Neutrophil extracellular traps in ischemia-reperfusion injury-induced myocardial no-reflow: therapeutic potential of DNase-based reperfusion strategy

Lan Ge,1,* Xin Zhou,1* Wen-Jie Ji,1 Rui-Yi Lu,1 Yan Zhang,2 Yi-Dan Zhang,1 Yong-Qiang Ma,1 Ji-Hong Zhao,1 and Yu-Ming Li1

1Tianjin Key Laboratory of Cardiovascular Remodeling and Target Organ Injury, Institute of Cardiovascular Disease and Heart Center, Pingjin Hospital, Logistics University of the Chinese People’s Armed Police Forces, Tianjin, China; and 2Institute of Molecular Medicine, Peking University, Peking, China

Submitted 4 June 2014; accepted in final form 17 December 2014

Neutrophil extracellular traps (NETs) are a weblike structure composed of decondensed chromatin (DNA backbone) and antimicrobial proteins that are released from neutrophils in response to microorganisms, activated platelets (8), activated endothelial cells (22), placental microdebris (21), and proinflammatory cytokines (44). The process by which NETs are formed is termed NETosis (63). Mounting evidence shows that NETs play an important role in the innate immune response against microbial infections by trapping, immobilizing, and killing the invading pathogens with the aid of locally high concentrations of antimicrobial proteins (7, 26).

Recent evidence also implicates a potential role of NETs in linking sterile inflammation with thrombosis (5, 17, 18, 26, 38). As a consequence of NETosis, externalized nucleosomes, in conjunction with neutrophil elastase and cathepsin G, can induce intravascular thrombus formation without a pathogen challenge (39). In this way, NETs would serve as a platform that allows the carrying cytotoxic proteases in contact with endothelial cells (22, 51). In addition, NETs provide a scaffold for trapping red blood cells (RBCs), contributing to RBC-rich thrombi, especially in deep-vein thrombosis (17, 18). Moreover, previous work has shown that neutrophils are involved in the pathogenesis of myocardial ischemia-reperfusion (I/R) injury (25, 29), which is one of the major reasons accounting for the “no-reflow” phenomenon observed in clinical practice when epicardial coronary artery patency is restored by reperfusion strategies (43). In patients with coronary heart disease, the elevated levels of circulating DNA and chromatin, markers for NET formation, are associated with the prothrombotic state and the occurrence of adverse cardiac events (4).

Because DNA is the backbone of NETs, which could be degraded by deoxyribonuclease I (DNase I) (17), Savchenko and coworkers (52) recently reported that DNase I could cleavage extracellular DNA and limits infarct size in a mouse model using a 1-h ischemia/23-h reperfusion protocol. On the other hand, coronary microthrombosis has been recognized as an important pathophysiological process contributing to I/R injury-induced no-reflow (13, 41, 55). However, tissue-type plasminogen activator (t-PA), a thrombolytic agent, has no proven efficacy on coronary no-reflow (28). A recent work suggested that the combination of DNase I and recombinant t-PA (rt-PA), but not either agent alone, can degrade NET-induced thrombus in vitro (17). It remains unclear whether DNase I, or the combined use of DNase I and t-PA, could have therapeutic potential on coronary no-reflow. Therefore, the present study was designed to demonstrate: 1) the involve-
ment of NETs in myocardial microcirculation obstruction induced by I/R injury; and 2) the therapeutic potential of DNase-based strategies (DNase I alone or in combination with t-PA) on I/R injury-induced myocardial “no reflow”, as well as their long-term benefit on cardiac function.

METHODS

Following the guidelines from the National Institutes of Health, this study was approved by our Institutional Animal Care Use Center and Use Committee.

