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Immunohistochemistry of connexin43 and zonula occludens-1 in the myocardium as markers of early ischemia in autopsy material

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Summary. Immunohistochemistry of the terminal complement complex (C5b-9) and fibronectin (FN) is useful to detect myocardial ischemia preceding necrosis in the postmortem diagnosis of sudden cardiac death. The present immunohistochemical study examined connexin43 (Cx43) and zonula occludens-1 (ZO-1) as markers of early myocardial ischemia in addition to the above-mentioned markers, using forensic autopsy cases of acute deaths due to myocardial infarction (MI, n=15) and acute ischemic heart disease (AIHD) without apparent myocardial necrosis (n=8), compared with those of acute mechanical asphyxiation (As, n=24) and drowning (D, n=10) as controls. Immunopositivities of each marker in the myocardium were semi-quantitatively graded by scoring. ZO-1, C5b-9 and FN were detected in the myocardial cytoplasm, whereas Cx43 and non-phosphorylated (np) Cx43 showed varied localizations at the intercalated disc, in the cytoplasm and along the lateral cell border. ZO-1 and FN showed a tendency to be detected more intensely in MI and IHD than in As and D. C5b-9 showed specific staining at the site of ischemia in MI (n=10/15) and AIHD (n=6/8), while the distribution of npCx43 was different in most cases of MI (n=14/15) and AIHD (n=5/8), compared with As and D; npCx43 positivity score was higher in the cytoplasm than at the intercalated disc, indicating redistribution due to myocardial ischemia. Such findings were detected in a few cases of As (n=3/24). These findings suggest that the combination of npCx43 and C5b-9 immunohistochemistry is useful for detecting early lesions of myocardial ischemia in sudden cardiac death.

Key words: Forensic pathology, Myocardial ischemia, Immunohistochemistry, Connexin43, Zonula occludens-1

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Introduction

Autopsy diagnosis of sudden cardiac death (SCD), involving differentiation from mechanical asphyxiation or drowning, is a major issue in forensic and clinical pathology, since detection of early myocardial changes due to ischemia is often difficult by conventional procedures (Brinkmann et al., 1993; Ortmann et al., 2000; Ribeiro-Silva et al., 2002; Campobasso et al., 2008). Previous studies have shown that immunohistochemistry of the terminal complement complex (C5b-9) and fibronectin (FN) is useful to visualize early ischemic myocardial damage after the onset of symptoms (Thomsen and Held, 1995; Hu et al., 1996; Kossmehl et al., 2005; Fracasso et al., 2011a-c). C5b-9 is one of the complement system proteins involved in cytolysis in the inflammatory process or immune response, and is detected in ischemic areas of the myocardium by immunostaining around 30-40 min after the beginning of ischemia (Thomsen and Held, 1995; Ferreira et al., 1998; Yasojima et al., 1998; Busche and Stahl, 2010; Fracasso et al., 2011a-c). FN is an extracellular matrix protein that plays an important role in repairing injured tissues and can also be detected immunohistochemically in the myocardium after ischemia (Hu et al., 1996; Trial et al., 2004; Kossmehl et

al., 2005). Previous studies showed that C5b-9 was specific for detecting the myocardial ischemic area; however, the positive rate was not high (about 40%) (Fracasso et al., 2011a-c).

Recently, connexin43 (Cx43) and zonula occludens-1 (ZO-1) were studied in animal models of acute myocardial ischemia and hypoxia using immunohistochemistry (Beardslee et al., 2000; Turner et al., 2004; Matsushita et al., 2006). Cx43 is a phosphoprotein, which is a major gap junction protein in human cardiomyocytes of the ventricular myocardium, and regulates gap-junctional intercellular communication (Laird, 2006). In the normal heart, Cx43 is phosphorylated and localized at the intercalated discs (ID); however, stimuli such as ischemia, hypoxia and hypothermia induce non-phosphorylation and redistribution to the cytoplasm (CP) and/or lateral cell border (LCB) of cardiomyocytes (Beardslee et al., 2000; Barker, 2002; Turner et al., 2004; Sasano et al., 2007; Hesketh et al., 2010). Decreased Cx43 and increased non-phosphorylated (np) Cx43 at ID were detected 15 min after the beginning of ischemia and became more evident with prolonged ischemia (Beardslee et al., 2000). In hypoxia, npCx43 immunoreactivity at ID increased after 20 min (Matsushita et al., 2006); the response was slower than in ischemia. ZO-1, which was originally identified as a tight junction protein, co-localized with Cx43 at ID, plays a role in anchoring Cx43 to the cytoskeleton (Gonzalez-Mariscal et al., 2000; Palatinus et al., 2011). Animal studies have shown that ZO-1 binding to Cx43 regulates gap junction size and stability and does not change its localization at ID in hypoxic conditions (Barker 2002; Hunter et al., 2005; Matsushita et al., 2006; Laird 2006). These observations suggest that Cx43 and ZO-1 are potential markers of early myocardial ischemia. Recent reports presented imaging-based quantitative methods to analyze the spatial distribution of Cx43 in living and fixed myocytes in animal models (Lasher et al., 2009; Lackey et al., 2011). A previous study using explanted human hearts from transplant patients demonstrated increased ZO-1 with a negative correlation to Cx43 as well as increased co-localization of Cx43 with ZO-1 at ID in the failing ventricle, including ischemic myopathy (Bruce et al., 2008); however, there have been no published data on autopsied material.

