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ORIGINAL ARTICLE

Molecular pathology of pulmonary edema after injury in forensic autopsy cases

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Abstract The lung is vulnerable to trauma; pulmonary edema starts quickly as part of the systemic responses involved in shock. The present study investigated the molecular pathology of posttraumatic alveolar damage and responses involving pulmonary edema in forensic autopsy cases of injury (n=66) compared with acute cardiac death cases (n=13). Intrapulmonary mRNA and immunohistochemical expressions of matrix metalloproteinases (MMPs; MMP-2 and MMP-9), intercellular adhesion molecule-1, claudin-5, and aquaporins (AQPs, AQP-1 and AQP-5) were examined. Subacute injury deaths showed an increase in lung weight similar to that in acute cardiac death, but relative mRNA quantification using the Taqman real-time PCR assay demonstrated different findings among the causes of death; higher expressions were detected for all markers, except for AQP-5 in sharp instrument injury, for MMP-2 in blunt brain injury, and for MMP-9 in non-brain blunt injury, but these expression levels were lower in acute cardiac death. In immunostaining, only MMPs showed differences among the causes of death: MMP-2 expression was evident in most subacute deaths due to blunt brain injury and sharp instrument injury, whereas MMP-9 was intensely positive in those of non-brain blunt injury and sharp instrument injury. These findings suggest significant differences in the mechanism of pulmonary edema among fatal injuries and acute cardiac death, especially between blunt and sharp instrument injury. Systematic analysis of gene expressions using real-time PCR in combination with immunohistochemistry may be useful in evaluating pulmonary damage and responses after injury in death investigations, especially in connection with posttraumatic shock.

Keywords Forensic molecular pathology · Pulmonary edema · Injury · Aquaporin · Matrix metalloproteinase · Claudin-5 · Intercellular adhesion molecule-1

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Introduction

In forensic pathology, pulmonary edema is a major finding, which is commonly involved in most traumatic deaths, especially in connection with shock of various types. It is theoretically classified into four main categories on the basis of the initiating mechanism: (a) imbalance of Starling forces (e.g., cardiogenic/hemodynamic origin), (b) altered alveolar-capillary membrane permeability, (c) lymphatic insufficiency, and (d) unknown or incompletely understood (e.g., neurogenic origin) [1]. However, it is difficult to explain the pathogenesis of pulmonary edema by means of macro/micromorphological evidence alone. In molecular biology, spectrums of alveolar-capillary components are involved in maintaining structures. Matrix metalloproteinases (MMPs), representing a group of neutral proteinases involved in the breakdown of most components of the extracellular matrix (ECM), participate in tissue remodeling associated with a variety of physiological and pathological conditions [2]. MMP-2 and MMP-9, also called gelatinases, which degrade almost all basement membrane constituents, are involved in the repair of alveolar epithelia [3, 4].

Intercellular adhesion molecule-1 (ICAM-1) is an adhesion molecule expressed in both alveolar epithelia and vascular endothelia [5] and plays important roles in neutrophil recruitment and trafficking into the lung [6]. Claudin-5 (CLDN-5) is a critical component of endothelial tight junctions that control the pericellular permeability of ions and molecules [7, 8]. Aquaporins (AQPs) are a family of small integral membrane proteins involved in water homeostasis; they increase cell membrane permeability for water and offer transport pathways for excess fluid, among which major contributors are AQP-1 and AQP-5 in the lung [9–11]. With consideration of the recent advances in forensic molecular pathology [12–19], systematic analysis of these biomarkers may be useful in investigating the pathogenesis of alveolar damage involving pulmonary edema with regard to the cause of death.

The present study analyzed intrapulmonary gene expressions and immunohistochemistries of MMP-2, MMP-9, ICAM-1, CLDN-5, AQP-1, and AQP-5 to investigate the pathogenesis of alveolar damage involving pulmonary edema with special regard to fatal injury in forensic autopsy cases for possible practical application.

