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Nardilysin is a promising biomarker for the early diagnosis of acute coronary syndrome

Po-Min Chen [a,1], Mikiko Ohno [a,1], Takaki Hiwasa [b], Kiyoto Nishi [a], Sayaka Saijo [a], Jiro Sakamoto [a], Yusuke Morita [a], Shintaro Matsuda [a], Shin Watanabe [a], Yasuhide Kuwabara [a], Koh Ono [a], Masao Imai [a], Katsumi Inoue [c], Tatsuya Murai [d], Tsukasa Inada [e], Masaru Tanaka [e], Toru Kita [f], Takeshi Kimura [a], Eiichiro Nishi [a,g,*,2]

[a] Department of Cardiovascular Medicine, Graduate School of Medicine, Kyoto University, Kyoto, Japan
[b] Department of Biochemistry and Genetics, Graduate School of Medicine, Chiba University, Chiba, Japan
[c] Kokura Memorial Hospital, Kitakyushu, Japan
[d] Sakakibara Memorial Hospital, Fuchu, Japan
[e] Osaka Red Cross Hospital, Osaka, Japan
[f] Kobe City Hospital Organization, Kobe, Japan
[g] Shiga University of Medical Science, Otsu, Japan

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ABSTRACT

Background: Biomarkers for detection of transient myocardial ischemia in patients with unstable angina (UA) or for very early diagnosis of acute myocardial infarction (AMI) are not currently available.
Methods and results: We performed two sequential screenings of autoantibodies elevated shortly after the onset of acute coronary syndrome (ACS), and focused on metalloendopeptidase nardilysin (NRDC) among 19 identified candidate antigens. In a retrospective analysis among 93 ACS and 117 non-ACS patients, the serum level of NRDC was significantly increased in patients with ACS compared with that in patients with non-ACS (2073.5 ± 189.8 pg/ml versus 775.7 ± 63.4 pg/ml, $P < 0.0001$). The area under the curve of NRDC for the diagnosis of ACS was 0.822 by the receiver operating characteristic curves analysis. In the time course analysis in 43 consecutive ACS patients (AMI: $N = 35$ and UA: $N = 8$), serum concentration of NRDC was significantly increased even in UA patients with a peak serum NRDC levels reached at admission both in AMI and UA patients. In a mouse model of AMI, we found an acute increase in serum NRDC and reduced NRDC expression in ischemic regions shortly after coronary artery ligation. NRDC expression was also reduced in infarcted regions in human autopsy samples from AMI patients. Moreover, the short treatment of primary culture of rat cardiomyocytes with H_2O_2 or A23187 induced NRDC secretion without cell toxicity.
Conclusion: NRDC is a promising biomarker for the early detection of ACS, even in UA patients without elevation of necrosis markers.

1. Introduction

Acute coronary syndrome (ACS), mainly caused by disruption of atherosclerotic plaques and thrombosis, is one of the leading causes of cardiovascular disease-related death. Timely and accurate diagnosis of ACS is critical because delay in the diagnosis and initiation of the treatment may increase the risk of fatal complications. ACS consists of acute myocardial infarction (AMI) and unstable angina (UA), which are defined by the presence or absence of myocardial cell necrosis. Cardiac troponin (cTn) is the preferred biomarker for the detection of cardiomyocyte necrosis, and thus for the diagnosis of AMI. The latest generation of high-sensitivity cTn assays can detect cTn in >95% of a reference population, enabling the diagnosis of AMI even with very small amounts of necrosis [1–4]. On the other hand, however, biomarkers for detection of UA or transient myocardial ischemia with high sensitivity and specificity are not currently available. Moreover, as complete necrosis of myocardial cells requires 2–4 h or even longer after the onset of ischemia, biomarkers of necrosis, such as cTn, are not ideal for the diagnosis of very early phase ACS.

Therefore, we investigated possible biomarkers of ACS with pathophysiological backgrounds other than cell necrosis. Several lines of

* Corresponding author at: Department of Cardiovascular Medicine, Graduate School of Medicine, Kyoto University, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan.
E-mail addresses: nishi@kuhp.kyoto-u.ac.jp, enishi@belle.shiga-med.ac.jp (E. Nishi).
[1] These authors contributed equally to this work (alphabetically ordered).
[2] Present address: Department of Pharmacology, Shiga University of Medical Science, Seta Tsukinowa-cho, Otsu, Shiga 520-2192, Japan.

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evidence suggest that some autoantibodies are involved in the pathogenesis of ACS [5]. Given that immune-mediated inflammation plays critical roles in the formation and rupture of atherosclerotic plaques, we hypothesized that factors specifically induced in vulnerable plaques or secreted by small ruptures may cause the primary formation of autoantibodies before the onset of ACS. Autoantibody formation may also be induced by sequestered antigens in cardiomyocytes, of which secretion is induced by transient ischemia. While it takes several days for the primary formation of antibodies, the second and subsequent exposures to antigen produce more immediate and stronger immune responses. Therefore, screening of autoantibodies with high titer in ACS patients shortly after onset might lead to the discovery of biomarkers useful for the early detection or even prediction of ACS.