From the observations described above, the present study evaluated Cx43 and ZO-1 as immunohistochemical markers of early myocardial ischemia by semi-quantitative scoring, compared with C5b-9 and FN, using forensic autopsy cases of SCD due to myocardial infarction (MI) and acute ischemic heart disease (AIHD) without apparent necrosis established by autopsy findings and circumstantial evidence.

Materials and methods

Materials

Serial forensic autopsy cases at our institute (n=57: 43 males and 14 females; 19–87 years (median, 63.5) of age; acute death with survival within 30 min; postmortem time <48h) were examined. These cases included SCD as a result of ischemic heart disease due to coronary lesions (n=15), which was classified into myocardial infarction (MI), comprising cases without pathological evidence of old infarction (acute and recurrent MI: AMI, n=8 and RMI, n=7, respectively), and cases without apparent myocardial necrosis (AIHD; n=8). In these SCD cases, ischemic myocardial lesions were identified in anterior and interventricular walls of the left ventricles in accordance with the respective coronary lesions. Acute mechanical asphyxiation (As; n=24), including hanging (n=11) and strangulation (n=13), as well as drowning (D; n=10: fresh water, n=5 and salt water, n=5) were used as controls. Case profiles are shown in Table 1. For these groups, typically representative cases without significant complications, where the causes of death were established on the basis of autopsy findings and circumstantial evidence, excluding other causes of death and hospital deaths after critical medical care, were collected; extensive

Table 1. Case profiles (n=57).

Cause of death	n	Male/Female	Age (years) Range (median)	Postmortem time[a] (hours) Range (median)
Myocardial infarction	15	14/1	39-85 (62)	16-34 (22)
Acute myocardial infarction	8	7/1	39-85 (58.5)	18-30 (26)
Recurrent myocardial infarction	7	7/0	44-82 (65)	16-34 (21)
Acute ischemic heart disease	8	8/0	38-68 (51.5)	11-35 (21.5)
Mechanical asphyxiation	24	14/10	26-87 (64.5)	9-46 (23.5)
Hanging	11	9/2	26/75 (61)	12-46 (30)
Strangulation	13	5/8	36-87 (66)	9-37 (21)
Drowning	10	7/3	19-79 (60)	7-38 (23.5)
Salt water	5	3/2	19-79 (50)	7-37 (23)
Fresh water	5	4/1	51-74 (61)	20-38 (29)
Total	57	43/14	19-87 (63.5)	7-46 (23.5)

[a]: Estimated interval from death to autopsy.

exclusions in particular were involved in the diagnosis of AIHD. Postmortem time was defined as the estimated time from death to autopsy and survival time was the estimated period from the onset of the fatal insult to death. There was no significant difference in subject age or estimated postmortem interval among cause-of-death groups.

Methods

Immunostaining

Routine heart tissue specimens (anterior wall of the left ventricle, LV; free wall of the right ventricle, RV; and interventricular septum, IS) were taken during autopsy and immediately fixed in 10% formaldehyde in phosphate-buffered saline (PBS) (pH 7.4), embedded in paraffin, and then sectioned at a thickness of 4-μm. Serial sections were used for hematoxylin-eosin (H-E) and azan staining to evaluate the quality of fixed tissue specimens, followed by immunostaining. Primary antibodies were rabbit polyclonal anti-Cx43 antibody (Sigma-Aldrich, St. Louis, MO, code C6219, diluted 400-fold), mouse monoclonal anti-npCx43 antibody (Life Technologies Japan, Tokyo, Japan, code 13-8300, clone CX-1B1, isotype IgG1, diluted 100-fold), mouse monoclonal anti-ZO-1 antibody (Life Technologies Japan, Tokyo, Japan, code 339100, clone ZO1-1A12, isotype IgG1, diluted 100-fold), mouse monoclonal anti-C5b-9 antibody (Dako, Tokyo, Japan, code M0777, clone aE11, isotype IgG2a, diluted 25-fold) and rabbit polyclonal anti-FN antibody (Dako, Tokyo, Japan, code A0245, diluted 800-fold). Rabbit polyclonal anti-Cx43 antibody detects phosphorylated (p) Cx43 and npCx43, and mouse monoclonal anti-npCx43 antibody identifies npCx43 specifically (Matsushita et al., 2006). Antigen retrieval techniques were as follows: microwave (5 min) for Cx43, npCx43 and C5b-9, and 0.1% proteinase K (15 min) for C5b-9. Tissue sections were blocked by 3% hydrogen peroxidase (H_2O_2) for 15 min. Following overnight incubation with the primary antibody described above at room temperature, immunoreactions were visualized using the polymer method (ChemMate Envision; Dako, Tokyo, Japan, code k5027), followed by color development with 3,3'-diaminobenzidine tetrahydrochloride (DAB liquid system; Dako, Tokyo, Japan, code k3466), according to the manufacturer's instructions (counterstaining with hematoxylin). For a control study to confirm the specificity of immunostaining, normal rabbit serum or mouse IgG, and PBS were substituted for the primary antibody.