Materials and methods

Materials

Medicolegal autopsy cases (n=79; within 48 h postmortem with a median of 21 h; survival time, <0.5–48 h with a median of <0.5 h) at our institute were examined. Cases comprised 66 males and 13 females, between 20 and 91 (median, 58) years of age. Causes of death were determined on the basis of autopsy examination, including macromorphological, histological, and biochemical analyses, as follows: acute cardiac death (ACD, n=13), blunt brain injury (n=23: acute death, n=11; subacute death, n=12), non-brain blunt injury (n=22), including acute death (n=10: chest trauma involving cardiac/aortic rupture and pulmonary contusions/lacerations with massive hemothorax, n=5; abdominal trauma involving hepatic/renal lacerations, n=2; and multiple trauma involving the thoracic and abdominal viscera, n=3) and subacute death (n=12: traumatic shock due to massive subcutaneous hemorrhages and multiple fractures without other visceral injury, n=6; hemoperitoneum due to abdominal visceral injury, n=2; pulmonary contusions/lacerations, n=1; gastric rupture, n=1; abdominal aortic rupture, n=1; and mesenteric lacerations, n=1), and sharp instrument injury (n=21: acute death, n=10; subacute death, n=11); cases with any preexisting pulmonary pathologies were excluded. Details are shown in Table 1. In the present study, clearly accountable cases without any other complications that may have contributed to the death, supported by well-established circumstantial evidence, were collected. ACD cases included those due to acute ischemic heart disease with or without myocardial infarction without any evidence of cause of death other than a cardiac attack. Postmortem interval was defined as the estimated time from death to autopsy, and survival time was the estimated period from the onset of fatal insult to death; these were estimated on the basis of autopsy findings and circumstantial evidence recorded in autopsy documents. Acute and subacute death cases were those of survival time of <30 min and 3 h–2 days, respectively [20, 21]. Sample collections and analyses described below were performed within the framework of our routine casework, following the autopsy guidelines (2009) and ethical guidelines (1997 and 2003) of the Japanese Society of Legal Medicine, approved by our institutional ethics committee.

Methods

Gene expression

Tissue specimens were taken from consistent sites in the upper lobe of the left lung (anterior segment) at autopsy, then immediately submerged in 1 ml of RNA stabilization solution (RNAlater™, Ambion, Austin). In pulmonary injury cases, lung specimens distant from the primary injury site were examined. Total RNA was isolated with ISOGEN (Nippon Gene, Toyama) according to the manufacturer's instructions. cDNA copies of total RNA were obtained using a High Capacity RNA-to-cDNA kit (Applied Biosystems Japan, Ltd.). Nine-microliter samples of total RNA were subjected to 20-μl reactions (containing 10.0 μl of 2× RT buffer and 1.0 μl of 20× RT enzyme mix). Conditions for reverse transcription were 37 °C for 60 min and 95 °C for 5 min. PCR primers and probes (TaqMan Gene Expression Assay) were purchased from Applied Biosystems, Inc. (Carlsbad, CA, USA). A total of 20 μl, containing 10.0 μl of TaqMan Gene Expression Master Mix (2×), 1.0 μl of TaqMan Gene Expression Assay (20×), 2 μl of cDNA, and 7 μl of H_2O, was applied to a fast 96-well reaction plate (0.1 ml). RT-PCR was performed with primers for human MMP-2 (Taqman assay ID: Hs01548727_m1), MMP-9 (Taqman assay ID: Hs00234579_m1), ICAM-1 (Taqman assay ID: Hs00164932_m1), CLDN-5 (Taqman assay ID: Hs01561351_m1), AQP-1 (Taqman assay ID: Hs00166067_m1), and AQP-5 (Taqman assay ID: Hs00387048_m1) using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) [22]. Preliminary screening of endogenous references (housekeeping genes) in serial autopsied human lung tissue specimens showed high and equivalent correlations (r=0.97–0.98, p <0.0001) among beta-2-microglobulin (B2M, Taqman assay ID: Hs99999907_m1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH,

Table 1 Case profiles (n=79)

Cause of death	n	Male/Female	Age, years (median)	Survival time, h (median)	PMI, h (median)	CLW/H ratio, g/cm mean ± SEM
Acute cardiac death	13	13/0	43–67 (61)	<0.5–3 (<0.5)	14–46 (23)	7.18±0.45
Brain injury						
Acute death	11	8/3	29–71 (39)	<0.5	13–35 (18)	5.04±0.56
Subacute death	12	8/4	35–86 (56)	3–48 (8)	7–38 (23)	7.20±0.88
Non-brain injury						
Acute death	10	9/1	45–69 (58)	<0.5	11–39 (22)	4.11±0.27
Subacute death	12	10/2	20–90 (68)	6–12 (6)	11–34 (20)	6.54±0.91
Sharp instrument injury						
Acute death	10	9/1	29/65 (50)	<0.5	13–25 (15)	4.14±0.41
Subacute death	11	9/2	46–91 (61)	3–6 (3)	9–35 (19)	5.61±0.63
Total	79	66/13	20–91 (58)	<0.5–48 (<0.5)	7–46 (21)	5.82±0.27