In the current study, we performed two sequential screenings of autoantibodies using serum from healthy volunteers and ACS patients. As a result, we identified 19 candidate antigens including nardilysin (N-arginine dibasic convertase; NRDC). NRDC is a metalloendopeptidase of the M16 family, which was identified as a binding protein of HB-EGF [6]. NRDC is secreted from cells and enhances ectodomain shedding of multiple membrane proteins such as TNF-α, HB-EGF, NRG1, and APP [7–9]. NRDC has thus far been suggested to have important roles in inflammatory diseases [10,11]. NRDC is also expressed in the nucleus and plays important roles as a transcriptional coregulator in several biological processes such as thermogenesis and glucose homeostasis [12,13]. We demonstrate here that NRDC could be a biomarker for the early detection of ACS, even in UA patients with no significant elevation of biomarkers for myocardial necrosis such as cTn.

2. Results

2.1. Screenings of autoantibodies in ACS patients

To identify autoantibodies elevated in patients with ACS, we performed two sequential screenings (Fig. 1A). For the first screening, we compared the pattern of autoantibodies in the serum of 6 ACS patients and 6 healthy volunteers using protein arrays, on which >9000 purified proteins are immobilized. In this screening, the titer of autoantibodies against 44 antigens was significantly elevated in ACS patients compared with healthy volunteers. Among these candidate antigens, 32 full-length proteins and 96 peptides corresponding to the predicted regions for MHC Class-II binding were prepared for the measurement of autoantibodies in the second screening using AlphaLISA (Amplified Luminescence Proximity Homogenous Assay). Furthermore, 5 recombinant proteins, which were not included in the protein array but implicated in atherosclerosis, were also applied to the second screening. In the second screening, titers of autoantibodies against 37 proteins and 96 peptides in total were measured in sera from 94 healthy volunteers and from 61 ACS patients, including 41 AMI patients and 20 UA patients. Autoantibodies against 10 full-length proteins and 13 peptides were found to be significantly elevated in ACS patients compared with healthy volunteers (Supplementary Table 1). For three antigens (GDNF, PIK3CD, and FAM125B), titers of autoantibodies were elevated for both full-length protein and peptide fragments. Collectively, we selected autoantibodies against 19 molecules as candidate predictive markers of ACS. Among these candidates, we proceeded to further analyze NRDC, whose autoantibody was significantly elevated in AMI and tended to be increased in UA patients (Fig. 1B).

2.2. Serum concentration of NRDC is significantly increased in patients with ACS

NRDC was measured in serum using a highly sensitive sandwich enzyme-linked immunosorbent assay (ELISA) system [14].

In study 1, we analyzed the serum concentration of NRDC in 210 patients with cardiovascular diseases (144 male and 66 female, mean age; 68 years, 93 ACS patients and 117 non-ACS patients), who admitted to the Department of Cardiovascular Medicine in Kyoto University Hospital (Supplementary Tables 2 and 3). As shown in the one-way layout, serum concentration of NRDC was increased in patients with ACS compared with that in non-ACS patients (2073.5 ± 189.8 pg/ml versus 775.7 ± 63.4 pg/ml, $P < 0.0001$) (Fig. 1C, Supplementary Table 3). Especially, serum NRDC in ACS patients is significantly higher than that in patients with stable coronary artery disease (SCAD; $n = 54$) (2073.5 ± 189.8 pg/ml versus 630.3 ± 74.2 pg/ml, $P < 0.0001$). Measurement of serum NRDC in 170 ACS patients, who admitted to Osaka Red Cross Hospital (Supplementary Table 4), revealed a similar value (2332.6 ± 213.8 pg/ml). Collectively, serum NRDC was significantly elevated in patients with ACS compared with that in patients with non-ACS cardiovascular diseases (Fig. 1D). To assess the diagnostic accuracy of NRDC in the detection of ACS, we performed receiver operating characteristic (ROC) curves analysis and found that the area under the curve (AUC) was 0.822, which was as high as that of troponin T (Fig. 1E) [15]. Sensitivity, specificity, and positive and negative predictive values for NRDC were 75.2%, 76.1%, 71.4%, and 79.5%, respectively. We also analyzed correlations of serum NRDC with serum biomarkers at admission (Supplementary Table 5). White blood cell count showed mild correlation with serum NRDC in all diagnosis groups, while CK-MB showed mild correlation with NRDC in AMI patients.

