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Spaciotemporal Alteration of 8-hydroxy-2′-deoxyguanosine Levels in Cardiomyocytes After Myocardial Infarction in Rats

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Temporary or persistent heart failure is one of the major complications after myocardial infarction (MI). In order to elucidate the pathogenesis of MI, we studied the spaciotemporal alteration of 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels in cardiomyocytes in a rat model of ligation of the left anterior descending branch of the coronary artery. The lethality in this model was 18%. Hearts were dissected at 0, 3, 6, 12, 24, 48 h, and 1, 2, 4, 6 weeks after the operation. The cardiac level of 8-OHdG was evaluated biochemically as well as by immunohistochemistry with monoclonal antibody N45.1. Three to 6 h after ligation, the 8-OHdG levels were increased in the cardiomyocytes of MI (six-fold) and peri-MI (four-fold) areas. After 24 h, the myocardium in the MI area was necrotized, and thereafter the 8-OHdG level decreased. 8-OHdG levels in the myocardium of peri-MI areas returned once to a normal level, but were significantly increased at 2–4 weeks along with the appearance of apoptotic cardiomyocytes in this area. The heart after MI has been generally considered as clinically stable after four weeks. However, cardiomyocytes near the infarcted area were oxidatively stressed even after four weeks when the affected lesion was extensive. The present data support the use of supplementary antioxidant therapies to save functional myocardium after MI. (213 words)

Keywords: Myocardial infarction; 8-Hydroxy-2′-deoxygunosine; Oxidative stress; Apoptosis; Immunohistochemistry

INTRODUCTION

Temporary or persistent heart failure is one of the major complications after myocardial infarction (MI), and may lead to a grave prognosis. Loss of functioning cardiomyocytes due to MI results in a chronic increase in the working load of the remaining functional myocardium. Recently, it was shown that heart failure subsequent to MI is associated with a deficit in antioxidative protection mechanisms such as those involving catalase, superoxide dismutase, and glutathione peroxidase, which results in increased thiobarbituric acid-reactive substances, and that these changes were in parallel with changes of hemodynamic function.[1] On the other hand, Ide *et al.* showed that in the failing myocardium, hydroxyl radicals were produced as a reaction product of superoxide and H_2O_2, which might play an important role in left ventricular failure.[2] However, there is a scarcity of data on when and which part of the myocardium is oxidatively stressed after MI.

DNA is one of the major targets of reactive oxygen species. Hydroxylation of the C-8 position of 2′-deoxyguanosine 8-hydroxy-2′-deoxyguanosine, (8-OHdG) by ascorbic acid in the presence of oxygen was first reported by Kasai and Nishimura in 1984 in a study of the isolation of mutagens and carcinogens from heated glucose.[3] It has been established that either hydroxyl radical, singlet oxygen or direct photodynamic action is responsible for the formation of 8-OHdG (currently one of the most commonly used markers for assessing oxidative DNA damage).[4] It was also shown that peroxynitrite

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can induce 8-OHdG through an active intermediate similar to hydroxyl radical.[5]

This DNA base-modified product has been frequently used as a marker because of the ability to detect it with high sensitivity by high performance liquid chromatography with an electrochemical detector (HPLC-ECD).[6,7] Recently, a specific monoclonal antibody to 8-OHdG (N45.1) has been developed for use in immunohistochemistry, using which the localization of 8-OHdG can be studied.[4,8] This immunohistochemical method has been used to clarify the involvement of reactive oxygen species in a variety of pathologic situations such as iron overload[8] and arsenic-induced carcinogenesis,[9] as well as in lectin-like oxidized low-density lipoprotein receptor-1-induced apoptosis in cultured neonatal rat cardiac myocytes.[10] However, as far as we know, the level of 8-OHdG in the myocardium after MI has not been evaluated.