CLW/H ratio showed significant differences among the causes of death on ANOVA (p<0.05). All acute injury death groups had lower CLW/H ratios. Significantly higher CLW/H ratios were detected in subacute blunt brain injury death and acute cardiac deaths than in acute deaths due to non-brain blunt injury and sharp instrument injury on Tukey tests (p<0.05)

PMI postmortem interval, *CLW/H* combined lung weight/height, *SEM* standard error of mean

Taqman assay ID: Hs99999905_m1), and beta-actin (Taqman assay ID: Hs99999903_m1), as shown in a previous study [15]; thus, B2M was used as an endogenous reference in the present study. Thermal cycling conditions included one cycle at 50 °C for 2 min, one cycle at 95 °C for 10 min, followed by 40 cycles of amplification at 95 °C for 15 s and 60 °C for 1 min. The threshold cycle (Ct) was calculated by the instrument software (7500 Fast system ver. 1.3.1) automatically (threshold value at 0.2). The expression levels for mRNA transcripts are described as the ratios of the targets normalized to the endogenous reference (B2M), using the $2^{-\Delta\Delta Ct}$ method [23], and as the ratios for fold change relative to the calibrator, which was from a case of peracute death due to open brain injury (35-year-old male; 18 h postmortem). Negative controls were included in each run. For each assay, a fivefold serial dilution of the calibrator case was used to create a standard curve and calculate amplification efficiency using the equation: efficiency=$10^{(-1/\text{slope})}-1$ [23].

Immunostaining

Serial sections (5-μm thick) were prepared from routine formalin-fixed paraffin-embedded lung tissue specimens and were used for routine hematoxylin–eosin and immunostaining. Rabbit polyclonal anti-MMP-2 antibody (Abcam, Cambridge, code ab79781, diluted 200-fold), rabbit polyclonal anti-MMP-9 antibody (Abcam, Cambridge, code ab38898, diluted 5000-fold), mouse monoclonal anti-ICAM-1 antibody (Santa Cruz Biotechnology, Santa Cruz, code sc-8439, diluted 200-fold), rabbit polyclonal anti-CLDN-5 antibody (Abcam, Cambridge, code ab53765, diluted 500-fold), mouse monoclonal anti-AQP-1 antibody (Abcam, Cambridge, code ab9566, diluted 200-fold), and rabbit monoclonal anti-AQP-5 antibody (Abcam, Cambridge, code ab92320, diluted 200-fold) were used. Following overnight incubation with the primary antibodies described above at room temperature, immunoreactions were visualized by the polymer method (ChemMate Envision, Dako, Tokyo, code k5027), and color was developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB liquid system, Dako, Tokyo, code k3466) according to the manufacturer's instructions (counterstaining with hematoxylin).

Statistics

Regression equation analysis was used to examine the relationship between pairs of parameters. One-way analysis of variance (ANOVA) with repeated measures, followed by post hoc Tukey tests, was used for comparisons of multiple groups. These analyses were carried out using SPSS 16.0 statistical package (SPSS Inc., Chicago, IL, USA) and StatView (version 5.0; SAS Institute Inc., Cornelius, NC, USA). A p value less than 0.05 was considered significant. The line in each box represents the median, and the lines outside each box represent the 90 % confidence interval in Fig. 1.

Results

Gene expression

All assays for targets and the endogenous reference (B2M) to estimate amplification efficiency showed excellent correlations of duplicates with R^2>0.99. The efficiencies of target

