Forensic Science International 232 (2013) 218–228

Contents lists available at ScienceDirect

Forensic Science International

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

Studies on RNA integrity and gene expression in human myocardial tissue, pericardial fluid and blood, and its postmortem stability

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ARTICLE INFO

Article history:
Received 30 November 2012
Received in revised form 24 June 2013
Accepted 3 August 2013
Available online 15 August 2013

Keywords:
Gene expression
RNA integrity
Post-mortem interval
Myocardial zones
Blood
Pericardial fluid

ABSTRACT

Analyses of gene expression of ischemic myocardial injury and repair related proteins has been carried out for the first time in samples from five specific sites of the myocardium, pericardial fluid and blood from thirty cadavers in relation to post-mortem interval (PMI). RNA integrity was evaluated by RNA integrity number (RIN), with values ranging from 6.57 to 8.11; sufficiently high levels of integrity to permit further gene amplification. No significant correlations between RIN and PMI in any samples were detected. Prior to target gene expression analysis, a normalization strategy was carried out to assess candidate reference gene stability, involving the analysis and comparison of four common housekeeping genes (Glyceraldehide-3-phosphate dehydrogenase, beta-actin, TATA box binding protein and Cyclophilin A). Gene expression of cardiac troponin I (TNNI3), myosin light chain 3 (MYL3), matrix metalloprotease 9 (MMP9), transforming growth factor beta 1 (TGFB1), and vascular endothelial growth factor A (VEGFA) in myocardial zones and body fluids were subsequently studied by real-time quantitative PCR. Expression levels of all the proteins studied in cardiac zone samples were similar. No statistical differences for expression were detected among proteins taken from any myocardial area. No significant differences were detected for TNNI3 and TGFB1 gene expressions when compared with samples at or under 12h-PMI or over 12h-PMI. However, differences in MYL3, MMP9, and VEGFA gene expression in body fluids were found at PMI periods of over 12 h. These interesting results may contribute to the refinement of current knowledge regarding cardiac metabolism and improve understanding of the underlying mechanisms involved in myocardium ischemia and its repair.

1. Introduction

Messenger RNA (mRNA) is an intermediate molecule in protein synthesis, offering insight into gene expression patterns of any given tissue at any given time. Comparisons between patterns of physiological expression in healthy and pathological tissue can reveal the causes of death, given that changes in mRNA occur in accordance with the physiological needs of cells [1,2]. Although gene expression is already a well established aspect of modern molecular medicine, its study and application in forensic pathology remains at an early stage. However, within the forensic community there is growing interest in the possibilities that this area of research may offer [3–11].

RNA is an extremely labile molecule that is prone to damage from either intrinsic factors, such as enzymatic degradation by RNases, or external factors such as light, humidity, or high temperatures [2]. In addition, postmortem factors, such as post-mortem interval (PMI), cause of death and body storage conditions may alter mRNA integrity [6,7,11]. Terminal coma and hypoxia [1], medication, pyrexia, dehydration, drug and alcohol abuse, and various types of stress [8,9] have also been associated with low mRNA integrities. Furthermore, several studies have shown that sufficiently high quality mRNA permits quantitative gene expression analyses in post-mortem tissue, even after periods of several days [6,10,11].

Prior to gene expression determination, photometric analysis of mRNA quantity, purity and integrity is essential. Spectrometric determination of mRNA concentration can be carried out by measuring absorbance at 260 nm, while the ratio of readings at 260 nm and 280 nm provides a reliable estimation of purity. When analyzing postmortem samples, caution should be taken in the interpretation of quantitative gene expression, given that low mRNA quality and the wide range of possible biological samples may lead to erroneous results [7,12–15]. In addition to mRNA

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http://dx.doi.org/10.1016/j.forsciint.2013.08.001

concentration, mRNA integrity determination is crucial to effective quantitative gene expression analysis, and to an even greater extent in postmortem samples. The use of mRNA samples with the highest possible RNA integrity number (RIN) is therefore highly recommended. The method of choice for mRNA integrity estimation is chip-based capillary electrophoresis [7,12,13,15–18]. Although postmortem mRNA degradation is a complex process, requiring further systematic study, the verification of mRNA integrity prior to gene amplification is essential.

