Title

Insufficient production of urinary trypsin inhibitor for elastase release promotes organ failure following cardiac arrest

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Running head

Insufficient of UTI following cardiac arrest

ABSTRACT – To investigate the relationship between the inflammatory responses and post-resuscitation syndrome, we prospectively examined the serial changes of neutrophil elastase (NE), urinary trypsin inhibitor (UTI) and tumor necrosis factor-α (TNF-α) in successfully resuscitated patients following out-of-hospital cardiac arrest. This study included 36 patients with out-of-hospital cardiac arrests that were admitted to our intensive care unit after return of spontaneous circulation (ROSC). The twenty-two patients who died within 3 days after ROSC were defined as nonsurvivors. The fourteen patients who survived for more than 3 days after ROSC were defined as survivors. Eight healthy volunteers served as control group. Daily plasma levels of NE, UTI, and TNF-α were measured from days 1 to 5 after ROSC. Persistently high levels of TNF-α and NE were observed in both the survivors and nonsurvivors. In the two groups, the levels of UTI were significantly high and increased as time progressed. NE/UTI ratios were significantly higher in the nonsurvivors than in

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the survivors, especially on day 1. The nonsurvivors showed statistically higher scores according to the Sequential Organ Failure Assessment and they also had more organ failure than the survivors. In conclusion, an insufficient production of UTI for NE release and persistent high levels of TNF-α may contribute to the pathogenesis of post-resuscitation syndrome following out-of-hospital cardiac arrest.

KEYWORDS – ischemia, neutrophil, out-of-hospital, protease inhibitor, reperfusion, resuscitation, tumor necrosis factor-α, ulinastatin

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INTRODUCTION

Cardiac arrest truly involves whole-body ischemia and maximal stress in both humans and animals. A resuscitation and return of spontaneous circulation (ROSC) allow reperfusion after global ischemia. Following ROSC, various pathophysiological disturbances are observed as post-resuscitation syndrome (1). Post-resuscitation syndrome is the greatest ischemia-reperfusion injury in humans and is recognized as microcirculation impairment and tissue damage in many vital organs (1-6). The microcirculation impairment and tissue damage after reperfusion may affect the neurological outcome and prognosis in patients following cardiac arrest (2-7).

Ischemia-reperfusion injury is a complex phenomenon associated with a multitude of molecular networks (7,8). Tumor necrosis factor-α (TNF$-\alpha$) plays the most significant role in regulating the ischemia-reperfusion molecular

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pathway (6,8). The increase in TNF-α contributes to cell death, apoptosis, and several organ dysfunctions (6). Endothelial adhesion molecules induce neutrophil accumulation both within the microvessels and extravascular tissues (3,7). The accumulated neutrophils release elastase, which injures endothelial cells and induces systemic inflammation (2-4,7,9). Neutrophil elastase (NE) appears to have the greatest potential among other neutrophil proteases, such as matrix metalloproteases and cathepsin G (9). Such evidence suggests that NE and TNF-α play pivotal roles in ischemia-reperfusion injury (2-4,6-10).

Urinary trypsin inhibitor (UTI) is a protease inhibitor discovered in human urine, which is also termed as bikunin or HI-30 (11,12). UTI is an acidic glycoprotein that is cleaved from inter-α-trypsin inhibitor by NE (9,12,13). UTI suppresses neutrophil infiltration and decreases neutrophil-mediated endothelial injury by inhibiting elastase activity (9). In addition, UTI decreases TNF-α production of lipopolysaccharide-stimulated monocytes by the inhibition

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of either translation or secretion of TNF-α from monocytes (14). Although many reports investigated UTI levels in urine, few investigations have been reported regarding the UTI levels in plasma (15).

In this study, we hypothesized that an insufficient production of UTI for NE release and persistent high levels of TNF-α lead to post-resuscitation syndrome following out-of-hospital cardiac arrest. To test this hypothesis, we measured the serial changes of UTI, NE and TNF-α in successfully resuscitated patients following out-of hospital cardiac arrest.