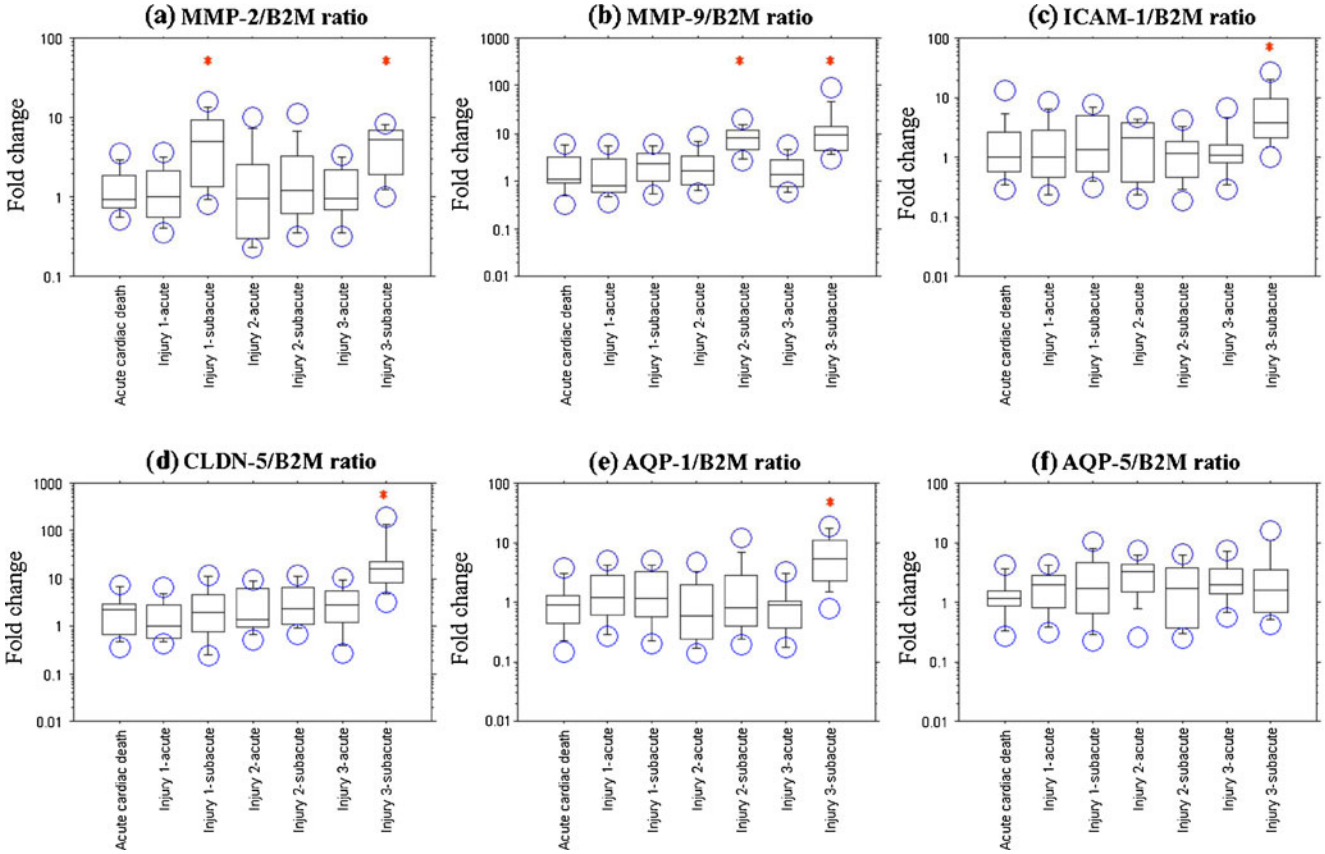

Fig. 1 MMP-2 (**a**), MMP-9 (**b**), ICAM-1 (**c**), CLDN-5 (**d**), AQP-1 (**e**), and AQP-5 (**f**) mRNA/B2M mRNA ratios with regard to the cause of death. **a** Significant differences were detected on ANOVA ($p<0.05$): the *asterisk* indicates significantly higher ($p<0.05$), injury 1-subacute and injury 3-subacute vs. injury 1 and 3-acute as well as acute cardiac death (ACD) on Tukey tests. **b** Significant differences were detected on ANOVA ($p<0.05$): the *asterisk* indicates significantly higher ($p<0.05$), injury 2-subacute vs. injury 1 and 2-acute as well as ACD, injury 3-subacute vs. injury 1, 2 and 3-acute as well as ACD on Tukey tests. **c** Significant differences were detected on ANOVA ($p<0.05$): the *asterisk* indicates significantly higher ($p<0.05$), injury 3-subacute vs. injury 1 and 3-acute, injury 2-subacute as well as ACD on Tukey tests. **d** and **e** Significant differences were detected on ANOVA ($p<0.05$): the *asterisk* indicates significantly higher ($p<0.05$), injury 3-subacute vs. others on Tukey tests. **f** There were no significant differences among groups on ANOVA ($p>0.05$). Injury 1, blunt brain injury; injury 2, non-brain blunt injury; and injury 3, sharp instrument injury

amplification (MMP-2, 97.41 %; MMP-9, 100.83 %; ICAM-1, 100.40 %; CLDN-5, 97.78 %; AQP-1, 99.25 %; AQP-5, 99.93 %) were close to the endogenous control (B2M, 97.07 %), which were consistent with the declaration of Applied Biosystems (100±10 %). There were no gender-related differences, or age or postmortem interval dependences in these mRNA expression levels ($p>0.05$), where postmortem intervals showed no significant differences among all causes of death ($p>0.05$).