Determination of mRNA using quantitative real-time PCR (qPCR) is a commonly used in gene expression level analysis. However, prior to any gene expression study, a normalization strategy is required in order to validate the stability of candidate endogenous control genes [4,5,19–21]. The ideal housekeeping gene should be easy to detect and should be expressed at a constant level among different tissues of an organism when exposed to the same experimental protocol as the genes under study [4,20,21]. For precise data normalization, current literature proposes several specific endogenous reference genes. Nevertheless, expression levels for certain housekeeping genes are known to vary in accordance with pathological or experimental conditions and types of tissue [15,19]. In an attempt to select the most suitable endogenous control gene in our postmortem samples, four genes commonly reported as stable reference genes in forensic studies were selected: Glyceraldehide-3-phosphate dehydrogenase (GAPDH), beta-actin (ACTB), TATA box binding protein (TBP), and Cyclophilin A (CYCA) [4,5,15,19,21].

Given the likely importance of gene expression levels in the sequence of pathological events, gene expression studies in human cardiac zones and fluids are becoming ever more necessary to understand the underlying mechanisms involved in myocardium ischemia and its repair. Our primary objective was to perform expression studies, in human myocardial zones and body fluids, on several genes associated with ischemic myocardial injury and its repair, and their postmortem stability, involving: cardiac troponin I (TNNI3), myosin light chain 3 (MYL3), matrix metalloprotease 9 (MMP9), transforming growth factor beta 1 (TGFB1), and vascular endothelial growth factor A (VEGFA). Troponin is a contractile protein of the thin filament of striated muscle, consisting of three subunits: Troponin C, Troponin T, and Troponin I as the inhibitory subunit of the troponin complex. At basal levels of calcium, Troponin I inhibits actin-myosin cross-bridges. In systole, as calcium binds to the regulatory site of Troponin C, the inhibitory subunit (Troponin I) is released, thereby activating muscle contraction [22]. The expression of cardiac Troponin I isoform (TNNI3), a protein encountered in adult human hearts, is tissue-specific and was selected for study given that the determination of blood levels of this protein is already a commonly used method in clinical practice in the diagnosis of acute myocardial infarction [23,24]. MYL3 is a ventricular muscle and embryonic isoform of alkali light chain, and is considered an important structural protein in cardiac muscle [25–29]. MMP9 plays an important role in vessel matrix changes, contributing to left ventricle re-modeling by altering the basement membrane which promotes molecular traffic around myocardiocytes and vascular permeability. MMP9 also plays an important role in post-myocardial infarction and left ventricle re-modeling [30–32], while TGFB1 in the vascular system appears to produce a protective effect, attributed to its anti-inflammatory and postulated anti-atherogenic action. TGFB1 is thought to act as a vasoprotective and plaque-stabilizing cytokine. Low levels of TGFB1 activity are believed to contribute to atherosclerotic plaque destabilization [33]. The potent effect of these proteins plays a central role in infarct healing, cardiac repair and left ventricular re-modeling [34]. VEGFA is critical for initiating early cellular responses to myocardial hypoxia [35] and is considered a specific endothelial mitogen that plays an important role in myocardial angiogenesis [36].

The aim of this study was to shed light on the stability of endogenous control genes, and several target genes associated with ischemic myocardial injury and its repair and their relationship with post-mortem interval, as well as the correlation between RNA integrity and quantitative expression data. Analyses were carried out on overall mRNA integrity and mRNA expression levels in samples from heart (five myocardial sites) and body fluids (femoral vein blood and pericardial fluid) from 30 cadavers. We believe that the study of these gene expressions may prove highly significant in the interpretation of pathological mechanisms in myocardial ischemia after testing the possible effect of PMI.