Scoring of immunopositivity

Immunopositivities of each marker in the myocardium were semi-quantitatively graded by scoring (Table 2) (Ortmann et al., 2000). Cx43 and npCx43 positivity at ID and along LCB: score 0, negative; score 1, thin linear or intermittent positivity; score 2, dense but intermittent positivity; score 3, dense band-like positivity. Cx43 and npCx43 positivity in CP: score 0, negative; score 1, scattered positivity; score 2, patchy positivity (intermediated between score 1 and 3); score 3, diffuse positivity. The redistribution grade (Δ) of Cx43 and npCx43 from ID to CP or from ID to LCB was calculated as the difference between the respective scores: Δ(CP-ID)=score in CP – score at ID and Δ(LCB-ID)=score along LCB - score at ID, respectively. The sum total score was also calculated: Σ=score at ID + score along LCB + score in CP. ZO-1 positivity in CP: score 0, negative; score 1, weak and patchy positivity; score 2, dense but patchy positivity; score 3, totally dense positivity. C5b-9 positivity in CP: score 0, negative; score 1, single cell positivity; score 2, cell group positivity; score 3, diffuse positivity. FN positivity in CP: score 0, negative; score 1, focal weak positivity; score 2, diffusely weak and partially strong positivity; score 3, totally strong positivity.

Statistics

The Mann-Whitney U test was used to compare the investigation groups and the Tukey test was used to compare multiple groups, including cases of negative positivity (score 0). A p value less than 0.05 was considered significant. These analyses were carried out

Table 2. Semi-quantitative scoring of immunopositivity.

Score	Cx43 and npCx43		ZO-1	C5b-9	FN
	ID and LCB	CP	CP	CP	CP
0	negative	negative	negative	negative	negative
1	Thin linear or intermittent positivity	Scattered positivity	Weak and patchy positivity	Single cell positivity	Focal weak positivity
2	Dense but intermittent positivity	Patchy positivity (intermediate between score 1 and 3)	Dense and patchy positivity	Cell group positivity	Diffuse weak and partially strong positivity
3	Dense band-like positivity	Diffuse positivity	Totally dense positivity	Diffuse positivity	Totally strong positivity

Cx43, connexin43; npCx43, non-phosphorylated connexin43; ZO-1, zonula occludens-1; C5b-9, the terminal complement complex; FN, fibronectin; ID, intercalated disc; LCB, lateral cell border; CP, cytoplasm.