2.3. Serum NRDC is increased in patients with UA patients

In Study 2, to explore the time course of serum NRDC value after the onset of ACS, serial blood samples were obtained from 43 consecutive ACS patients (33 male and 10 female, mean age: 70 years, 35 AMI and 8 UA) at admission and every 6 h until creatine kinase (CK) reached a peak (Supplementary Tables 6 and 7). Importantly, the serum NRDC levels were similarly elevated in both UA and AMI patients at admission (4845.9 ± 920.5 pg/ml versus 5998.4 ± 696.6 pg/ml, $P = 0.350$) (Fig. 2A). At admission, the positive rates of NRDC, HFABP, and TnT in AMI patients were 95.3%, 78.6%, and 59.5%, respectively, whereas the positive rates of NRDC and HFABP in UA patients (TnT-negative) were 87.5% and 42.9%, respectively. Notably, NRDC levels at admission were elevated, irrespective of the time of blood sampling after onset (Fig. 2B). Moreover, positive rates of NRDC, HFABP, and TnT in 15 patients, whose blood was taken within 3 h of onset, were 100%, 73.3%, and 46.7%, respectively. In time course analysis, serum NRDC level in UA patients had already peaked at admission, which gradually declined in a time-dependent manner until 12 h after the admission (Fig. 2C). The serum NRDC level in AMI patients had also peaked at admission when the CK level did not reach a peak, and was maintained at peak level for at least 12 h (Fig. 2D).

2.4. NRDC protein expression is reduced in necrotic myocardial regions in humans

To gain mechanistic insights into the elevation of serum NRDC in ACS, we analyzed NRDC protein expression in the autopsy hearts obtained from patients who had died of AMI. Fig. 3A shows the heart of a patient who died from cardiac rupture 24 h after the onset of AMI. Immunostaining of NRDC in the autopsy sample demonstrated that NRDC is markedly reduced in the necrotic lesion, which clearly contrasts with the surrounding viable regions (Fig. 3B, D). A similar expression pattern of NRDC was confirmed in 3 other autopsy samples from patients who died of AMI (Fig. 3E). Interestingly, the contrast in NRDC expression was already clear in a patient who died shortly after the onset (4 h), and appeared to become more obvious in patients who died later after the onset (24 h and 4 days). On the other hand, NRDC was homogeneously expressed in the myocardium of a patient who died of non-cardiac disease (Fig. 3B, C). Inflammatory cell infiltration is one of the histological characteristics of myocardial infarction. Immunofluorescent double staining demonstrated that NRDc is highly expressed in neutrophils infiltrated in the infarcted area (Supplementary Fig. 1).