MATERIALS AND METHODS

Approval of this study was obtained from our Institutional Review Board. This study was performed from July 2000 to December 2001. We studied 36

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resuscitated patients following out-of-hospital cardiac arrest. Informed consent for this study was obtained from their next of kin. Cardiac arrest was defined as the absence of a palpable pulse confirmed by an emergency medical service. Patients were excluded if they were under 18 years of age, had a terminal illness or history of trauma-induced arrest. The cardiopulmonary resuscitation was performed in accordance with guidelines 2000 for cardiopulmonary resuscitation and emergency cardiovascular care (16). After ROSC, the patients were admitted to our intensive care unit. Exogenous UTI was not administered. Induced hypothermia therapy was not performed in any included patient. Patients who died within 3 days after ROSC were defined as nonsurvivors. Patients who survived for more than 3 days after ROSC were defined as survivors.

Blood samples were collected into a test tube containing EDTA-2Na using an arterial catheter within 30 minutes after ROSC (day 1) from the

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survivors and nonsurvivors. Samples were collected daily on day 2 through day 5 from the two groups. Eight healthy volunteers served as control group. The blood samples were promptly centrifuged and the separated plasma was frozen at –80 ^{o}C until analysis. The plasma levels of NE, UTI, and TNF-α were determined by enzyme immunoassay (granulocyte elastase EIA, SANWA KAGAKU KENKYUSHO CO., LTD., Nagoya, Japan), (UTI measurement Kit, MOCHIDA PHARMACEUTICAL CO., LTD., Tokyo, Japan), and (Quantikine HS Human TNF-α Immunoassay, R&D SYSTEMS, Minneapolis, MN, USA), respectively. The Sequential Organ Failure Assessment (SOFA) scores were calculated daily (17). We defined organ failure as a SOFA score ≥3 in each included organ. A cerebral performance category was evaluated at discharge from our hospital (18).

The StatView 5.0 statistical software package (SAS Institute Inc., Cary, NC, USA) was used for all statistical calculation analyses. Comparisons

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between the groups were made using the paired or unpaired Student's t-test and the Chi-square test. The time course of measured variables was compared by one-factor repeated-measures analysis of variance and the Dunnett's multiple comparison was used as a post-hoc test. A *P* value of < 0.05 was considered statistically significant. All data were expressed as the means ± SEM.

RESULTS

Thirty-six patients restored spontaneous circulation and were included in this study. Twenty-two patients died within 3 days after ROSC (nonsurvivors). Fourteen patients survived for more than 3 days after ROSC (survivors). Etiologies of the cardiac arrest are shown in Table 1. Table 2 presents the characteristics of patients in the two groups. The duration of cardiac arrest was

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estimated based on information reported by an emergency medical service. The estimated duration of cardiac arrest of the nonsurvivors was significantly longer than that of the survivors (47.8 ± 3.9 versus 29.7 ± 4.2 min., $P = 0.004$). The cerebral performance category of the survivors showed good performance in three patients, moderate disability in one patient, severe disability in one patient, coma or vegetable in seven patients, and death in two patients.

Fig. 1 (top) presents NE levels. In both the survivors and nonsurvivors, NE levels were significantly higher than those of the control group throughout the observation period ($P < 0.05$). Fig. 1 (middle) presents UTI levels. In the two groups, UTI levels were significantly higher than those of the control group throughout the observation period ($P < 0.05$). On day 2 in nonsurvivors and after day 3 in survivors, UTI levels significantly increased in comparison to day 1 in each group ($P < 0.05$). Fig. 1 (bottom) presents NE/UTI ratios. Ratios of NE/UTI showed a significant decrease after day 2 in comparison with day 1 in

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both of the two groups ($P < 0.05$). We found statistical significance in the NE/UTI ratios on days 1 and 2 between the survivors and nonsurvivors ($P = 0.044$ and $P = 0.047$). Fig. 2 presents TNF-α levels. In both the survivors and nonsurvivors, persistently high levels of TNF-α remained throughout the observation period with statistical significance in comparison to the control group ($P < 0.05$).

Table 3 presents daily changes in SOFA scores and the number of failed organs in both the survivors and nonsurvivors. All of the patients showed high SOFA scores and had multiple organ failure. There were significant differences in SOFA scores and in the number of failed organs between the survivors and nonsurvivors on days 1 and 2 ($P < 0.05$).

