Forensic Science International 255 (2015) 118–122

Contents lists available at ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forsciint

Tissue-dependent VEGF and GLUT1 induction in a rat hemorrhage model: With regard to diagnostic application of mRNA quantification in forensic pathology

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ARTICLE INFO

Article history:
Available online 31 August 2015

Keywords:
Forensic pathology
Hemorrhage
Rat
mRNA quantification
Real-time PCR

ABSTRACT

Systemic hypoxia is inevitably involved in the death process to a varying extent. Hypoxia-response factors proved useful in forensic pathology in previous studies; however, fundamental investigations using animal models are expected to reinforce the findings from autopsy practice. An animal experiment using a rat model of fixed-volume hemorrhage was performed to apply basic insight into quantitative mRNA analyses in forensic pathology. Male Sprague–Dawley rats ($n = 5$) were anesthetized, bled from the femoral artery (24 ml/kg; about 30% of total circulating blood volume), and decapitated after 1 or 2 h. Tissue samples of the heart, brain (hippocampus), kidney, liver, lung and skeletal muscle were collected for RNA and protein analyses. Quantitative analyses of VEGF, GLUT1 and GAPDH mRNAs were performed with TaqMan real-time RT-PCR assay. In the sham control without bleeding, mRNA quantification revealed the tissue-dependent mRNA levels in physiological condition. Relative quantification of VEGF and GLUT1 showed significant inductions under hemorrhage at the mRNA level, using GAPDH as endogenous reference. In conclusion, tissue-dependent induction patterns of VEGF and GLUT1 were revealed in the volume-fixed hemorrhage rat model. This study could practically guide the selection of mRNA markers and tissue samples in forensic pathology related to tissue ischemia and cellular hypoxia for autopsy cases.

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1. Introduction

Systemic hypoxia and tissue ischemia are inevitably involved in the death process as episodes of various extents and characteristics, which are closely related to the cause and circumstance of death [1]. Mammalian cells express multiple gene products in response to cellular hypoxia, including hypoxia-inducible factor 1 (HIF-1α) and its down-stream factors, such as vascular endothelial growth factor (VEGF) and glucose transporter 1 (GLUT1), which collectively maintain cellular metabolic adaptation to decreased oxygen availability [2–4]. Benefiting from technological advances in fluorescence real-time PCR, our previous studies showed that mRNA quantitative analysis of hypoxia-response factors in autopsy tissue samples could be helpful in investigating the cause of death, especially for traumatic injuries [1,5], which have attracted the attention of forensic pathologists and biologists [6–8].

In the present study, a rat model of fixed-volume hemorrhage was developed to produce tissue ischemia and cellular hypoxia. Fundamental insights from such an animal model are expected to be helpful to evaluate the data consistency of hypoxia-response factors in human autopsy and animal experiments, and to direct the selection of mRNA markers and tissue samples for forensic pathology.

2. Materials and methods

2.1. Rat hemorrhage model

Male Sprague–Dawley rats (10 weeks old, $n = 5$ for each group; Crj Inc.) were anesthetized (intraperitoneal sodium pentobarbital, 50 mg/kg body weight), blood was removed with a syringe from the femoral artery (24 ml/kg body weight; about 30% of total

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http://dx.doi.org/10.1016/j.forsciint.2015.08.007
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Table 1
Primers and TaqMan probes for gene expression assay.

mRNA	Full name	[a]Assay code no.
VEGF	Vascular endothelial growth factor	Rn00582935_m1
GLUT1	Glucose transporter 1	Rn01417099_m1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Rn01417099_m1

[a] Primers and TaqMan probe for each assay were purchased from Life Technologies.

circulating blood volume) [9], and they were decapitated after maintaining the hemorrhagic status for 1 or 2 h. Control rats ($n = 5$) were sham-operated without bleeding. All procedures were approved by the animal facility of our institution.

2.2. Tissue sample collection and RNA extraction

Tissue samples were collected from consistent sites in the heart (left ventricle), hippocampus of the brain, kidney, liver, lung (upper lobe) and skeletal muscle (forelimb) of rats immediately after decapitation, then submerged in RNA stabilization solution (RNAlater™; Ambion, Austin, TX) and stored at 4 °C for less than 1 week until RNA extraction. Total RNA was isolated with ISOGEN (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions, treated with TURBO DNA-free™ kit (AM1907; Ambion), measured by a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and stored at −80 °C until use. The quality of RNA extracts was assessed by electrophoresis in agarose gels stained with ethidium bromide to visualize 18S and 28S rRNA bands under UV illumination.