MMP-2 mRNA expression levels were significantly higher in subacute deaths due to blunt brain injury and sharp instrument injury than in acute death groups of these injuries as well as ACD (control group) (Fig. 1a). MMP-9 mRNA expression levels were higher in subacute non-brain blunt injury death than in acute blunt injury death groups and ACD, and higher in subacute sharp instrument injury death than in all acute injury death groups and ACD (Fig. 1b). ICAM-1 mRNA expression levels were significantly higher in subacute sharp instrument injury death than in acute deaths due to blunt brain injury, sharp instrument injury, subacute non-brain blunt injury death, and ACD (Fig. 1c). CLDN-5 and AQP-1 mRNA expression levels were significantly higher in subacute sharp instrument injury death than in other groups (Fig. 1d, e). There were no significant differences in AQP-5 mRNA expression among all groups (Fig. 1f). These findings were independent of the combined lung weight/height ratio, which was generally lower in all acute injury death groups, partly showing significant differences: the ratios were lower in acute deaths due to non-brain blunt injury and sharp instrument injury than in subacute blunt brain injury death and ACD (Table 1).

Immunostaining

Immunostaining showed substantial interindividual variations in each group; however, the following differences were

detected. MMP-2 was detected mainly in epithelial cells, and some interstitial and intra-alveolar inflammatory cells also showed positivity. MMP-2 was more intensely positive in most subacute deaths due to blunt brain injury and sharp instrument injury (Fig. 2a, b). MMP-9 was detected in interstitial cells, mainly in macrophages, and showed more intense staining in subacute deaths due to non-brain blunt injury and sharp instrument injury (Fig. 2c, d). ICAM-1 was detected diffusely in alveolar walls, predominantly along the basement membranes and in capillary endothelia, showing no significant differences in distribution or intensity among the causes of death, including sharp instrument injury (Fig. 2e, f). CLDN-5 was strongly positive in bronchial epithelia and capillary endothelia, and no significant differences in distribution or intensity were detected among the causes of death (Fig. 2g, h).

AQP-1 immunostaining was clearly detected in all vascular endothelial cells but not in bronchial epithelia, and no significant differences in distribution or intensity were detected among the causes of death (Fig. 2i, j). AQP-5 positivity was mainly demonstrated in a linear pattern in type I alveolar epithelial cells, in bronchial epithelial cells, and sporadically in interstitial macrophages (Fig. 2k, l).

Discussion

The essential tasks of forensic pathology involve investigating the cause and process of death, especially in traumatic and unexpected sudden deaths, largely including unwitnessed deaths [12]. In forensic practice, however, the interpretation of trauma-specific damage to life-supporting organs, such as posttraumatic pulmonary edema, may be obstructed in conventional pathological observations of traumatic shock without apparent structural damage; furthermore, shock is a functional alteration of the microcirculation with poor or nonspecific morphological findings [24]. Previous studies have suggested that postmortem biochemistry can detect systemic functional alterations depending on survival time in these fatalities [25–28]. In addition, possible application of relative mRNA quantification has been reported for molecular biological analysis of the pathophysiology of injury death [12, 13].

In the present study, subacute injury death cases had higher lung weights than those of acute death cases, representing posttraumatic shock as previously reported [29], but there were no characteristic findings regarding the types of injury, including blunt brain injury, non-brain injury, and sharp instrument injury, or that of ACD. However, in