Animal model. Male Wistar rats (body weight 250–300 g) were used in this study. All rats received humane care in compliance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication no. 85-23, revised 1996). The myocardial I/R injury model with coronary no-reflow was created as described previously (49, 64). Briefly, the rats were anesthetized by intraperitoneal injection of pentobarbital sodium solution (40 mg/kg body wt; Sigma-Aldrich, St. Louis, MO) and then intubated with polyethylene tubing and placed on artificial ventilation (75 strokes/min, tidal volume = 8 ml/kg) using a volume-controlled small-animal respirator (Inspira 7059; Harvard Apparatus, Holliston, MA). Following a left thoracotomy, the left anterior descending (LAD) coronary artery was ligated. After LAD ligation, ischemia was induced for 45 min. Five minutes before reperfusion, intervention drugs were administered by intravenous injection. The control group received an equal amount of vehicle (physiological saline). A successful operation was confirmed by the appearance of a deep S wave, subsequent ST segment elevation, and ventricular arrhythmia on continuous ECG monitoring (MP150, Biopac Systems, Goleta, CA). Postsurgical analgesia was provided by intramuscular injection of buprenorphine (0.1 mg/kg). The sham-operated group received the same surgical procedure, except LAD was not occluded. The mortality rate during operation was ~30%, and no difference was observed among the different groups.

Research protocols. To evaluate the effects of DNase-based interventions on in situ NET formation, neutrophil infiltration, myocardial myeloperoxidase (MPO) activity, no-reflow area, and infarct size, the following five groups of survival rats were used: I/R control (n = 33) group, DNase (DNase I, Sigma-Aldrich) group (n = 35), DNase plus rt-PA treatment group (n = 33), and sham control group (n = 32). Immediately after 3 h of reperfusion, some rats in each group (n = 22–27 for each group) were euthanized, and their hearts were harvested for histological analyses (see below), and some rats in each group (n = 5–6 for each group) were used for myocardial MPO activity assays. The dose for DNase I was 20 μg/rat. Assuming that an adult rat has a blood volume of ~20 ml, a bolus injection of 20 μg would yield a transient plasma level of ~1.0 μg/ml. This concentration was shown having an in vitro capability of reducing sputum viscosity induced by NET formation (54). The bolus injection dose for rt-PA was 420 μg/rat, producing a transient plasma level of ~20 μg/ml rt-PA, similar to the study by Fuchs and colleagues (17), which showed that rt-PA in a concentration of 25 μg/ml, in combination with DNase I, can degrade NET-induced thrombus in vitro.

To evaluate the long-term left ventricular (LV) functional and structural changes after DNase-based treatments, the remaining rats in the above five groups (n = 5 for each group), as well as another three groups (sham plus DNase, sham plus rt-PA, and sham plus DNase and rt-PA), which were used to exclude the direct effects of DNase, rt-PA, and their combination on LV remodeling (n = 5 for each group), were subjected to LV echocardiographic, invasive LV hemodynamic and LV histological analyses 45 days after operation. A graphic description of the research protocol is shown in Fig. 1.

Detection of myocardial neutrophil infiltration and NET formation. After 3 h of reperfusion, the rat hearts were fixed with paraformaldehyde, embedded in paraffin, and sectioned at 5-μm intervals. In situ triple-immunofluorescence (DNA, histone, and neutrophil markers) staining was performed, as previously described (27). Briefly, the slices were treated with citrate buffer for antigen retrieval, subsequently blocked with donkey serum, and incubated with a mixture of primary antibodies against histone H2B (marker for decondensed DNA, 1:200, Abcam) and MPO (marker for neutrophil, 1:200, Abcam) in a humidified chamber at 4°C overnight. Tissue sections were then rinsed using 0.01 M phosphate-buffered saline (PBS), and species-specific secondary antibodies were applied for 1 h at room temperature. Finally, the sections were washed again with 0.01 M PBS and incubated with 4,6-diamidino-2-phenylindole (DAPI) stain and mounted. The stained slices were scanned and digitized using a Zeiss LSM 700 confocal microscope. The acquired images were analyzed using the Zeiss ZEN software.

To quantify the number of infiltrating neutrophils and NET density, the regions from the LV ischemic area and the corresponding LV free wall (sham group) were randomly selected for image analyses. Cells colocalized with DNA and MPO were recognized as neutrophils. Cells colocalized with DNA, histone, and MPO, or cell clusters with a mesh-like structure that was colocalized with DNA, histone, and MPO, were considered as NETs. Neutrophil and NET density was expressed as the number of neutrophils and NETs per ×200 field. In each animal, five random fields were counted and averaged. The hearts from sham-operated rats were used as controls for neutrophil infiltration and NET formation.