DISCUSSION

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In the present study, we demonstrated significant increases in levels of TNF-α and NE in both the survivors and nonsurvivors during the post-resuscitation period. Although on days 1 and 2 the UTI levels significantly increased in both the survivors and nonsurvivors, the NE/UTI ratios on days 1 and 2 in the nonsurvivors showed higher levels than those of the survivors. This finding suggests that UTI production is not balanced with NE release in nonsurvivors. The nonsurvivors showed higher SOFA scores and had more organ failure than the survivors. The results suggest that an insufficient production of UTI for NE release and persistently high levels of TNF-α may therefore play an important role in the occurrence of post-resuscitation syndrome following out-of-hospital cardiac arrest.

A primary function of UTI is to inhibit serine proteases, especially NE (11,12). UTI suppresses neutrophil infiltration and decreases

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neutrophil-mediated endothelial injury by inhibiting elastase activity (9). In addition, UTI directly decreases the production of cytokines such as TNF-α (14). Although α_1-proteinase inhibitor has been shown to be a strong inhibitor of the NE activity, the enzyme is inactivated by neutrophil oxidants and it cannot suppress the NE activity in a case of severe inflammation (10). In such a case of severe inflammation, however, UTI can suppress NE regardless of neutrophil oxidants (19). Therefore, UTI plays a pivotal role to control the systemic inflammation in post-resuscitation syndrome following cardiac arrest. Until recently, only the urine UTI levels have been measured, however, no correlation between the plasma and urine UTI levels has been elucidated (15). We believe that the direct-measured plasma UTI accurately reflects the UTI activity. In spite of high UTI levels, we found a continuously marked increase in the levels of TNF-α and NE during the study period. Furthermore, statistically higher NE/UTI ratios were found in both the survivors and nonsurvivors than those of the

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control group, thus suggesting that the UTI production was not balanced with the NE release, especially in the nonsurvivors. These results also indicate the presence of more severe sustained systemic inflammation in the nonsurvivors than those in the survivors.

In our previous study, we could not detect TNF-α following out-of-hospital cardiac arrest, while neutrophil and endothelial activation were evident (3,4). Using a swine model, Niemann et al (6) found that TNF-α increases during the early post-resuscitation period, and they suggested that high TNF-α may play a role in post-resuscitation myocardial dysfunction. Adrie et al (5) showed that detectable levels of TNF-α were found within the first 2 days in only 54% of resuscitated patients following cardiac arrest. The presence of TNF-α was associated with a significantly higher mortality in their study (5). In the present study, we demonstrated persistently high levels of TNF-α in all of the patients throughout the observation period using highly

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sensitive measuring methods in comparison to our former studies (3,4).

In addition to the results of our previous studies (3,4), we herein demonstrated that NE levels remained elevated until 5 days after ROSC. We have already reported the elevated NE levels associated with neutrophil and endothelial activation followed by endothelial injury in patients following out-of hospital cardiac arrest (3,4). The NE release as well as the TNF-α production observed in our study suggest that systemic inflammation occurs in patients following out-of-hospital cardiac arrest. Such evidence of activated inflammation plays an important role in both human whole-body ischemia and reperfusion. Our NE assay measures a complex of NE and α_1-proteinase inhibitor, however, high levels of the complex have been confirmed as results of neutrophil activation and NE release (3,4).

In our study, all patients showed high SOFA scores and had multiple organ failure in the post-resuscitation period. The nonsurvivors showed

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statistically higher SOFA scores and had more organ failure than those of the survivors on days 1 and 2. These results demonstrate post-resuscitation syndrome in the patients following out-of-hospital cardiac arrest. Ultimately, our study suggests that the sustained systemic inflammation evoked by high TNF-α and massive NE release not balanced by UTI production may therefore play important roles in the pathogenesis of post-resuscitation syndrome. Stronger inflammation in the nonsurvivors, which was evaluated by NE/UTI ratios, implies that systemic inflammation may lead to a poorer prognosis in patients with post-resuscitation syndrome.