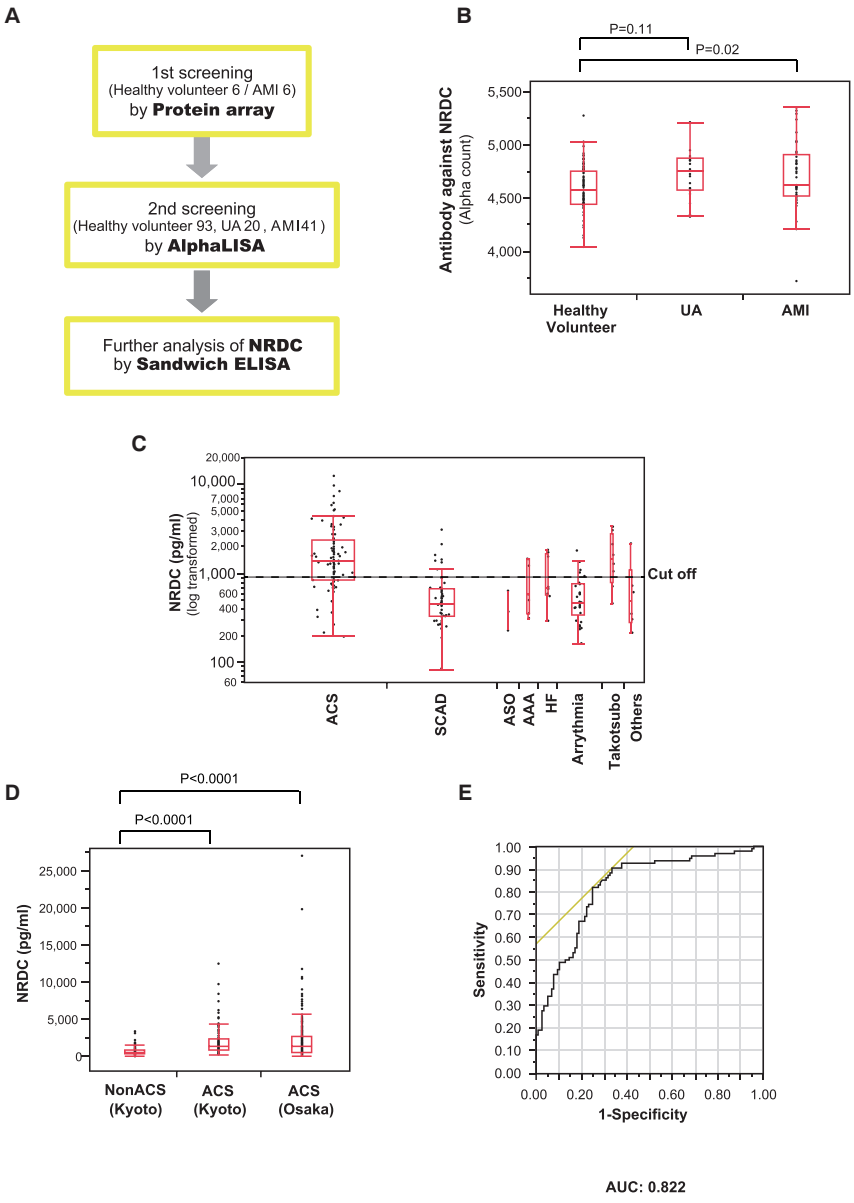

Fig. 1. Serum concentration of NRDC is significantly elevated in patients with ACS. (A) Flowchart shows the methods and numbers of patients used for the screening of autoantibodies. (B) Serum levels of antibody against NRDC in healthy volunteers and patients with UA and AMI. Antibody levels were examined by AlphaLISA, shown in box and whisker plots. Boxes represent interquartile ranges and whiskers display the 10th and 90th percentiles. AlphaLISA indicates amplified luminescence proximity homogeneous assay; AMI, acute myocardial infarction; ELISA, enzyme-linked immunosorbent assay; NRDC, nardilysin; and UA, unstable angina. (C) One-way layout of serum concentration of NRDC in 210 patients with known cardiovascular diseases. Others include dilated cardiomyopathy, hypertrophic cardiomyopathy, valvular heart diseases, and cardiac sarcoidosis. (D) Serum concentrations of NRDC in ACS patients and non-ACS patients who were admitted to Kyoto University Hospital are shown. NRDC levels in ACS patients admitted to another institute (Osaka Red Cross Hospital) are also shown. (E) Area under the receiver operating characteristic (ROC) curve for NRDC at presentation for the diagnosis of ACS. AAA indicates abdominal aortic aneurysm; ACS, acute coronary syndrome; ASO, arteriosclerosis obliterans; AUC, area under the curve; HF, heart failure; NRDC, nardilysin; SCAD, stable coronary artery disease; and Takotsubo, Takotsubo syndrome.

2.5. Serum NRDC is acutely increased in a mouse model of AMI

To examine whether acute elevation of serum NRDC is reproduced in a mouse model of ACS, we performed a conventional left anterior descending coronary artery (LAD) ligation in wild-type mice. As mouse NRDC cannot be measured by ELISA, we performed immunoprecipitation and immunoblot for the detection of mouse serum NRDC. Blood samples were obtained from mice with either LAD ligation, sham operation, or no treatment 1.5 h after the operation. As shown in Fig. 4A and B, serum NRDC was clearly increased in mice with LAD ligation compared with that in control mice.

We examined NRDC expression in hearts from mice with sham operation or LAD ligation. Similar to the findings of human autopsy samples, NRDC expression was reduced in the infracted area compared to that in the viable region. Additionally, NRDC was homogeneously expressed in the myocardium of mice that underwent sham operations (Fig. 4C). We divided left ventricles into the infarcted region, the viable region remote from the infracted area, and the border zone between infracted and viable regions, and extracted proteins from each region. Immunoblot of extracted proteins with anti-NRDC antibody revealed that the NRDC protein level is markedly reduced in the infarcted area compared to that in the remote viable region or the border zone