In the present study, we therefore studied the spaciotemporal alterations in the levels of 8-OHdG in three distinct portions (infarcted portion, peri-infarction portion, and non-infarction portion) of the myocardium after MI. Further, we analyzed the population of apoptotic cardiomyocytes in the acute and chronic stages of MI. Our data show for the first time that the levels of 8-OHdG in the myocardium are closely associated with the pathological sequence and chronic heart failure of MI.

MATERIALS AND METHODS

Animal Model and Experiments

A total of 66 10-week-old Sprague-Dawley rats (Shizuoka Laboratory Animal Center, Shizuoka, Japan) weighing 310–360 g were divided into 11 groups, which were assigned to different time-course groups as described below ($N = 6$ each group). Six animals served as unoperated controls for evaluating weight changes. The rats were orally intubated, placed on a volume-cycled ventilator for small animals (Rodent Ventilator, Model 683, Harvard, MA) under the induction of general anesthesia using diethylether (99.5%), and were maintained under anesthesia with 1–2% isoflurane as previously described.[11] Thoracotomy was done at the 4th intercostal space to reach the heart during surgery. The left anterior descending branch of the coronary artery in the rats were ligated with a 5–0 polypropylene suture (Prolene®, Ethicon, Inc., NJ) according to a previously described method[12,13] with slight modifications.[14,15] This model causes medium-sized MI: approximately one-third of the left ventricle (LV) is affected. The animals were maintained under close observation after the operation, and killed under anesthesia at 0, 3, 6, 12, 24, 48 h, or 1, 2, 4, 6 weeks after the ligation. The heart was dissected immediately, and divided into the three areas described above using a scalpel as described below. All the animal experiments in the present study were performed according to the institutional guidelines on animal experimentation of Kyoto University, which conform to the law "Guidance for the Care and Use of Laboratory Animals" in Japan.

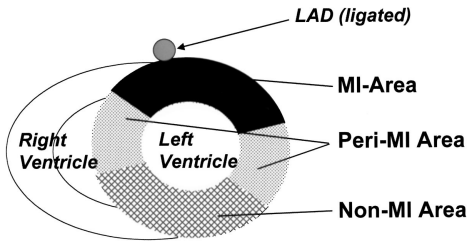

FIGURE 1 Definition of area in a transverse section of heart. Left ventricle excluding MI area was divided into peri-MI and non-MI areas. LV, left ventricle; RV, right ventricle; MI, myocardial infarction.

Definition of Areas in the Left Ventricle

The heart was at first dissected transversely in the middle; the chephalic portion was used for immunohistochemistry, and the caudal portion for the determination of 8-OHdG by HPLC-ECD as follows. The caudal part of the LV excluding the MI area was divided into four blocks of approximately the same size: two peri-MI areas and two non-MI areas as shown in Fig. 1.

Measurement of Left Ventricular Function

A two-dimensional targeted M-mode echocardiogram using a 7.5 MHz phased-array transducer (Hewlett Packard Sonos 1000®, Andover, MA) was obtained along the short-axis view of the LV at the level of the papillary muscles under mild anesthesia with sodium pentobarbital (15 mg/kg i.m.).[16,17] The left ventricular internal dimension at end diastole (LVIDd) and fractional shortenings (FS) were measured as previously described.[18,19] This procedure was performed immediately before killing each animal.

Measurement of 8-OHdG by HPLC-ECD

Cardiac tissues were immediately frozen after dissection in liquid nitrogen, and were preserved at −80°C until use. For quantitation of 8-OHdG, frozen samples (approximately 100 mg) were finely cut with a razor blade on dry ice, followed by manual homogenization using a Potter-type homogenizer with 1.5 ml of cold 0.15 M NaCl/0.1 M EDTA (pH 8.0)