Determination of MPO activity. Myocardial MPO activity was quantified according to previous work (9, 42). Briefly, the heart tissue from the ischemic region in LV free wall was homogenized for 30 s on ice in hexadecyltrimethylammonium bromide (HETAB; from Sigma Aldrich; 1 ml HETAB to 100 mg heart tissue) in 50 mM potassium phosphate buffer (pH 6.0). Then, samples were centrifuged (13,000 g for 20 min at 4°C) to remove debris. Protein concentrations in the supernatant were determined using a biocinchoninic acid protein

Fig. 1. Research protocols. TTC, 2,3,5-triphenyl tetrazolium chloride; I/R, ischemia-reperfusion; LV, left ventricular.
assay kit (Pierce, Rockford, IL). Fifty microliters of supernatant of each sample were added to 50 μl of o-dianisidine dihydrochloride (0.025% in sodium citrate buffer with 0.5% HETAB) in a 96-well plate. After the addition of 50 μl of 0.01% hydrogen peroxide, the change of optical density was measured using a microplate spectrophotometer (xMark, Bio-Rad, Hercules, CA) at 510 nm over 3 min. A standard curve was established using dilutions of a human neutrophil MPO (Sigma-Aldrich). One unit of MPO activity was defined as when there was an increase in optical density of one per minute at pH 7.0 at 25°C with the adding of guaiacol substrate (Sigma-Aldrich). The MPO activity was normalized to supernatant protein concentration, as expressed as MPO unit per 100 milligrams protein.

**Evaluation of anatomic no-reflow area.** After 3-h of reperfusion, 4% fluorescent dye thioflavin S (1 ml/kg, Sigma-Aldrich) saline solution was perfused retrogradely through the carotid artery to delineate the area of reflow (49). The LAD was then religated at the original site, and 2% Evans blue dye (0.4 ml/kg, Sigma-Aldrich) saline solution was perfused to outline the area at risk (AAR). The heart was excised, sliced transversely into six slices, and photographed using a fluorescence microscope (Eclipse 80i, Nikon, Tokyo, Japan). Under ultraviolet light, Evans blue stains the perfused myocardium dark blue, and thioflavin S stains the perfused myocardium bright blue, whereas the zone of anatomical no-reflow remains dark. Sham-operated rats injected with either thioflavin S or Evans blue served as controls.

**Evaluation of ischemic AAR and infarct size.** After 3 h of reperfusion, the thoracotomy was reopened under general anesthesia, and the LAD was reoccluded in situ, and 2% Evans blue dye were perfused retrogradely in the carotid artery to delineate the AAR (Evans blue nonperfused region) from the nonischemic zone. The heart was cut in six transverse slices. The slices were then incubated for 15 min in 1% 2,3,5-triphenyl tetrazolium chloride (TTC) in 0.1 M PBS (pH = 7.4, 37°C) to evaluate the infarct area (IA). The IA, AAR, and total cross-sectional heart area (TA) were measured using Image Pro Plus software (version 4.5, Media Cybernetics). The myocardial infarct size is presented as the IA divided by the AAR (IA/AAR).

**Cardiomyocyte hypertrophy and fibrosis.** For the determination of cardiomyocyte hypertrophy in the non-ischemic region in hearts 45 days postoperation, heart tissue was fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 7-μm sections. After incubation with tetramethylrhodamine isothiocyanate-labeled wheat germ agglutinin (1:200), as previously described (62, 65), the wheat germ agglutinin-stained sections were then counterstained with DAPI. The images were acquired and analyzed using Image Pro Plus software to calculate myocardial cross-sectional area. Masson’s trichrome staining was used to reveal the LV fibrotic area in the hearts 45 days postoperation, as previously described (37). The LV collagen volume fraction and LV index of expansion (IE) were determined using Image Pro Plus software (version 4.5, Media Cybernetics).

**RESULTS**

**Detection of NET formation in I/R-challenged myocardium.** As shown in Fig. 2A, the LV ischemic region with increased neutrophil (MPO positive) density following 45-min ischemia/3-h reperfusion also presented with intense immunostaining for NETs, which were characterized by colocalization of DNA (DAPI, blue), histone (H2B; FITC-labeled, green), and MPO (rhodamine-labeled, red), with a decondensed extracellular weblike structure (white arrows).

