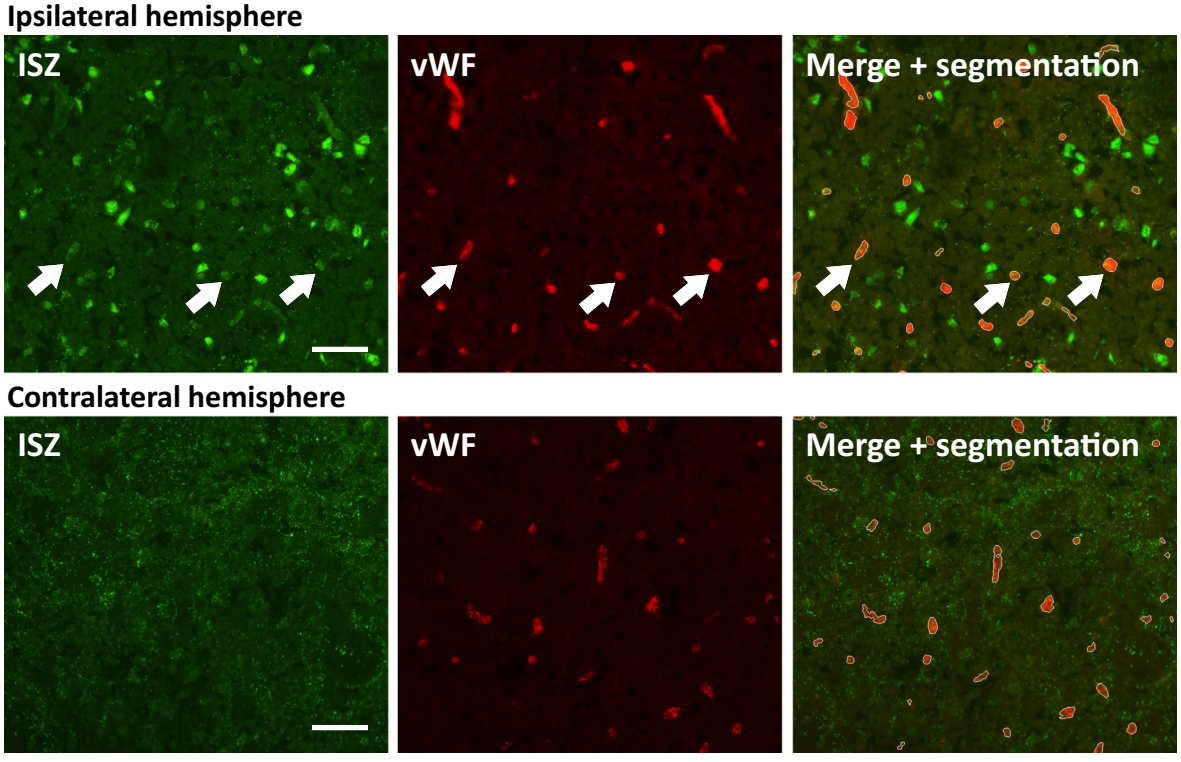

Fig. 1. Microvascular gelatinase activity in the ipsilateral and contralateral brain hemispheres after 1 h of ischemia and 3 h of reperfusion. ISZ, vWF and merged images are shown, together with segmentation from the automated analysis workflow. Although some longitudinally cut vessels are noticeable in the ISZ image, active perpendicularly cut vessels can only be identified using the vWF image as reference (white arrows). Only weak microvascular activity was seen in the contralateral hemispheres. The ISZ and imaging protocols were optimized for microvascular analysis. Scale bars = 50 μm.