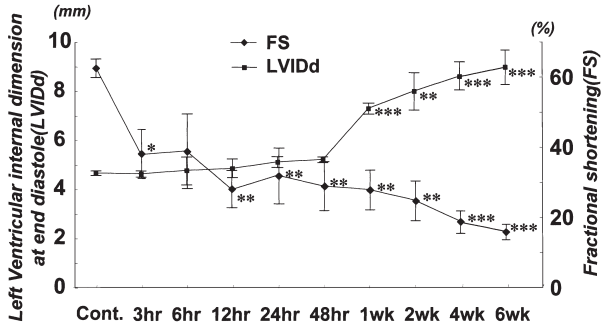

FIGURE 2 Alteration of left ventricular function after myocardial function. LV IDD, left ventricular internal dimension at end diastole; FS, fractional shortenings. Refer to Materials and Methods for details.

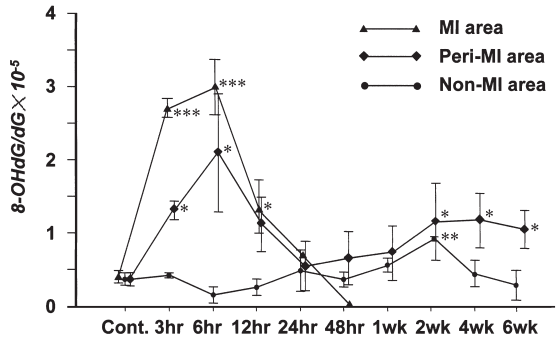

FIGURE 3 Levels of 8-hydroxy-2′-deoxyguanosine determined by high performance liquid chromatography and electrochemical detector. MI, peri-MI and non-MI areas are defined as in Fig. 1.

saturated with argon gas. DNA was extracted by the NaI method using a kit (DNA extractor WB kit; Wako, Osaka) according to the manufacturer's instructions. DNA was suspended in 100 μl of cold 1 mM EDTA (pH 8.0), and denatured by heating at 95°C for 5 min. DNA was then digested with nuclease P_1 (Sigma, St. Louis, MO) and thereafter with alkaline phophatase as previously described.[8] Finally, the solution was centrifuged at 10,000*g* for 10 min, and the supernatant filtered through a 0.22 μm filter (Millipore, Bedford, MA) was used for HPLC-ECD analyses.[8] 8-OHdG and 2′-deoxyguanosine used as standards were obtained from Sigma.

Immunohistochemistry

Cardiac tissues were fixed overnight in Bouin's solution[20] immediately after dissection, and then dehydrated sequentially with 50 and 70% ethanol for 24 h each. The avidin–biotin complex method was performed as previously described.[8] Briefly, after deparaffinization of the specimens, normal rabbit serum (diluted 1:75; Dako Japan Co., Ltd, Kyoto) for the inhibition of nonspecific binding of the secondary antibody, a purified mouse monoclonal antibody against 8-OHdG (N45.1, 10 μg/ml; Japan Institute for the Control of Aging, Fukuroi, Shizuoka, Japan), biotin-labeled rabbit anti-mouse IgG serum (diluted 1:100; Dako), and avidin–biotin complex (diluted 1:100; Vector Laboratories, Burlingame, CA) were sequentially applied. The substrate for alkaline phosphatase (black) was obtained from Vector. No nuclear counterstaining was performed.

Detection of Apoptotic Cardiomyocytes

The TUNEL (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling) assay[21] was performed using a commercial kit (apoptosis *in situ* detection kit; Wako, Osaka) according to the manufacturer's instructions.

Statistical Analysis

All the data are shown as means ± SEM. Statistical analyses were performed using an analysis of variance (ANOVA) and an unpaired *t*-test (StatView®, SAS Institute Inc., CA). Differences were considered as statistically significant at a p value of less than 0.05.

RESULTS

Weight Changes and Lethality

The body weight of the rats after the operation did not increase as much as that of control rats. Four weeks after the operation, the body weight increase of MI groups ranged from 342 ± 4.4 to 371 ± 6.3 g, whereas that of unoperated control animals ranged from 344 ± 6.2 to 410 ± 8.3 g ($p = 0.014$). The lethality after the ligation was 18%.

