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Forensic diagnosis of ante- and postmortem burn based on aquaporin-3 gene expression in the skin

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ABSTRACT

In order to diagnose death associated with fire, it is essential to show that the person was exposed to heat while still alive. We investigated both AQP1 and AQP3 expression in the skin of an experimental burn model, as well as in forensic autopsy cases, and discuss its role in the differential diagnosis of ante- and postmortem burns. In animal experiments, there was no difference in AQP1 gene expression among four groups ($n = 4$): antemortem burn, postmortem burn, mechanical wound, and control. However, AQP3 expression in the antemortem burn was increased significantly compared with that of the other groups even at 5 min after burn. Water content of the skin was decreased significantly by the burn procedure. Consistent with animal experiments, AQP3 gene expression in the skin of antemortem burn cases was increased significantly compared with postmortem burns, mechanical wounds, and controls ($n = 12$ in each group). These observations suggest that dermal AQP3 gene expression was increased to maintain water homeostasis in response to dehydration from burn. Finally, our results suggest that AQP3 gene expression may be useful for forensic molecular diagnosis of antemortem burn.

1. Introduction

During forensic autopsy examination of a burned body, pathologists must deliberate carefully to determine the cause of death. In order to diagnose death due to burn, it is essential to show that the person was exposed to heat while still alive. At present, external findings proving the exposure are erythema and blister, although they can be produced postmortem [1,2]. Significant internal observations are soot deposits within the respiratory tracts, as well as the elevated blood values of carbon monoxide–hemoglobin. However, these findings may be unremarkable or even absent in cases with a very short survival time after fire exposure or in outdoor open-air cases [3]. In recent years, several studies have shown immunoreactivity of heat shock protein-70, fibronectin and P-selectin increased in the lungs of fire fatalities [4–6]. However, much less reliable new techniques have been developed in forensic practice.

Aquaporins (AQPs) are a family of small, homologous water channels that are involved in fluid transport and are expressed in various epithelial and endothelial cell types, as well as keratinocytes, adipocytes and astrocytes [7–13]. In the forensic field, recent studies reported that the expression of AQPs in organs, and gene variations of AQPs were suitable as diagnostic markers for the differentiation between fresh and salt water drowning [14–16], and as predisposing factors for sudden infant death syndrome [17], respectively. As the main AQPs in the skin, AQP1 is expressed in the dermal capillaries and AQP3 is expressed in the epidermis [13,18,19]. In the present study, we investigated both AQP1 and AQP3 expression in the skin of an experimental burn model, as well as in forensic autopsy cases, and discuss its role in the differential diagnosis of ante- and postmortem burns.

2. Materials and methods

2.1. Animal experiments

2.1.1. Mice

Pathogen-free 8–9-week-old male BALB/c mice were obtained from SLC (Shizuoka, Japan). All mice were bred and housed in a temperature-controlled (23 ± 2 °C) environment with a 12 h light/12 h dark cycle. They were fed with standard feed and given water ad libitum. All animal experiments were approved by the Ethics Committee for Animal Experimentation at Kagoshima University.

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2.1.2. Burn injury procedure

Full-thickness burns were induced as described previously [20,21]. Briefly, under inhalation anesthesia, the dorsal skin of mice was exposed to a heated (100 °C) circular metal plate (20 mm in diameter) for 5 s. The mice were resuscitated immediately with an intraperitoneal injection of 0.05 ml/g body weight of saline. At 5 min after burn (the usual time interval between the exposure of the fire and the death in fire fatality cases), mice were sacrificed by deep anesthesia, and surrounding areas of burned skin (within 2 mm from the edge of burned area) were collected. As a postmortem burn model, the dorsal skin of other mice was exposed to the heated metal plate for 5 s at 15 min after being sacrificed by deep anesthesia, and skin samples were collected. Control skin was collected from non-burned mice ($n = 4$ in each group). The skin samples were immersed in RNA Later (Perkin–Elmer Applied Biosystems, Foster City, USA) and stored at −80 °C for the extraction of RNA. A part of each sample was fixed in 4% formaldehyde buffered with phosphate-buffered saline (PBS; pH 7.2) for histopathological analyses.

2.1.3. Mechanical (punch-out) wound procedure

Full-thickness skin wounds were inflicted as described previously [22]. Briefly, under inhalation anesthesia, the dorsal skin was picked up at the midline and two layers of skin were punched through with a sterile disposable biopsy punch (4 mm in diameter; Kai medical, Japan). This procedure generated two excisional full-thickness wounds with one on each side of the midline. The same procedure was repeated 3 times, generating 6 wounds on each animal. At 5 min after wounding, mice were sacrificed by deep anesthesia, and surrounding areas of wounded skins (within 2 mm from the edge of the wound) were collected ($n = 4$). Skin samples were stored for extraction of RNA.