**DNase-based interventions reduce I/R-induced neutrophil accumulation, NET formation, and MPO activity.** As shown in Fig. 2B and C, myocardial neutrophil (MPO-positive cells) accumulation (per ×200 field) was significantly decreased by DNase I-based interventions (21.0 ± 7.9 in the DNase I-treated group; and 24.0 ± 9.0 in the DNase I plus rt-PA group), compared with the I/R control (113 ± 21, all P < 0.001) and rt-PA (63.5 ± 8.7, all P < 0.05) groups. Accordingly, the amount of NET structure (per ×200 field) was also significantly reduced by DNase-based interventions (26.9 ± 3.1 in the I/R control group, 8.9 ± 2.2 in the DNase I group, and 8.4 ± 2.0 in the DNase I plus rt-PA group; P < 0.001 vs. I/R controls). Of note, rt-PA treatment had no obvious effect on the NET counts. No NET-like structure was detected in the sham control group. Consistent with the changes of neutrophil density, myocardial MPO activity presented with parallel changes, which were significantly reduced by DNase I-based interventions (Fig. 2C).

**DNase I combined with rt-PA reduces myocardial I/R-induced anatomic no-reflow.** Using a well-characterized method (thioflavin S plus Evans blue under ultraviolet light) for the detection of myocardial anatomic no-reflow, we showed that, in this model, the ratio of AAR to TA of the heart transverse section was comparable, indicating similar magnitude of ischemic insult among all groups (Fig. 3H). Based on this condition, we found that the combined intervention by DNase I and rt-PA significantly decreased the no-reflow area in the ischemic region, compared with the I/R controls (15.2 ± 5.6 vs. 34.6 ± 5.0%, P < 0.05). No significant difference in no-reflow area size was observed in DNase I- or rt-PA alone-treated groups compared with the I/R control group (all P > 0.05) (Fig. 3I).

**DNase I combined with rt-PA limits infarct size.** To examine the effect of DNase-based interventions on early myocardial salvage, we used Evans blue plus TTC staining. Representative heart sections are shown in Fig. 4A. As shown in Fig. 4B, no significant difference was found in the ratio of AAR to TA between the groups (Fig. 4B). Similar to the result of myocardial no-reflow area, there was a significant decrease in the IA-to-AAR ratio in the DNase I + rt-PA group compared with the I/R control group (38.4 ± 5.0 vs. 71.0 ± 3.5%; P < 0.001). No significant difference in IA-to-AAR ratio was observed in either the DNase I- or rt-PA-treated groups, compared with the I/R control group (Fig. 4C).
DNase I combined with rt-PA improves long-term postinfarction LV remodeling. We then evaluated the effect of DNase-based interventions on long-term LV functional changes by transthoracic echocardiography (Fig. 5), and by invasive LV hemodynamics (Fig. 6) at time of death on day 45 postoperation. The transthoracic echocardiographic analyses revealed that, compared with the I/R control group, combined treatment of DNase I and rt-PA could attenuate postinfarction LV remodeling, as shown by decreased LV end-systolic volume and LV end-diastolic volume, as well as improved LV ejection fraction (64.22 ± 3.37 vs. 33.81 ± 2.98%, P < 0.05) and LV fraction shortening (Figs. 5, A and B).

The invasive LV hemodynamic measurements are shown in Fig. 5C. In accordance with echocardiographic results, the combination of DNase + rt-PA led to a significant improvement in LV systolic (+dP/dt max: 3,785 ± 216 vs. 2,596 ± 299
mmHg/s, \( P < 0.05 \) and diastolic \( (-\frac{dP}{dt}_{\text{min}}; -3.409 \pm 154 \text{ vs. } -2.303 \pm 260 \text{ mmHg/s, } P < 0.05) \) functions, compared with the I/R control group. Additionally, to exclude the possible influence of DNase-based interventions on normal cardiac function, we also analyzed transthoracic echocardiography and invasive LV hemodynamics in DNase I-, rt-PA-, and their combination-treated sham animals. The results excluded potential impact of these interventions on LV function (Fig. 5).