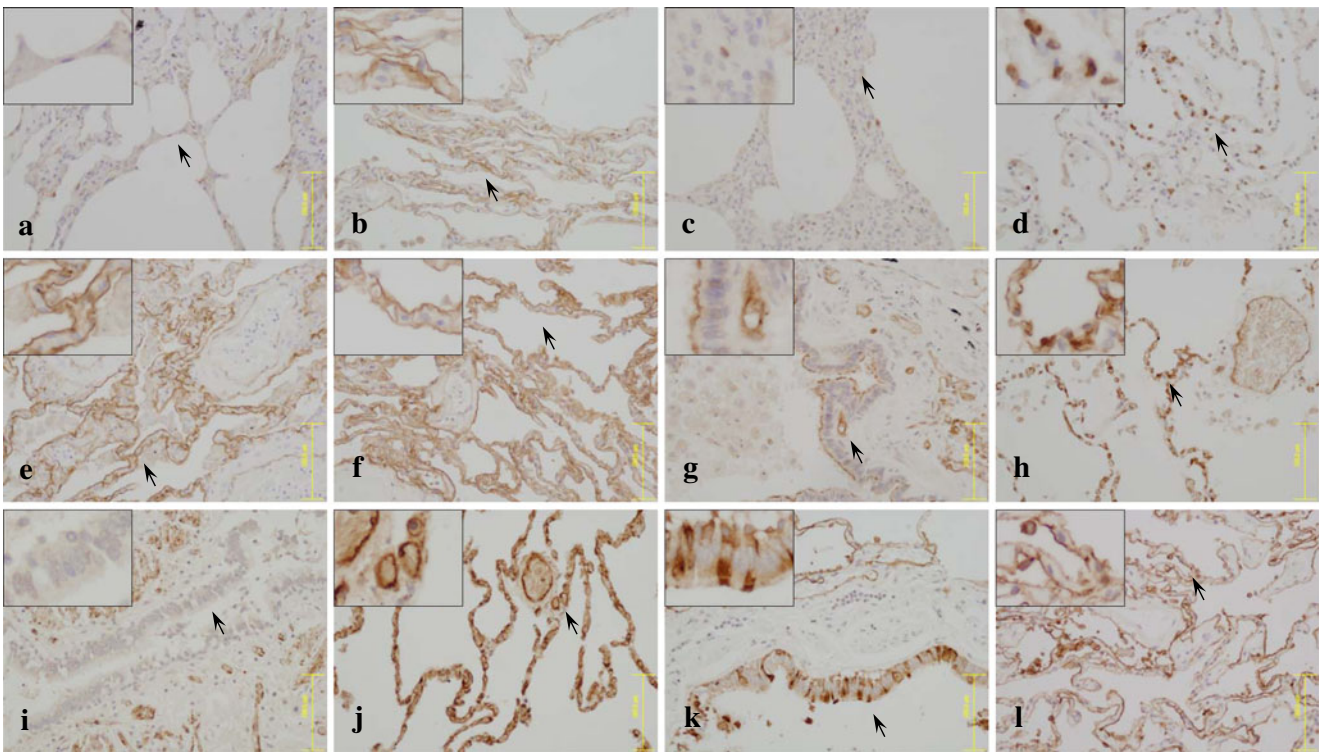

Fig. 2 Immunostaining of MMP-2 (**a** and **b**), MMP-9 (**c** and **d**), ICAM-1 (**e** and **f**), CLDN-5 (**g** and **h**), AQP-1 (**i** and **j**), and AQP-5 (**k** and **l**) in the lung. Acute death due to sharp instrument injury (**a**, **c**, and **e**), a 53-year-old male, survival time <0.5 h, 19 h postmortem. Subacute death due to sharp instrument injury (**b**, **d**, and **f**), a 62-year-old male, survival time 6 h, 24 h postmortem. Acute cardiac death (**g**–**l**), a 63-year-old male, survival time <0.5 h, 24 h postmortem. *Arrows* indicate typical findings, which are enlarged in *insets*

quantitative analyses of intrapulmonary gene expressions of MMP-2, MMP-9, ICAM-1, CLDN-5, AQP-1, and AQP-5, injury type-specific findings were detected in subacute deaths, demonstrating higher expressions of all markers except for AQP-5 in sharp instrument injury, MMP-2 in blunt brain injury, and MMP-9 in non-brain blunt injury. These findings suggest the severest alveolar damage due to fatal circulating blood loss in sharp instrument injury as well as partial differences between brain and non-brain blunt injuries, as discussed below. In contrast, all these biomarkers remained low in ACD cases, which also had higher lung weights, suggesting the major contribution of the hemodynamic factor to development of pulmonary edema. These findings in subacute injury deaths were partly validated by immunostaining of MMPs, which showed intense positivity for MMP-2 in blunt brain injury and sharp instrument injury as well as for MMP-9 in non-brain blunt injury and sharp instrument injury, although a small hemilateral portion of the lung was analyzed by means of mRNA quantification in each case in the present study; however, substantial overlapping among the causes of death suggest that there are continuums of pulmonary damage after trauma. These findings showed no relationship with lung weights at autopsy, possibly due to other factors contributing to increased lung weight, including gender and age of subjects [29]. Multiple sampling and site-to-site comparisons may be needed to establish the findings in individual case studies in practical application [15].