In the present study, few patients showed good levels in the cerebral performance category. A long duration of cardiac arrest before ROSC may contribute to the poor neurological outcome. However, neurological damage following cardiac arrest is determined by primary brain ischemia as well as microcirculation impairment and tissue damage resulting from

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post-resuscitation syndrome (2,7,20,21). There may be a possibility that the systemic inflammation observed in the present study has a pivotal role in secondary brain damage following out-of-hospital cardiac arrest. Primary brain ischemia induced by cardiac arrest is determined by ischemic duration. However, secondary brain damage resulting from post-resuscitation syndrome may be improved by several treatments in a hospital. Our previous report showed that the administration of exogenous UTI suppressed the increase of NE following out-of-hospital cardiac arrest (4). The results from both our previous and present studies imply that sufficient suppression of NE by exogenous UTI may improve the clinical outcome after ROSC by reducing microcirculation impairment and tissue damage resulting from post-resuscitation syndrome (2-4,14).

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CONCLUSIONS

We demonstrated systemic inflammatory responses in successfully resuscitated patients following out-of-hospital cardiac arrest. The main findings were: [1] the release of massive amounts of NE from activated neutrophil just after ROSC; [2] the insufficient production of UTI for NE release in the post-resuscitation period; [3] the sustained systemic inflammation (highly elevated TNF-α); and [4] high SOFA scores in the early post-resuscitation period, especially among the nonsurvivors. These findings suggest that the systemic inflammation evoked by the insufficient production of UTI for NE release, accompanied by persistent high TNF-α levels may play important roles in the pathogenesis of post-resuscitation syndrome following out-of-hospital cardiac arrest.

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TABLE 1. **The etiologies of cardiac arrest**

	Survivors (n = 14)	Nonsurvivors (n = 22)
Cardiovascular	9	11
Respiratory	5	6
Neurogenic	0	4
Undetermined	0	1

TABLE 2. **Characteristics of the patients**

	Survivors (n = 14)	Nonsurvivors (n = 22)	*P* value
Age (years)	65 ± 3	70 ± 3	NS
Gender (male/female)	7/7	14/8	NS
Witnessed arrest (yes /no)	13/1	16/6	NS
Bystander CPR (yes/no)	9/5	7/15	NS
Primary rhythm (VF/PEA/asystole)	5/7/2	1/8/13	0.009
ROSC before arrival to emergency department (yes/no)	4/10	3/19	NS
Epinephrine (mg)	3.1 ± 1.0	3.8 ± 0.5	NS
Duration of cardiac arrest (min)	29.7 ± 4.2	47.8 ± 3.9	0.004
APACHE II score	24.8 ± 2.3	29.3 ± 2.8	NS
CPC (1/2/3/4/5)	3/1/1/7/2	0/0/0/0/22	< 0.0001

CPR, cardiopulmonary resuscitation; VF, ventricular fibrillation; PEA, pulseless electrical activity; ROSC, return of spontaneous circulation; APACHE II, Acute Physiology and Chronic Health Evaluation II; CPC, Cerebral Performance Categories; CPC 1, good performance; CPC 2, moderate disability; CPC 3, severe disability; CPC 4, coma/vegetable; CPC 5, death; NS, not significant.

TABLE 3. **Sequential Organ Failure Assessment (SOFA) score and the number of failed organs in the patients**

	Day 1	Day 2	Day 3	Day 4	Day 5
Survivors (n = 14)					
SOFA score	8.6 ± 0.7	7.1 ±0.8	7.3 ±0.8	6.6 ± 1.0	6.4 ±1.0
Number of failed organs	2.4 ± 0.3	2.1 ± 0.3	2.3 ±0.3	2.0 ± 0.4	1.9 ±0.4
Nonsurvivors (n = 22)					
SOFA score	11 ± 0.3*	13.0 ± 0.6^{+}			
Number of failed organs	2.9 ± 0.1*	3.7 ± 0.3*			

*, $P < 0.05$; $^{+}$, $P < 0.001$ vs. the survivors.

FIGURE LEGENDS

FIG. 1. **Levels of neutrophil elastase (top), urinary trypsin inhibitor (middle), and ratios of neutrophil elastase/urinary trypsin inhibitor (bottom) after return of spontaneous circulation.**

Data are presented as the means ± SEM. * $P < 0.05$ vs. the control subjects. $^{+}$ $P < 0.05$ vs. the nonsurvivors on day 1. $^{\#}$ $P < 0.05$ vs. the survivors on day 1.

FIG. 2. **Levels of tumor necrosis factor-α after return of spontaneous circulation.**

Data are presented as the means ± SEM. * $P < 0.05$ vs. the control subjects.