2.3. TaqMan RT-PCR and quantification of mRNA

Quantification of mRNAs of VEGF, GLUT1 and GAPDH was performed by the TaqMan real-time RT-PCR system with inventory primers and probes purchased from Applied Biosystems (Foster City) (Table 1). TaqMan RT-PCR was performed using the High Capacity cDNA Reverse Transcription kit (4368814; Applied Biosystems) and TaqMan Gene Expression Master Mix (4369016; Applied Biosystems) containing the same amount (45 ng) of total RNA extracts for each sample in a 20 μl PCR system on an ABI PRISM 7700 Sequence Detector (Applied Biosystems). The contents of the amplification mix and the thermal cycling conditions were set according to the accessory protocols. GAPDH housekeeping gene mRNA was used as endogenous references for relative quantification.

According to the manufacturer's instructions and previous reports [10], purified RT-PCR products of VEGF, GLUT1 and GAPDH were used as samples to establish standard curves for absolute quantification of the mRNA copy number. The relative quantification of VEGF and GLUT1 was expressed as a ratio of the target normalized against an endogenous reference (GAPDH), and then ratios for fold change relative to a calibrator sample were obtained. The calibrator was the mean value of each related sham-operating group. Relative quantification determines the changes in steady-state mRNA levels of genes across multiple samples and expresses it relative to the levels of GAPDH that was previously proved stable in tissue ischemia [11]. GAPDH as a housekeeping gene is consistently expressed among the samples, and can be co-amplified in the same tube in a multiplex assay.

2.4. Statistical analyses

Regression equation analysis was used to study the relationships between pairs of parameters. Comparisons between groups were performed using Student's t-test, figures for which present the mean value and the standard deviation, and $P < 0.05$ was considered significant.

3. Results

3.1. TaqMan real-time RT-PCR standard curves for convenient absolute quantification

Purified RT-PCR products of VEGF, GLUT1 and GAPDH mRNAs were used as standard samples to establish standard curves for quantification of the mRNA copy number and to determine the dynamic range of the real-time RT-PCR assays. Curves were generated by plotting the log of the starting quantity of the standard samples versus the threshold cycle values (CT). In the 20 μl real-time PCR system, 10^3 to 10^8 template copies of each standard sample were added, amplified and produced with variant CT values, which in theory negatively correlated with the originally added template copy number. All the standard curves constructed in this study were linear over 5 orders of magnitude with regression coefficients over 0.99 (Fig. 1). Two replicates were performed for each standard curve point. Electrophoresis of the RT-PCR products of total RNA validated the specificity of amplicons.

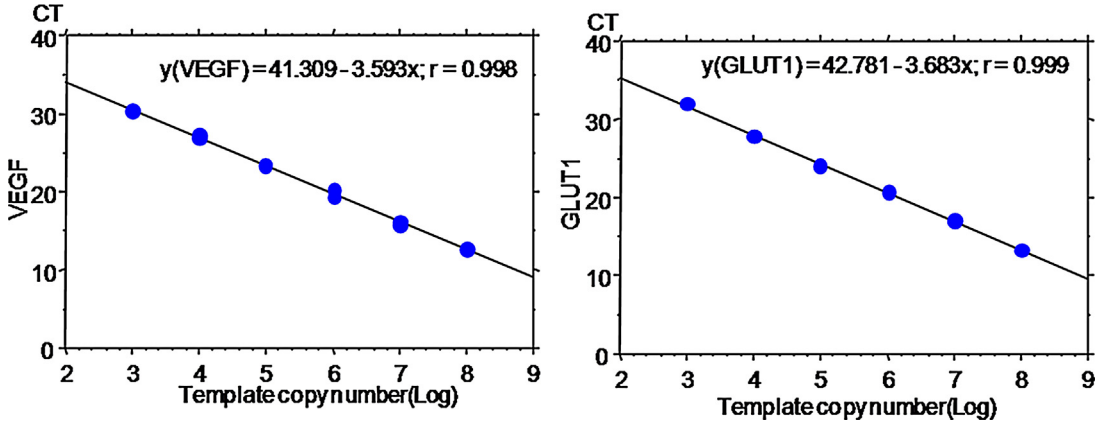

Fig. 1. Standard curves of TaqMan real-time RT-PCR. RT-PCR products were isolated after migration on agarose, purified with a QIAEX II Gel Extraction kit (Qiagen), quantified by spectrophotometry, and then used as standard samples to establish standard curves for convenient absolute quantitation and to determine the dynamic range of the real-time RT-PCR assay. Curves were generated by plotting the log of the starting quantity of the standard samples versus the threshold cycle (CT). Starting template copies of 10^8 for VEGF, GLUT1 RT-PCR products were serially diluted 10-fold for 5 orders of magnitude, respectively. Two replicates were performed for each standard curve point.

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