Consistent with LV functional changes, the heart morphological and histological analyses at 45 days after operation (Fig. 6) showed that the combined treatment with DNase I and rt-PA was associated with decreased LV chamber size enlarge-

![Fig. 3. DNase I combined with rt-PA attenuated I/R injury-induced myocardial anatomic “no-reflow.” A–E: representative thioflavin S and Evans blue staining under ultraviolet light. F and G: the schemes of how to recognize the no-reflow region and the Evans blue perfused region, respectively. H and I: the statistical comparisons of the ratio of the area at risk (AAR) to the total heart section area (TA) and the ratio of the no-reflow area (NR) to the AAR, respectively. The horizontal lines in the scatter plots indicate the means. * \( P < 0.05 \).

![Fig. 4. DNase I combined with rt-PA limits infarct size. A: representative TTC and Evans blue-stained transverse heart sections. The dark blue area indicates the Evans blue perfused regions. The non-Evans blue perfused region indicates the AAR. The red area indicates viable myocardium, whereas the white region within the AAR indicates the infarct area (IA). B and C: the statistical comparisons of the AAR to the TA, the index of ischemia magnitude, and the IA to AAR ratio, an index of myocardial salvage, respectively. The horizontal lines in the scatter plots indicate the means. *** \( P < 0.001 \).]

![Fig. 5. DNase I combined with rt-PA limits infarct size. A: representative TTC and Evans blue-stained transverse heart sections. The dark blue area indicates the Evans blue perfused regions. The non-Evans blue perfused region indicates the AAR. The red area indicates viable myocardium, whereas the white region within the AAR indicates the infarct area (IA). B and C: the statistical comparisons of the AAR to the TA, the index of ischemia magnitude, and the IA to AAR ratio, an index of myocardial salvage, respectively. The horizontal lines in the scatter plots indicate the means. *** \( P < 0.001 \).]
NETs and Myocardial I/R Injury

**DISCUSSION**

Myocardial I/R injury-induced alterations, including cytokine upregulation (6), autophagy activation (50), and ROS generation, are essential for NETosis (23). In anoxic myocardial microcirculation, the formation of NETs not only provides a scaffold for thrombosis, but also can aggravate endothelium injury, both of which are key pathological features of coronary no-reflow. In this study, we demonstrated the existence of NETs in rat myocardium during I/R injury and found that a DNase-based regimen, which targets NETs (by DNase I) and fibrin (by rt-PA), could reduce NET density, improve coronary microvasculature patency, limit infarct size, and attenuate long-term postinfarction LV remodeling. Thus these results provide experimental evidence for the therapeutic potential of NET-targeted intervention in myocardial I/R injury and coronary “no reflow.”

Our study highlights the synergistic effect of DNase I and rt-PA in the treatment of myocardial I/R injury-induced microcirculation obstruction. In line with a previous report using a canine model (28), we also confirmed the ineffectiveness of rt-PA in ameliorating coronary no-reflow. In terms of DNase I alone-treated group, we did not observe a protective effect, which is inconsistent with recently published data by Savchenko and colleagues (52). It seems that...
the difference in DNase I may play a role in accounting for the differential outcome: we used one bolus of intravenous DNase I administration (20 μg/rat), whereas, in Savchenko’s work, DNase I was administered both intravenously (10 μg) and intraperitoneally (50 μg) twice (120 μg/mouse) in a mouse model using a 60-min, 23-h I/R protocol. However, the following reason should be taken into account. First, even using the relatively low dose (20 μg/rat), we did observe a reduced NET formation and neutrophil infiltration in DNase I alone treated group 3 h after reperfusion (Fig. 2). Our finding that the reduced density of neutrophils and NETs, as well as the decreased MPO activity after DNase I treatment, is not associated with a parallel benefit in infarct size and LV function is supporting the emerging concept that neutrophils per se are not likely playing a central role in I/R injury [with the exception of no-reflow phenomenon (3)], and their accumulation is determined by, rather than a cause of, the magnitude of infarct size (3, 10). In this regard, we noted that there was a difference in infarct size calculation method between our and Savchenko’s report. Because of the anatomic variations in coronary artery distribution, it is a routine procedure to use AAR to correct infarct size determined by TTC staining in each animal. However, this correction was not applied in the report by Savchenko et al. (52). Another reason we chose the low dose is based on the concern that DNase I is a proapoptotic agent (2, 31, 48, 66) and has been used in cancer treatment (35). In the setting of myocardial ischemia, apoptosis was thought to be an important contributor to cardiomyocyte death (60). Therefore, the long-term effect of high-dose DNase I on postinfarction LV remodeling warrants further study. As a fact, in our study, we did not observed a worsened LV function after a bolus injection of 20-μg DNase I in rat.