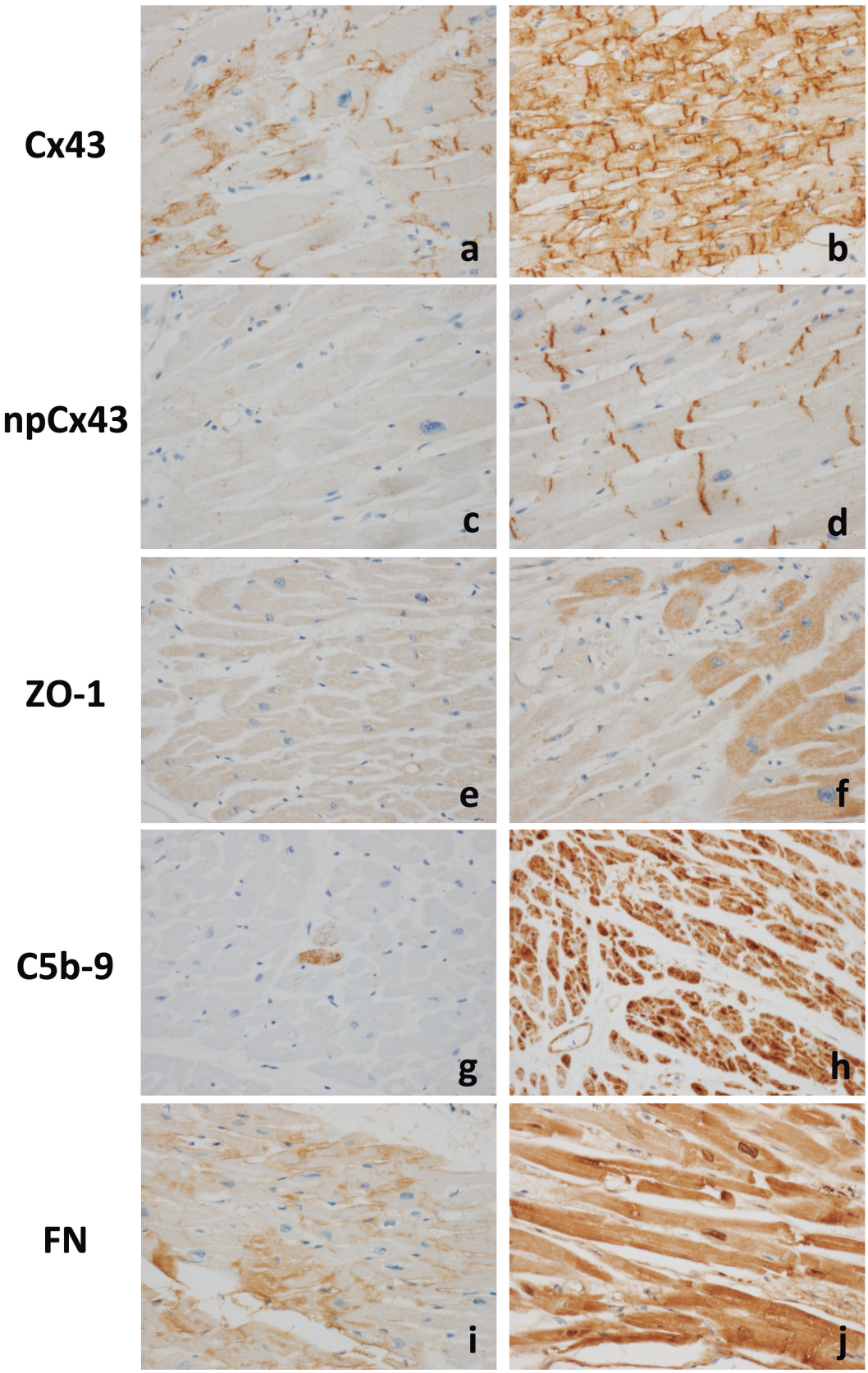

Fig. 1. Comparisons of immunohistochemical staining of connexin 43 (Cx43; **a and b**), non-phosphorylated Cx43 (npCx43; **c and d**), zonula occludens-1 (ZO-1; **e and f**), the terminal complement complex (C5b-9; **g and h**) and fibronectin (FN; **i and j**) in the myocardium. **a, c and f**. Recurrent myocardial infarction, an 81-year-old man, 19 h postmortem. In each figure, infarcted zone is located on the right side. **b**. Hanging, a 66-year-old man, 33 h postmortem. **d.** Hanging, a 35-year-old man, 23 h postmortem. **e.** Hanging, a 65-year-old man, 32 h postmortem. **g.** Acute ischemic heart disease, a 38-year-old man, 11 h postmortem. **h.** Recurrent myocardial infarction, a 44-year-old man, 34 h postmortem. **i.** Hanging, a 75-year-old man, 30 h postmortem. **j.** Acute ischemic heart disease, a 40-year-old man, 26 h postmortem. **a**. Score 2 at intercalated disc (ID), score 1 along lateral cell border (LCB) and score 1 in cytoplasm (CP). **b.** Score 3 at ID, score 3 along LCB and score 2 in CP. **c**. Score 1 at ID, score 0 along LCB and score 1 in CP. **d.** Score 3 at ID, score 0 along LCB and score 0 in CP. **e.** Score 1 in CP. f. Score 3 in CP. g. Score 1 in CP. **h.** Score 3 in CP. **i.** Score 1 in CP. **j.** Score 1 in CP. x 400

using the SPSS 15.0 statistical package (SPSS Inc., Chicago, IL, USA) and Stat View (Version 5.0; SAS Institute Inc., Cornelius, NC, USA).

Results

Immunohistochemical distribution

Immunohistochemical distribution of each marker is shown in Fig. 1 and is summarized in Table 3. C5b-9 was detected in the myocardial cytoplasm of LV and/or IS at the site of ischemia in more than half of all cases of MI (n=10/15; 66.7%) and AIHD (n=6/8; 75%), showing different distributions in LV and/or IS by case (n=4/4/2 in MI and n=1/3/2 in AIHD; Fig. 1g and h and Table 3), but was negative in all asphyxiation and drowning cases. ZO-1 and FN were stained diffusely in the myocardial cytoplasm to various intensities, showing a tendency to be more intensely positive in MI and AIHD than in controls (Fig. 1e,f,i,j, respectively, and Table 3). In two cases of RMI, ZO-1 positivity in CP was evidently weak in regions with moderate to strong C5b-9 and FN positivity. ZO-1 was not detected at ID. Cx43 and npCx43 were positive at ID, in CP and along LCB, and the distribution of npCx43 depended on the cause of death (Fig. 1a-d; Table 3): npCx43 positivity was mostly localized at ID in controls, but was more frequently and intensely detected in CP in MI and AIHD.

Semi-quantitative analysis of immunopositivity

Connexin43: The Cx43 positivity score in each myocardial region showed no significant difference among the causes of death, except that the score in CP of LV was significantly lower in D than other groups ($p<0.05$) (Fig. 2). The npCx43 positivity scores in CP of LV and IS were significantly higher in MI and AIHD than in controls ($p<0.05$). ΣCx43 and ΣnpCx43 in each myocardial region showed no significant difference among the causes of death.