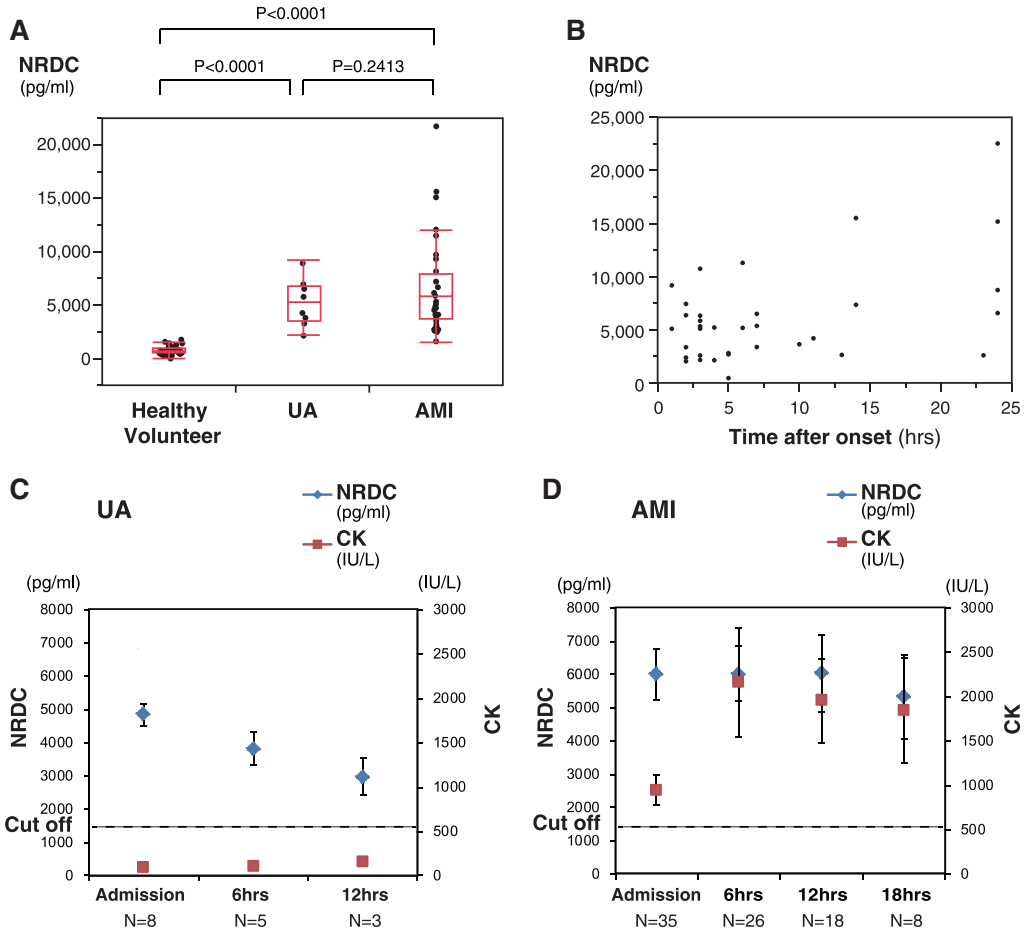

Fig. 2. Serum NRDC is significantly increased in patients with UA patients. (A) Serum concentration of NRDC in patients with UA and AMI at presentation. NRDC is significantly increased in both UA and AMI patients compared with that in healthy volunteers. (B) Scatter plotting of serum NRDC and the blood sampling time after the onset in patients with AMI at presentation in study group 2. (C, D) Time course analysis of serum NRDC and CK levels in UA (C) and AMI patients (D) Time 0 is at admission. All values represent mean ± standard error of mean. AMI indicates acute myocardial infarction; CK, creatinine kinase; NRDC, nardilysin; and UA, unstable angina.

(Fig. 4D). These findings strongly suggest that NRDC was released from the infarcted myocardium and secreted into serum after LAD ligation.

2.6. NRDC is secreted from primary culture of rat cardiomyocytes

To further confirm whether NRDC is indeed secreted from cardiomyocytes, we performed in vitro experiments using primary culture of rat ventricular cardiomyocytes. Rat cardiomyocytes were treated with hydrogen peroxide (H_2O_2) or calcium ionophore (A23187) for 2 or 24 h, and the secreted NRDC was analyzed in concentrated conditioned medium (CM) by immunoblot. As shown in Fig. 4E and F, the secretion of NRDC was markedly induced by both stimuli possibly in a distinct time course. Importantly, NRDC was clearly secreted in 2 h-treated cells with no significant toxicity, which was tested by lactate dehydrogenase (LDH) activity in the conditioned medium (Supplementary Fig. 2). On the other hand, cells were obviously injured after 24 h-treatment with H_2O_2 or A23187 (Supplementary Fig. 2), which might partly explain why NRDC was elevated in the CM (Fig. 4E and F).

3. Discussion

In this study, we attempted to identify novel biomarkers useful for the prediction of ACS. To this end, we screened autoantibodies in the sera of ACS patients and found that autoantibodies against 21 molecules were significantly elevated in ACS patients compared with that in healthy volunteers. Among 21 candidate antigens, we focused on NRDC and further examined its significance as a biomarker for ACS. Retrospective cohort studies demonstrated that 1) serum NRDC is a powerful diagnostic marker of ACS with an AUC value of 0.822, 2) serum NRDC is elevated in the very early phase of ACS, 3) serum NRDC is elevated in patients not only with AMI, but also with UA.

Elevation of NRDC in UA patients in whom neither cTn nor CK-MB was raised suggests that NRDC is a biomarker with a pathophysiological background independent of cell necrosis. NRDC in patients with AMI peaked at admission and peak levels were maintained for at least 12 h. This time course of serum NRDC is clearly distinct from that of CK, a typical necrosis marker, which was not significantly elevated at admission and peaked at 6–12 h after the admission. Consistently, there was no correlation between NRDC and CK, and a very weak correlation between NRDC and CK-MB in AMI patients. The results of cell-based experiments also suggest that NRDC is a non-necrosis marker, because the short treatment of rat cardiomyocytes with H_2O_2 or A23187 induced NRDC secretion without cell toxicity. While NRDC is widely expressed throughout the whole body, our findings from immunohistochemistry in human autopsy hearts (Fig. 3) and mouse ACS models (Fig. 4C) strongly suggest that the source of serum NRDC is cardiomyocytes. Immunoblot of mouse heart (Fig. 4D) and conditioned medium of cardiomyocytes (Fig. 4E, F) also support the hypothesis that NRDC is released from cardiomyocytes in response to ischemia. It is critical for future investigation to clarify the mechanisms and significance of ischemia-induced rapid disappearance of NRDC from cardiomyocytes.