Recently, the same group mentioned above demonstrated that NET-induced thrombus is resistant to rt-PA in an in vitro model (18). In addition, an extensive network of extracellular histone/DNA complexes was observed in the matrix of human ex vivo thrombi (46). In support of this evidence, the existence of NETs in coronary thrombusectomy specimens from patients who suffered from acute myocardial infarction was recently documented (12). These results are in accordance with our findings and highlight the synergistic effect of DNase I, in combination with thrombolysis, to target NET-mediated thrombosis. Taken together, our data suggest that NET-triggered thrombosis, not neutrophils per se, mediated the I/R injury-induced microcirculation obstruction.

The proposed mechanisms for NET-mediated microvascular thrombosis following myocardial I/R injury are shown in Fig. 7. Ischemic insult contributes to the release of proinflammatory cytokines, such as IL-8, as well as the generation of ROS, which are essential triggers for the induction of NETosis (6, 15, 34). The process of how NETs promote thrombosis coronary microcirculation involves the following mechanisms. First,
NETs can bind and activate factor XII (58) through contact pathway to facilitate the activation of coagulation cascade. Second, the exposure of NET-conjugated histones to circulation results in the binding and activation of platelets, leading to their cross-linking with plasma fibrinogen (16), and platelet-dependent thrombin generation (53), which would lead to the conversion of fibrinogen to fibrin. Third, NETs can bind to tissue factor and promote the activation of extrinsic pathway of coagulation (14). Forth, neutrophil elastase-associated NETs are capable of cleaving tissue factor pathway inhibitor, thus leading to the inactivation of endogenous anticoagulation (39). Consequently, once reperfusion is initiated, the above mechanisms would work in concert with NET- and fibrin-formed scaffold to trap RBCs and hence lead to the propagation of thrombus. Therefore, DNase I, which targets NETs, in combination with rt-PA, which targets fibrin, will be an ideal option to disrupt the NET- and fibrin-provided meshlike backbone structure of thrombus. On the other hand, it is theoretically feasible that upstream intervention targeting NET formation, i.e., the intracellular pathways implicated in the induction of NETs, might also be of therapeutic potential. Currently, these molecular pathways include peptidylarginine deiminase 4 (32, 59), the canonical Raf/MEK/ERK pathway (23), nitric oxide (47), Toll-like receptor-2 and -4 (8, 45, 53, 61), high-mobility group box 1 (20, 56), pentraxin 3 (11, 24), and mammalian targets of rapamycin/hypoxia-inducible factor 1-α (40).

Our study has several limitations. First, the potential pathogenesis of clinically observed no-reflow is multifactorial and may also involve the participation of coronary plaque debris, which could not be duplicated by our models. Second, the therapeutic responses were assessed at one time point following I/R 45 days after operation, and our study protocol included only one dose. Additional studies may be valuable to determine the optimal doses and optimal intervention times. Finally, in our study, the treatment of the NET-targeted regimen was longitudinally assessed using heart morphological and functional methods. Future studies should include the evaluation of the safety and pharmacokinetics of the NET-targeted regimen for clinical translation.

In conclusion, our study provides experimental evidence for the existence of NETs in I/R-challenged myocardium, and we have confirmed the long-term benefit of a novel DNase I-based reperfusion treatment strategy (DNase I + rt-PA) capable of attenuating experimental myocardial I/R injury and coronary “no reflow.” This work may be relevant when designing new treatment regimens for clinical practice.

GRANTS
This work was supported by National Natural Science Foundation of China (81070121, 81102088, and 81170328), Tianjin Municipal Science and Technology Committee (09CZDSP04200, 11JCYBJC12000, and 12JCYBJC16600), and intramural research program from Pingjin Hospital and Logistics University of the Chinese People’s Armed Police Forces (FYM201104 and WHM201218).

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).
AUTHOR CONTRIBUTIONS


REFERENCES


AJP-Heart Circ Physiol • doi:10.1152/ajpheart.00381.2014 • www.ajpheart.org