ΔnpCx43 (CP–ID) was positive in LV and/or IS in most cases of MI (n=14/15; 93.3%) and AIHD (5/8; 62.5%), and was significantly higher in LV than in RV of MI cases ($p<0.05$) (Table 3; Fig. 3). There was no significant difference between MI and AIHD or between AMI and RMI. In asphyxiation and drowning, ΔnpCx43(CP–ID) was 0 or negative with a few exceptions of asphyxiation (n=3/24) in elderly subjects (60-77 years of age). ΔLV Cx43 (CP-ID) was significantly lower in drowning (D) than in acute ischemic heart disease (AIHD) and asphyxiation (As). ΔLV npCx43 (CP-ID) and ΔIS npCx43 (CP-ID) were significantly greater in AIHD than in As and D ($p<0.05$). ΔLV npCx43 (CP-ID) was significantly higher in myocardial infarction (MI) than in As and D ($p<0.05$). ΔRV npCx43 (CP-ID) showed a tendency to be higher in SCD than in controls (insignificant).

Zonnula occludens-1: ZO-1 positivity score in CP of LV was significantly lower in As than in AIHD, and that of RV was significantly lower in D than in other groups ($p<0.05$) (Table 3; Fig. 2).

C5b-9: C5b-9 positivity was detected in LV and/or IS in most MI and AIHD cases as described above, showing a significant difference from controls, but was totally negative in RV (Table 3; Fig. 2).

Fibronectin: FN positivity score in CP in each myocardial region was significantly lower in D than in other groups ($p<0.05$), and that of IS was significantly higher MI than in As ($p<0.05$) (Table 3; Fig. 2).

Discussion

Previous studies demonstrated the efficacy of C5b-9 and FN as immunohistochemical markers of early myocardial lesions in acute ischemic heart disease in autopsy diagnosis (Thomsen and Held, 1995; Hu et al., 1996; Fracasso et al., 2011a-c), as well as their possible application to detect myocardial damage in acute pulmonary embolism and intoxication (Fracasso et al., 2011a-c); however, there remained false negative findings for C5b-9 and the influence of global hypoxia for FN (Ortmann et al., 2000; Fracasso et al., 2011a-2011c). The present study showed immunohistochemical findings similar to those in previous reports

Table 3. Positivities of immunohistochemical markers with regard to the cause of death.

Marker		MI (n=15)		AIHD (n=8)		As (n=24)		D (n=10)	
		LV and/or IS	RV	LV and/or IS	RV	LV and/or IS	RV	LV and/or IS	RV
npCx43	ID	7 (46.7%)	10 (66.7%)	5 (62.5%)	7 (87.5%)	13 (54.2%)	13 (54.2%)	6 (60.0%)	4 (40.0%)
	CP	15 (100%)	12 (80.0%)	8 (100%)	8 (100%)	8 (33.3%)	9 (37.5%)	2 (20.0%)	1 (10.0%)
Positive ΔnpCx43	CP-ID	14 (93.3%)	6 (40.0%)	5 (62.5%)	2 (25.0%)	3 (12.5%)	2 (8.3%)	0 (0%)	0 (0%)
ZO-1	CP	15 (100%)	14 (93.3%)	8 (100%)	8 (100%)	24 (100%)	20 (83.3%)	8 (80.0%)	6 (60.0%)
C5b-9	CP	10 (66.7%)	0 (0%)	6 (75.0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
FN	CP	15 (100%)	13 (86.7%)	8 (100%)	7 (87.5%)	24 (100%)	17 (70.8%)	3 (30.0%)	2 (20.0%)

MI, myocardial infarction; AIHD, acute ischemic heart disease; As, mechanical asphyxiation; D, drowning; npCx43, non-phosphorylated connexin43; ΔnpCx43, redistribution grade of npCx43; ZO-1, zonula occludens-1; C5b-9, the terminal complement complex; FN, fibronectin; ID, intercalated disc; LCB, lateral cell border; CP, cytoplasm.