In sharp instrument injury, upregulated mRNA expressions of intrapulmonary MMPs, ICAM-1, CLDN-5, and AQP-1 in subacute death suggest damage to whole alveolar structures, involving degradation of ECM components of the basement membrane and interstitium by activated MMPs (gelatinases) as well as altered permeability of alveolar epithelia and vascular endothelia, accompanied by activated ICAM-1, CLDN-5, and AQP-1. These findings were similar to those of hyperthermia (heat stroke), which involved multiple organ dysfunction syndrome (unpublished data). Experimental studies demonstrated increased ICAM-1 expression in acute lung injury [30], and elevated plasma levels of soluble ICAM-1 were associated with poor clinical outcomes in patients with acute lung injury [31]; thus, increased ICAM-1 mRNA levels in subacute sharp instrument injury deaths may indicate severe lung epithelial and endothelial injury in the death process [32]. Furthermore, breaching of endothelial barriers is a key event in the development of pulmonary edema during pulmonary injury. The loss of CLDN-5 in tight junctions can lead to disassembly of adhesive structures, endothelial barrier dysfunction, and ultimately increased vascular permeability [33]; however, upregulation of CLDN-5 expression in leaky rat lung endothelial cells can help restore paracellular barrier function [34]. Although the integrity of tight junctions was not examined in the present study, upregulated CLDN-5 may suggest a compensatory response to mend junctional complexes and restore barrier function. Of note, characteristic findings were detected for AQPs, which play important roles in regulating water homeostasis [10]; higher expression of AQP-1, but not AQP-5, was detected in subacute sharp instrument injury deaths, indicating an improvement in alveolar fluid reabsorption capacity, since an elevation of AQP-1 expression could prevent pulmonary edema in an acute lung injury rat model experiment [35], while AQP-5 may have different roles, including a response to alveolar hypoxia [22].

Differences in MMP-2 and MMP-9 expressions between blunt brain and non-brain injury indicate varied contributions of these gelatinases to alveolar damage in degrading ECM components of the basement membrane and interstitium despite their structural similarities, depending on the types of injury [4, 36]. MMP-2 may be involved in neurogenic pulmonary edema following brain injury, while MMP-9 may have another role in pulmonary edema in fatal blunt injury, independent of hypoxia-induced alterations, changes in pulmonary surfactant expressions, or activation of the cytokine system, which were detected irrespective of the type of injury [13, 15]. Further investigation is needed to elucidate these phenomena.

In the present study, the immunohistochemistry of these biomarkers did not detect evident differences in distribution or intensity among the types of injury, except for MMPs. These findings may be attributed to the lower sensitivity of immunostaining in detecting changes in gene products than that with quantitative analyses of gene expressions using real-time PCR [13]; however, the combined use of both procedures is useful to investigate the molecular pathology of the death process involving changes in gene expressions and gene products. For this purpose, quantitative mRNA analyses have been widely used in experimental models as well as human materials in forensic pathology [13, 15–18, 37, 38]. Such autopsy data are especially important for understanding and investigating systemic pathophysiological changes involved in human death. The use of multiple reference genes was suggested with consideration of possible variations of postmortem RNA degradation [14]. In the present study, B2M was used as the reference marker on the basis of a previous study using human postmortem lung tissue specimens, which showed good correlations among B2M, GAPDH, and beta-actin [15]. Further cooperation and investigation will be needed to establish the efficacy of individual endogenous references in practical application.

In conclusion, the present study demonstrated different patterns of pulmonary alveolar damage and biological responses with regard to the types of injury and survival time; there was a marked difference between blunt and sharp instrument injury as well as a partial difference between blunt brain and non-brain injury. Systematic analysis of

gene expressions using real-time PCR, combined with immunohistochemistry, may be useful to investigate the pathogenesis of alveolar damage involving pulmonary edema in forensic death investigation, especially in connection with posttraumatic shock.

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