Cardiac Dimension and Function

After the ligation of the left anterior descending branch, the size of the LV (left ventricle end-diastolic diameter; LVEDD) was significantly increased compared with the controls ($p < 0.001$) because of the impairment of cardiac function. Accordingly, FS was significantly decreased after the ligation ($p < 0.001$). A marked decrease in FS was seen as early as 3 h after the ligation. Dilatation of the LV was observed one week after MI (Fig. 2).

Determination of 8-OHdG

In untreated control cardiac muscle, the level of 8-OHdG was 0.39 ± 0.17 per 10^5 dG (Fig. 3). At this level, essentially no nuclear staining was observed in our immunohistochemical conditions (as in Fig. 4H). Three to 6 h after MI, the levels of 8-OHdG were increased most prominently (six-fold increase) in the areas of ischemia (Figs. 3s and 4A), and clear nuclear

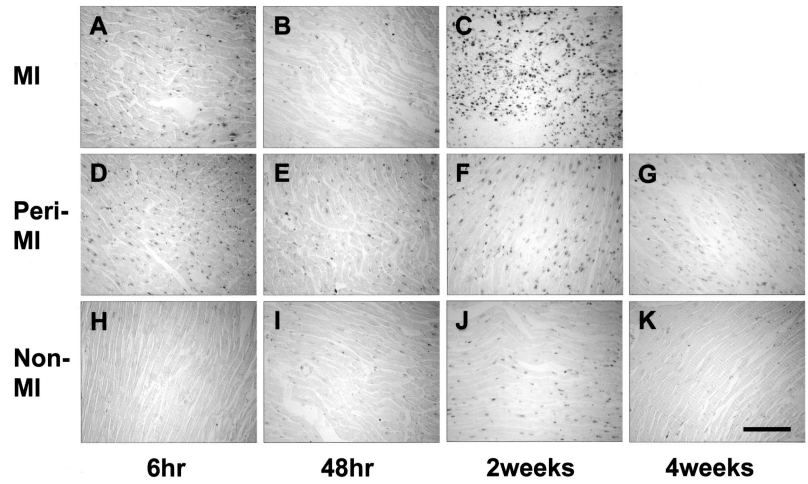

FIGURE 4 Immunohistochemical analysis of 8-hydroxy-2′-deoxyguanosine by the use of specific monoclonal antibody (N45.1). MI, peri-MI and non-MI areas are defined as in Fig. 1. Black substrate was used for final color presentation. Refer to Materials and Methods and Results sections for details. In C, a post-infarction scar area with inflammatory cells is shown. In other panels, immunostaining of cardiomyocytes are shown (bar = 100 μm, with no nuclear counterstaining).

staining of cardiomyocytes was seen with immunohistochemistry. After 12 h, cardiomyocytes in the MI area became gradually necrotized, and the levels of 8-OHdG were decreased (Figs. 3 and 4B).

In peri-MI areas, the level of 8-OHdG was increased in the acute phase in a similar pattern to that in the MI area (4-fold increase; Figs. 3 and 4D), and returned once to a near-normal level at 24 h. However, the levels of 8-OHdG was again increased two weeks after MI (Fig. 4F) whereas the increase was approximately half of that during the acute phase (2-fold increase; $p = 0.007$ *vs* untreated controls). At two weeks, the nuclei of infiltrating inflammatory cells were also stained, though the immunostaining of cardiomyocytes was more prominent (Fig. 4C). At four weeks oxidative stress was still at a high level ($p = 0.038$ *vs* untreated controls) and most of the immunostained nuclei were those of cardiomyocytes (Figs. 3 and 4G).

The level of 8-OHdG in non-MI area was not increased in the acute phase up to 48 h (Figs. 3 and 4H). However, the 8-OHdG level was significantly increased at two weeks (up to 1.5-fold increase; Figs. 3 and 4J). This was confirmed by the immunohistochemical staining of cardiomyocytes. However, 8-OHdG returned to a normal level at six weeks.