ACS is classified into two groups based on the ECG, ST-elevation MI (STEMI), and non-ST-elevation ACS (NSTE-ACS). NSTE-ACS is further

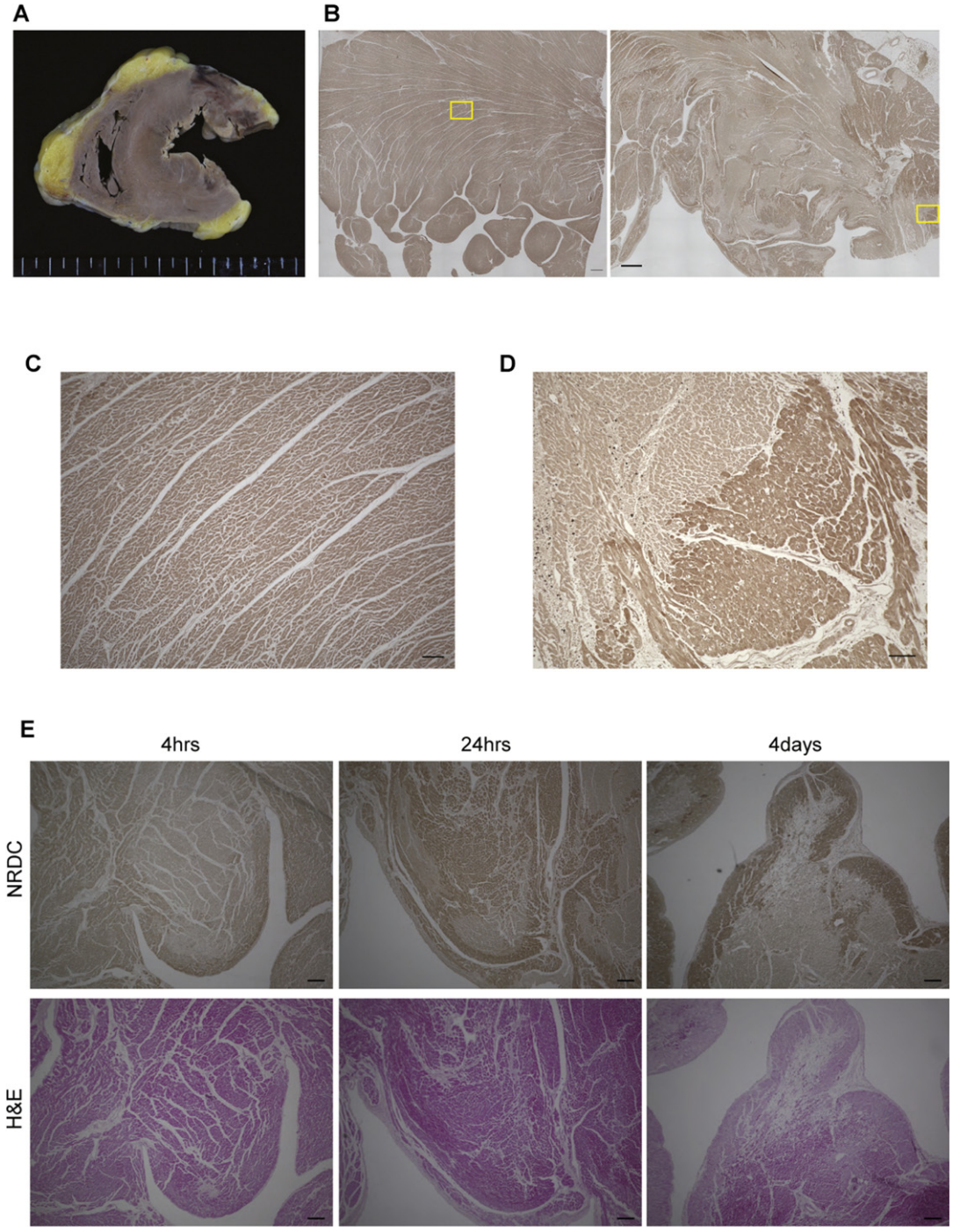

Fig. 3. NRDC protein expression is reduced in necrotic myocardial regions in human autopsy samples. (A) A heart from a patient who died from cardiac rupture 24 h after the onset of AMI. An incision was administered to explore the tear of the ruptured point of the left ventricular wall. Sections used for H&E and immunostaining were made from the site of incision of MI lesions. (B) Immunostaining of NRDC in the heart sections from a patient who died of a non-cardiac cause (left panel) and from an AMI patient (right panel) in lower magnification. Squares indicate the focused area described in C and D, respectively. Scale bar; 2 mm (C, D) Immunostaining of NRDC in the heart sections from a patient who died of a non-cardiac cause (C) and from an AMI patient (D) in higher magnification. Note that NRDC expression is dramatically reduced in necrotizing cardiomyocytes (D). Scale bar; 200 μm (E) Immunostaining of NRDC (Upper panels) and H&E staining (Lower panels) in heart sections from AMI patients who died 4 h, 24 h (different from the patient in A), or 4 days after the onset. Note that the contrast between the necrotized and viable regions 4 h after the onset is obscure in H&E staining, while it is clear in NRDC immunostaining. AMI indicates acute myocardial infarction; H&E, hematoxylin and eosin; and NRDC, nardilysin.