2. Materials and methods

2.1. Materials

Samples were taken from a total of 30 cadavers of known time of death. Autopsies were carried out at the Institute of Legal Medicine in Malaga, Spain, in accordance with European standards [37]. Prior to autopsy, all corpses had been maintained at 4 °C for periods ranging from 5 and 24 h after death. The mean age of the deceased subjects was 65.03 (±16.50) and consisted of 5 females and 25 males. Causes of death were classified as cardiac related in 13 cases (43%) by physiopathological similarity [37], while the remainder, 17 cases; (47%) were classified as other causes: craniocerebral trauma (3 cases); multiple trauma (5 cases); mechanical asphyxia (6 cases); and other natural deaths (3 cases). Hearts were dissected using the transverse slicing method [38–40]. Samples for molecular analysis were taken from the intermediate ventricular slice and frozen and stored for a maximum of six months at −80 °C before analysis. Pericardial fluid and femoral vein blood were in all cases collected in tubes suitable for the stabilization of intracellular RNA (PAXgene Blood RNA Tubes, PreAnanlytix), maintained for 2 h at room temperature before freezing to −80 °C. In total, 210 samples were analyzed, consisting of 150 myocardial tissue samples, and 30 blood and pericardial fluid samples, taken from 30 cadavers.

2.2. mRNA extraction

On the basis of information from previous studies [38,41,42] five specific sites of the myocardium were selected for molecular analyses: the anterior and posterior walls of the left ventricle, the interventricular septum, and the anterior and lateral walls of the right ventricle. Approximately 30 mg of tissue were taken from each myocardial site for molecular analyses. The frozen tissue samples were disrupted and homogenized using the TissueLyser LT (Qiagen, Gilden, Germany), and subsequently, mRNA was extracted using RNeasy Fibrous Tissue Mini Kit (Qiagen). Pericardial fluid and blood samples were thawed for 2 h before analysis and mRNA was extracted using PAXgene RNA Blood Kit (PreAnalityx). mRNA extraction procedures included a DNase treatment to eliminate residual genomic DNA. To minimize possible mRNA degradation during mRNA extraction, all materials and working surfaces were cleaned with RNase Away (Molecular Bioproducts).

2.3. Determination of mRNA quantity, quality and integrity

mRNA concentration was assessed by photometric analyses by measuring absorbance at 260 nm, using a NanoDrop 2000-C apparatus (Thermo Scientific, Wilmington, DE, USA). mRNA extraction yield was calculated from the nanograms of mRNA obtained from the mRNA isolate divided by the initial myocardium weight or fluid volume (ml), mRNA quality was estimated by measuring the ratio of absorbance at 260 nm and 280 nm. RNA integrity, expressed as RNA integrity number (RIN), which is an algorithm [14] for assigning integrity values to mRNA measurements, was assessed using chip-based capillary electrophoresis with Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA). mRNA samples were electrophoretically separated on microfluidic chips, and the electrophoregrams were observed using laser induced fluorescence by a Bio-Analyzer 2100 (Agilent Technologies). mRNA integrity was evaluated by calculating RIN from peak areas representing ribosomal RNA fragments (18S and 28S). RIN values from 1 to 10, represent completely degraded to completely intact RNA, respectively.

2.4. cDNA synthesis and quantitative real-time PCR

Reverse transcription quantitative real-time PCR (RT-qPCR) was conducted using a two-step protocol. QuantiTect Reverse Transcription kit (Qiagen) was used for synthesis of complementary DNA (cDNA). During the first stage, 500 ng of RNA from each sample was incubated for 2 min at 42 °C, with 2 μl of gDNA Wipeout Buffer to integrate the removal of genomic DNA contamination. At a later stage, 4 μl Quantiscript RT Buffer, 1 μl of RT Primer Mix and 1 μl of Quantiscript Reverse Transcriptase were added to a total volume of 20 μl, incubated for 15 min at 42 °C and then for a further 3 min at 95 °C. The resulting cDNA was diluted 1/10 with