2.2. Autopsy samples

Skin samples were obtained from 48 forensic autopsy cases (26 male and 22 female) at less than a 72 h postmortem interval. The age of the cases ranged from 20 to 88 years (median, 63.1 years). In each case, the cause of death was diagnosed carefully based on macroscopic, histopathological, and toxicological findings. The skin samples were divided into four groups as follows: antemortem burned skins, postmortem burned skins, mechanically wounded (abrasion and bruise) skins, and neither burned nor mechanically wounded skins (control) ($n = 12$ in each group). Antemortem burned skin samples were taken from the skin that displayed first to second degree burns (erythemas and blisters). Postmortem burned skin samples were taken from the skin in which the dermis of the blister bottom exhibited no flare and in which the protein concentration of the blister content measured less than 1.5 g/dl (blisters without vital reaction). Survival times after burn or wound were estimated to be less than several minutes. Skin samples were stored for extraction of RNA and histopathological analyses. Autopsy study was approved by the Ethics Committee of Kagoshima University and was conducted according to the Declaration of Helsinki Principles.

2.3. Histopathological analyses

Formaldehyde-fixed skin samples were embedded in paraffin. Six μm sections were cut and stained with hematoxylin and eosin (HE) or Masson's trichrome. Immunohistochemical analyses were performed for the evaluation of type of AQP1-, 3-expressing cells in the skin. Deparaffinized sections were immersed in 0.3% H_2O_2-PBS for 30 min to block endogenous peroxidase activity. The sections were incubated with mouse anti-AQP1 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:250) or rabbit anti-AQP3 polyclonal antibodies (Bioworld Technology, Inc., Minnesota, USA; 1:50) at 4 °C for 14 h. Thereafter, the sections were incubated with biotinylated goat anti-mouse IgG (Santa Cruz Biotechnology; 1:100) or goat anti-rabbit IgG (Santa Cruz Biotechnology; 1:100) at 18 °C for 1 h. After rinsing in PBS, the sections were incubated with LSAB2 (Labeled StreptAvidin–Biotin; Dako Cytomation) at 18 °C for 30 min, and positive signals were visualized using 0.02% 3,3′-diaminobenzidine, followed by nuclear staining with hematoxylin.

2.4. Extraction of total RNA, reverse transcription and quantitative real-time PCR

To examine the gene expression of AQP1 and AQP3 in both murine and human skin samples, quantitative real-time PCR analyses were performed as described previously [23]. Total RNA was extracted from the skin samples using ISOGEN (Nippon Gene, Toyama, Japan). One microgram of total RNA was reverse transcribed into cDNA using a PrimeScript™ RT Reagent Kit (Takara Bio Inc., Otsu, Japan). Thereafter, the generated cDNA was subjected to real-time PCR analysis using SYBR Premix Ex Taq (Takara Bio Inc.) with specific primer sets. Four candidates were selected as endogenous references for animal experiments (Pes1, 18S, Gapdh, B2m) and for autopsy cases (PES1, 18S, GAPDH, B2 M), respectively. To evaluate the gene expression stability of these endogenous references, BestKeeper analysis (one of the statistical algorithms for selecting stably expressed reference genes) was performed by RefFinder, which is a user-friendly web-based comprehensive tool developed for evaluating and screening reference genes from extensive experimental data sets (www.leonxie.com/referencegene.php) [24,25]. Because RefFinder uses 2 as the default exponential amplification value, Ct values corrected by their respective calculated amplification efficiencies (from Real-time PCR Miner) using GenEx were used as input data. The sequences of the primers are summarized in Table 1. The expression levels for target transcripts are given as the ratio of the target normalized against each endogenous reference.

Table 1
Sequences of the primers used for real time-PCR.

Transcript	Sequence	Product size (bp)
Mouse		
AQP1	F: 5′-CAATTCACTTGGCCGCAATG-3′	106
	R: 5′-GGTGGCCAGAACGCACAGTA-3′	
AQP3	F: 5′-CTGGATCAAGCTGCCCATCTA-3′	185
	R: 5′-TGACCATGTCCAAGTGTCCAGAG-3′	
Pes1	F: 5′-CTAAGCGCTTGGCCATCA-3′	132
	R: 5′-CAGCATCATCATGGGCTTTC-3′	
18S	F: 5′-TTCTGGCCAACGGTCTAGACAAC-3′	127
	R: 5′-CCAGTGGTCTTGGTGTGCTGA-3′	
Gapdh	F: 5′-TGTGTCCGTCGTGGATCTGA-3′	150
	R: 5′-TTGCTGTTGAAGTCGCAGGAG-3′	
B2m	F: 5′-TGCTACTCGGCGCTTCAGTC-3′	200
	R: 5′-AGGCGGGTGGAACTGTGTTAC-3′	
Human		
AQP1	F: 5′-CAGCCCAAGGACAGTTCAGAGA-3′	112
	R: 5′-GGCTAAGTGCACAGTGGTGGA-3′	
AQP3	F: 5′-CACAGCCGGCATCTTTGCTA-3′	107
	R: 5′-TGGCCAGCACACACACGATA-3′	
PES1	F: 5′-GAGGCTCACAGTCAATGAATCGTC-3′	198
	R: 5′-AAACGTTCGGGCTGCTGTAGA-3′	
18S	F: 5′-ACTCAACACGGGAAACCTCA-3′	123
	R: 5′-AACCAGACAAATCGCTCCAC-3′	
GAPDH	F: 5′-GCACCGTCAAGGCTGAGAAC-3′	138
	R: 5′-TGGTGAAGACGCCAGTGGA-3	
B2M	F: 5′-CGGGCATTCCTGAAGCTGA-3′	194
	R: 5′-GGATGGATGAAACCCAGACACATAG-3′	

F, forward primer; R, reverse primer.