divided into two categories, NSTE-MI and UA, by the presence or absence of cardiomyocyte necrosis. Myocardial necrosis is detected by a rise in necrosis marker, preferably cTn, above the 99th percentile upper reference limit (URL) [16]. The major weakness of necrosis markers is their delayed release after cell necrosis. Although the recent advance of immunoassay for cTn has enabled earlier detection of cell necrosis, multiple samplings are still often required for rule-in and rule-out of AMI in patients who present early (<3 h) after chest pain onset [17]. The latest generation of high-sensitivity cTn assays can detect cTn in >95% of a reference population, and the URL has markedly declined to 0.0028–0.01 ng/ml [18,19]. As predicted, the sensitivity of cTn assays for the diagnosis of AMI has improved, while the specificity has declined. The abnormal elevations of cTn have been observed in a wide variety of cardiac and non-cardiac conditions including stable

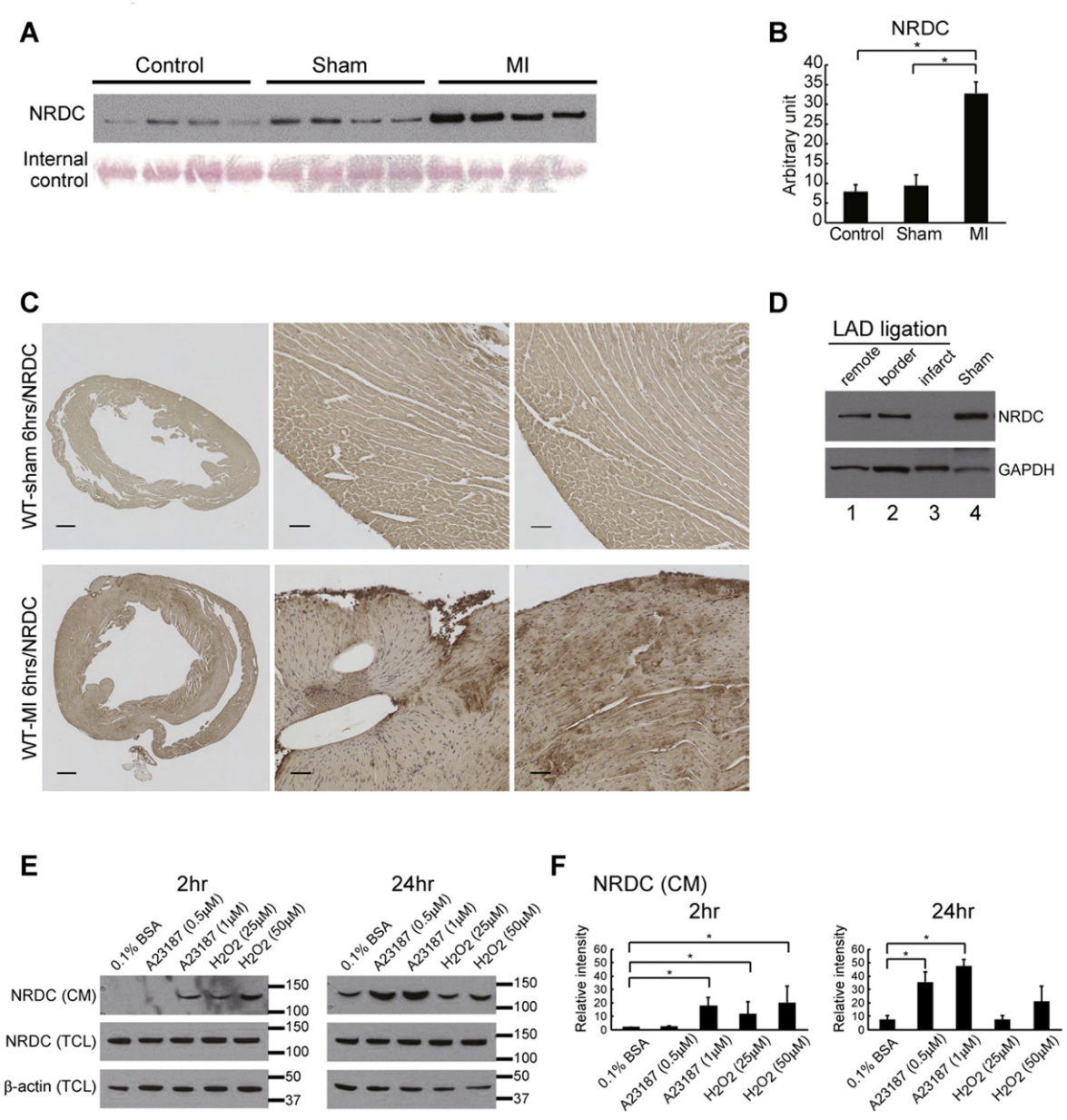

Fig. 4. Serum NRDC is acutely increased in a mouse model of MI, while NRDC is secreted from cultured cardiomyocytes. (A) Detection of mouse serum NRDC by the combination of immunoprecipitation and immunoblot. NRDC is clearly increased in the serum obtained at 90 min after LAD ligation in a mouse model of MI, but not in the serum from control or sham-operated mice (Sham). (B) Densitometric analysis of the signals of the immunoblot in A. The intensity of NRDC was divided by that of internal control by ponceau-S staining ($N = 4$/each group, $P < 0.05$). (C) Immunostaining of NRDC in heart sections from mice that underwent a sham operation (upper panel) and LAD ligation (lower panel). Hearts were obtained 6 h after the operation. Immunoreactivity of NRDC is diminished in the infarcted area in mice with LAD ligation. Scale bar; 500 μm (left column), 50 μm (middle and right column). (D) Immunoblot analysis of NRDC in heart extracts from mice with LAD ligation (Lane 1–3) or a sham operation (Lane 4). In mice with LAD ligation, the left ventricles were divided into the infarcted region (infarct), the viable region remote from the infracted area (remote) and the border zone between infracted and viable regions (border). H&E indicates hematoxylin and eosin; LAD = left anterior descending coronary artery; MI, myocardial infarction; and NRDC, nardilysin. (E) Immunoblot analysis of NRDC in the conditioned medium and total cell lysates (TCL) of rat cardiomyocytes. The conditioned medium and total cell lysates were collected after the treatment of cells with hydrogen peroxide (H_2O_2) or calcium ionophore (A23187) for 2h (right panel) or 24h (left panel). Comparable results were obtained in four independent experiments, and representative gels are shown. (F) Quantification of secreted NRDC (CM) in A by densitometriy. Intensity of signals relative to that of the control lane (Lane 1 of 2 h gel) is shown ($N = 4$, Mean + standard deviation).

angina, heart failure, tachyarrhythmia, renal failure, and even after exercise [20]. To overcome these problems in cTn testing, the simultaneous measurement of non-necrosis markers representing various pathophysiological pathways, such as inflammation, platelet activation, and ischemia, has been studied in several large trials [21,22,23,24]. Importantly, none of the markers were able to show incremental values for the early diagnosis of AMI. Given the possible detection of severe but transient ischemia by NRDC, serum NRDC might be useful for the early rule-in of ACS, especially in patients with a low probability of ACS in clinical findings. On the other hand, negativity of both NRDC and cTn may be helpful for the early and safe rule-out of ACS.