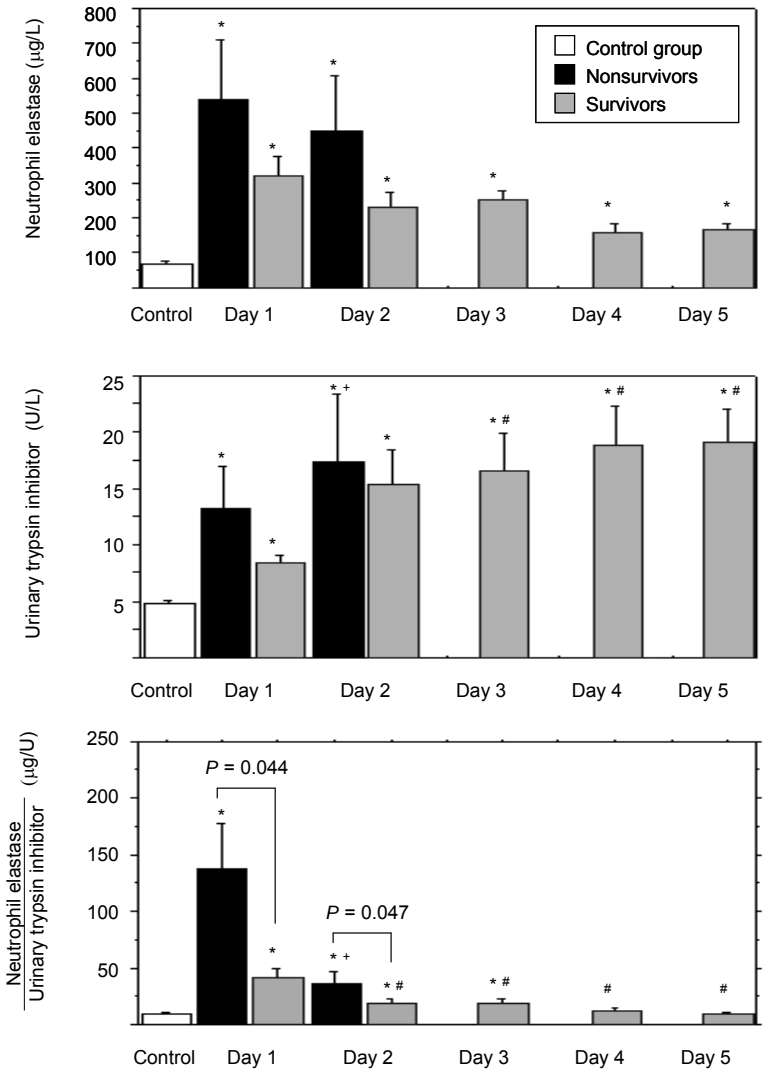
Neutrophil elastase (μg/L)
800
700
600
500
400
300
200
100
Control group
Nonsurvivors
Survivors
Control
Day 1
Day 2
Day 3
Day 4
Day 5
Urinary trypsin inhibitor (U/L)
25
20
15
10
5
Neutrophil elastase / Urinary trypsin inhibitor (μg/U)
250
200
150
100
50
P = 0.044
P = 0.047

Fig. 1

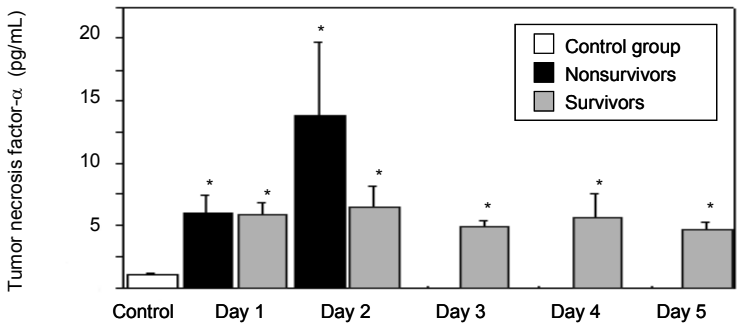
Tumor necrosis factor-α (pg/mL)
20
15
10
5
Control group
Nonsurvivors
Survivors
Control
Day 1
Day 2
Day 3
Day 4
Day 5

Fig. 2