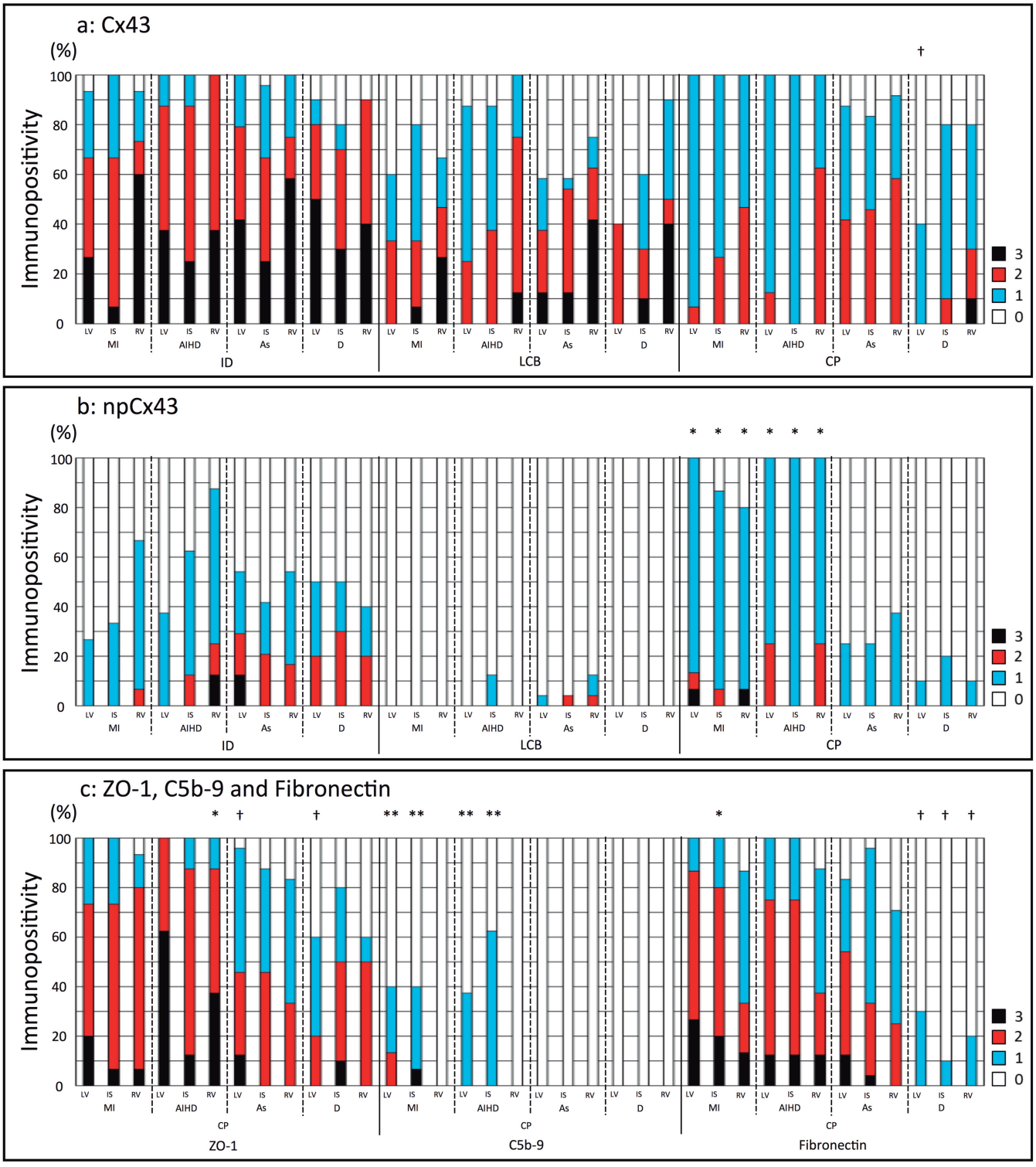

Fig. 2. Semiquantitative comparisons of immunopositivities of connexin43 (Cx43) **(a)**, non-phosphorylated (np) Cx43 **(b)**, zonula occludens-1 (ZO-1), the terminal complement complex (C5b-9) and fibronectin **(c)** among the causes of death. **a.** Cx43: †Positivity in the myocardial cytoplasm (CP) of the left ventricular wall (LV) was significantly lower in drowning (D) than in myocardial infarction (MI), acute ischemic heart disease (AIHD) and mechanical asphyxiation (As) ($p < 0.05$; Tukey test). **b.** npCx43: *Positivity in CP of each myocardial region was significantly higher in MI and AIHD than in As and D ($p < 0.05$; Tukey test). **c.** ZO-1: †Positivity in CP of LV was significantly lower in As than in AIHD ($p < 0.05$; Tukey test). †Positivity in CP of LV was significantly lower in D than in other groups ($p < 0.05$; Tukey test). *Positivity in CP of right ventricular wall (RV) was significantly higher in AIHD than in As and D ($p < 0.05$; Tukey test). C5b-9: **Positivity in CP of LV and IS was significantly higher in AIHD than in As and D ($p < 0.05$; Mann-Whitney U test). **Positivity in CP of LV and IS was significantly higher in MI than in As and D ($p < 0.05$; Mann-Whitney U test). Fibronectin: †Positivity in CP in each myocardial region was significantly lower in D than in MI, AIHD and As ($p < 0.05$; Tukey test). *Positivity in CP of IS was significantly higher in MI than in As ($p < 0.05$; Tukey test).

demonstrating specific C5b-9 positivity in SCD due to myocardial ischemia; however, the total positive rate including LV and IS (around 70%) was higher in the present study than in previous studies (around 40%) (Ortmann et al., 2000; Fracasso et al., 2011a-c), due to the frequent involvement of IS, suggesting myocardial ischemia caused by left anterior coronary lesions. A previous study showed C5b-9 positivity in RV of some MI cases (Fracasso et al., 2011c); however, such findings were not detected in the present study, although positive ΔnpCx43 (CP–ID) was found in RV of some MI and AIHD cases. This difference may depend on the site of coronary lesions and survival time after the onset of myocardial ischemia; the present study included cases of