Interestingly, serum NRDC positively correlated with white blood cell (WBC) number and C-reactive protein (CRP) in UA patients (Supplementary Table 5). The pro-inflammatory roles of NRDC have been

suggested in basic research studies. For example, TNF-α is activated by NRDC via enhancement of ectodomain shedding [10]. Moreover, NRDC-induced TNF-α activation was shown to be critically involved in the promotion of gastric cancer and steatohepatitis [11,14]. Transient ischemia-induced secretion of NRDC from cardiomyocytes may enhance inflammation in vulnerable plaques via TNF-α shedding in leukocytes. We demonstrate here that NRDC is highly expressed in accumulated leukocytes in injured cardiomyocytes (Supplementary Fig. 1), suggesting that the alternative source of serum NRDC is inflammatory leukocytes.

In this study, we initially screened autoantibodies elevated in patients with ACS in order to specify novel biomarkers for the prediction of ACS. As a result, autoantibodies against 19 molecules, including NRDC, were identified, and we confirmed that NRDC protein levels are elevated in the sera of patients with ACS. Given that the serum NRDC level peaked at admission in ACS patients, it is possible that NRDC is elevated before the onset of ACS. This hypothesis is supported by the fact that NRDC is elevated in patients with UA, several of whom developed AMI. In other words, our results indicate that the high throughput screening of autoantibodies may be a suitable way to identify novel biomarkers for ACS prediction. We are currently measuring titers of the autoantibodies identified in this study in a community-based large cohort to assess the capacity of those autoantibodies to predict ACS.

There are several limitations of this study. The present study was a single-center small retrospective study, and the results need to be replicated in larger prospective multicenter studies. We used conventional point-of-care testing for cTnT, in which the quantitative measuring range is 0.1–2 ng/ml. Thus, the discharge diagnosis of UA would be changed to NSTMI if a high-sensitivity assay for cTn measurement was used. Blood samples were collected at admission in most cases, whereas some of them were collected prior to urgent coronary angiography. As the chemiluminescent enzyme immunoassay for serum NRDC was improved between Studies 1 and 2, the cutoff values are different between the two studies. The cutoff values (Mean + 2SD) were determined by measuring the average (Mean) and standard deviation (SD) of two different reference populations (112 and 100 healthy volunteers); therefore, the values need to be determined by larger reference populations.

In conclusion, we demonstrate here for the first time that autoantibodies against NRDC and NRDC protein level are elevated in the sera of patients with ACS. NRDC is a promising biomarker for ACS, because serum NRDC is elevated at the very early phase of AMI and UA. NRDC has characteristics of a non-necrosis marker, thus may be useful for the diagnosis of ACS in combination with cTn.

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Disclosures

The authors report no relationships that could be construed as a conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijcard.2017.04.047.

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