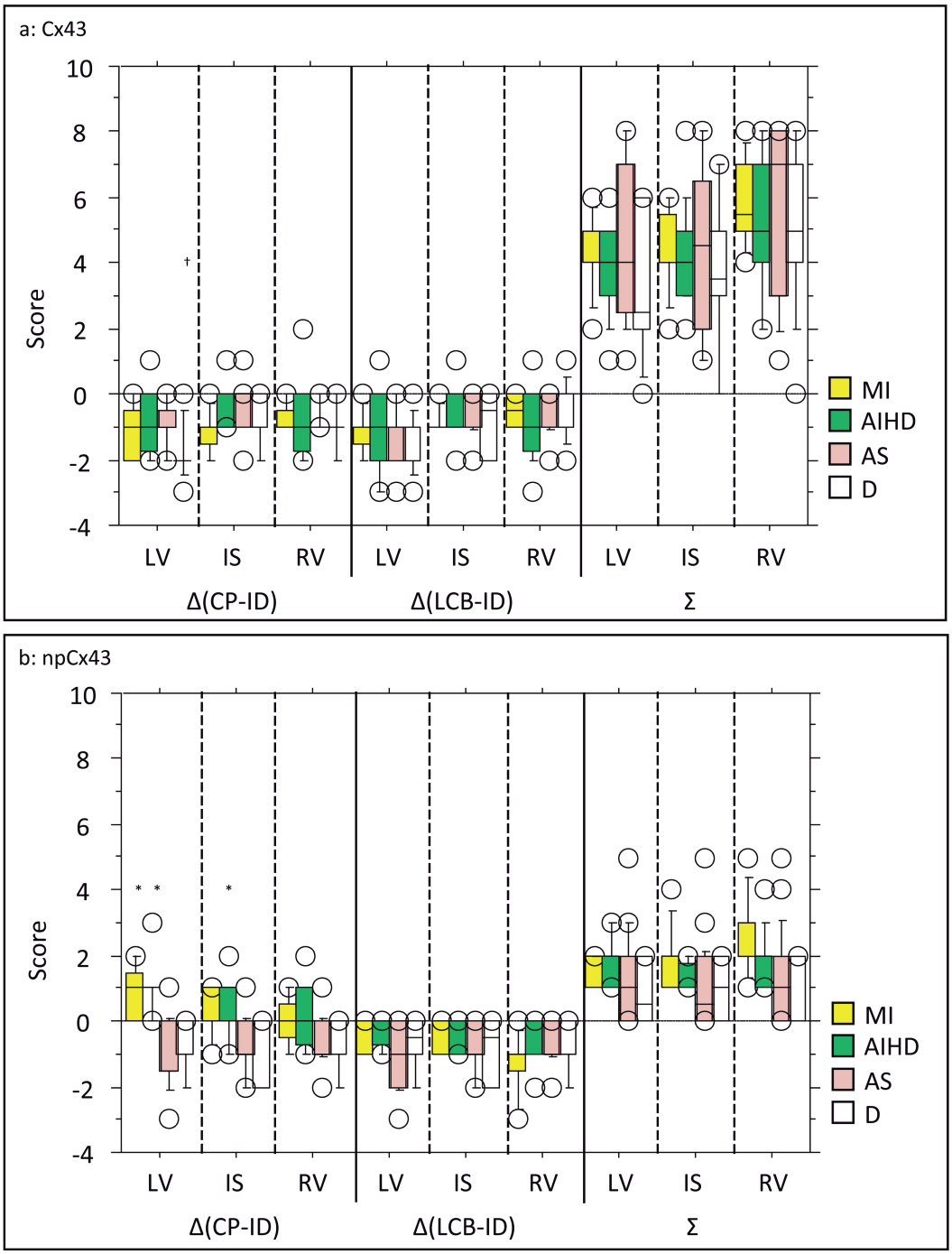

Fig. 3. Semi-quantitative comparisons of the redistributions and total scores of connexin43 (Cx43) **(a)** and non-phosphorylated (np) Cx43 **(b)** among the causes of death. **a.** Redistribution from the intercalated disc (ID) to the cytoplasm (CP) in the left ventricular wall (LV) (ΔLV Cx43 (CP-ID)) was significantly lower in drowning (D) than in acute ischemic heart disease (AIHD) and asphyxiation (As). **b.** Redistribution from ID to CP in LV and interventricular septum (IS) (ΔLV npCx43 (CP-ID) and ΔIS npCx43 (CP-ID)) was significantly greater in AIHD than in As and D. ΔLV npCx43 (CP-ID) was significantly higher in myocardial infarction (MI) than in As and D. The sum total score of npCx43 (npCx43) in the right ventricular wall was significantly higher in MI than in D. Asterisks indicate significantly higher scores ($p < 0.05$; Tukey test). LCB: lateral cell border, RV: right ventricular wall. Δ(CP-ID)=score in CP - score at ID, Δ(LCB-ID)=score along LCB - score at ID and Σ=score at ID + score along LCB + score in CP.

short survival period, with ischemic lesions predominantly in anterior and interventricular walls of the left ventricles. FN positivity was higher in such SCD cases but was also higher in mechanical asphyxiation than in drowning, which is believed to involve hypoxic myocardial damage (Szpilman et al., 2012; Miyazato et al., 2012); these findings suggest non-hypoxic/-ischemic myocardial damage in drowning.

Despite detailed animal studies on the functions of Cx43 and ZO-1 in myocardial ischemia and hypoxia, the published human data are poor (Beardslee et al., 2000; Turner et al., 2004; Matsushita et al., 2006; Bruce et al., 2008). The present study using human autopsy heart specimens demonstrated that specific redistribution of npCx43 from ID to CP, as indicated by positive ΔnpCx43 (CP–ID), could be detected in most SCD cases due to coronary lesions, including AMI and RMI as well as AIHD without evident pathological evidence of myocardial necrosis. The positivity using ΔnpCx43 (CP–ID) in MI (93.3%) was higher than that of C5b-9 (66.7%); however, both positivities were similar in AIHD (62.5% and 75%, respectively). These findings may depend on the different biological roles of Cx43 and C5b-9 in response to myocardial ischemia, involved in the remodeling of cardiac gap junctions related to functional disorder, including arrhythmia and the inflammatory process antecedent to tissue repair, respectively (Yasojima et al., 1998; Beardslee et al., 2000; Bruce et al., 2008; Busche and Stahl, 2010). In MI, prolonged myocardial ischemia leading to necrosis can rapidly aggravate the remodeling of cardiac gap junctions involving the redistribution of npCx43 from ID to CP before activating the inflammatory process. Combined positivity of these markers was high in AIHD (n=7/8; 97.5%), supporting the autopsy diagnosis in AIHD cases. In particular, positivity was higher in LV and IS, which were mainly involved in ischemic lesions in the present study groups, than in RV. These findings were consistent with the observations in the animal model, which suggested an earlier response of Cx43 (about 15 min after an ischemic insult), involving non-phosphorylation of Cx43 and redistribution of npCx43 from ID to CP; Cx43 can be an earlier marker of myocardial ischemia than C5b-9 (Thomsen and Held, 1995; Beardslee et al., 2000; Bruce et al., 2008). In asphyxiation, however, a few exceptions showed positive values of ΔnpCx43 (CP–ID). In these cases, positivity may indicate myocardial damage due to prolonged hypoxia; however, complications of ischemic heart attack during asphyxial death could not be excluded since these were elderly subjects over 60 years of age with age-related coronary atherosclerosis (Pomerance 1965). This should be considered in practical applications; npCx43 can be more sensitive but less specific than C5b-9.

Different from a previous report using frozen explanted human hearts (Bruce et al., 2008), ZO-1 as a tight junction protein could not be identified at ID but was diffusely detected in CP in the present study. Similar findings were seen in other causes of death, including hypothermia, hyperthermia and fatal intoxication, except for a few exceptions of fatal methamphetamine abuse (unpublished data). These findings suggest that ZO-1 may be easily dissociated from ID during agony or after death, possibly due to their weak affinity due to hydrogen bonds (Laing et al., 2007; Chen et al., 2008). In such conditions, ZO-1 positivity in CP showed a tendency to be higher in ischemic heart disease than in controls. In two RMI cases, ZO-1 positivity was evidently weak in the myocardium where C5b-9 and FN were intensely positive. This finding may represent an advanced ischemic change, although further investigation is needed using other immunohistochemical markers such as myoglobin and cardiac troponin (Brinkmann et al., 1993; Campobasso et al., 2008).

Major limitations of the present study include postmortem interference due to longer elapsed time after death and a lack of clinical history for forensic autopsy material; these should be supplemented using clinical cases as well as by reevaluation of postmortem changes before fixation and tissue fixation conditions using other myocardial markers, as described above. Postmortem interference was particularly evident for ZO-1 as described above; however, the present study did not include hospital deaths involving critical medical care that might have modified myocardial damage. In addition, it was demonstrated that a few cases did not show any positivity despite using multiple markers, suggesting the contribution of cardiac dysfunction without or prior to the remodeling of cardiac gap junctions; however, no particular finding was detected with regard to subject age except that a few elderly asphyxial death cases showed positive ΔnpCx43 (CP–ID), implying possible complications of ischemic heart attack during asphyxial death, as described above.

In conclusion, the present study using human autopsy material suggests that the combination of npCx43 and C5b-9 immunohistochemistry is useful for detecting early lesions of myocardial ischemia in SCD and demonstrating functional disorders and inflammatory responses in cardiomyocytes, respectively.

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