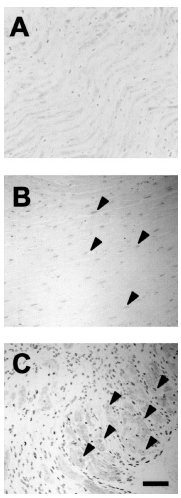

FIGURE 5 Detection of apoptotic cardiomyocytes. A, left ventricle of untreated animal, no apoptosis; B, MI area 6 h after MI, rare apoptosis; C, peri-MI area four weeks after MI; not only cardiomyocytes but also fibroblasts show apoptosis (bar = 50 μm).

Detection of Apoptotic Cardiomyocytes

Unoperated animals showed no apoptotic cardiomyocytes. Three hours after the ligation of the coronary artery, few apoptotic cardiomyocytes were observed in the MI area, and scarcely any were observed in any area from 6 h to one week. However, 2–4 weeks after the operation, cardiomyocytes in the peri-MI area showed apoptosis, suggesting that loss of cardiomyocytes occurred due to apoptosis induced by oxidative DNA damage (Fig. 5). Some of the fibroblasts in the post-infarction scar area showed apoptosis.

DISCUSSION

DNA is a major target of ROS, and approximately 100 kinds of oxidative modifications have thus far been reported.[22] 8-OHdG is one of the major products of oxidative DNA modifications, and thus have been commonly used as a marker of oxidative stress.[23] For example, formation of 8-OHdG is elevated in a variety of carcinogenic models.[7] Urinary excretion of 8-OHdG is elevated in the diabetic state, and as a result of smoking[24] and radiotherapy.[25]

8-OHdG may cause G:C to T:A transversions when present during DNA replication,[26] and high levels of 8-OHdG in nuclear DNA may be associated with cellular apoptosis and necrosis. Indeed, certain high levels of DNA damage lead to apoptosis via the p53 pathway.[27,28] Since cardiomyocytes do not divide once they become differentiated, it is important to evaluate whether high levels of 8-OHdG result in dysfunction and cellular death of the myocardium. Therefore, we assessed the spaciotemporal difference in oxidative stress of the myocardium after medium-sized MI by ligating the left anterior descending branch.

At an early stage up to 24 h, the levels of 8-OHdG in the nuclei of cardiomyocytes were increased in the MI and peri-MI areas. The 8-OHdG level in the MI area was decreased to a subnormal level thereafter because of the necrosis of the myocardium whereas 8-OHdG level in the peri-MI area decreased to a normal level. In the MI area, only rare apoptosis was observed, so high levels of 8-OHdG were associated with necrosis. The source of reactive oxygen species at this stage is not clear at present. A similar phenomenon was also observed in our previous experiments with ischemic skin flaps.[29] We believe that loss of antioxidants, and antioxidative and repair enzymes plays a major part in the increase in 8-OHdG. This is further supported by the recent finding that 8-OHdG is toxic to cultured leukemia cells when the repair enzyme for 8-OHdG is impaired.[30]

Echocardiography revealed an increase in the diameter of the LV one week after MI, suggesting an overload to the remaining cardiac muscle of the LV. Of note was the fact that 8-OHdG level was recurrently increased in the peri-MI area at two and four weeks as well as in the non-MI area at two weeks. Importantly, moderate levels of 8-OHdG were associated with apoptosis of cardiomyocytes at this stage. This second peak appears to be due to stress of myocardium by fibrosis as well as increased workload. Our present results agree well with a recently established concept that reactive oxygen species work as mediators of the reversible ventricular dysfunction that often accompanies reperfusion of the ischemic myocardium.[31]

Immunohistochemical analyses have an advantage for identifying cells with increased levels of 8-OHdG. This methodology was useful for evaluating different areas in the myocardium. 8-OHdG was detected in the nuclei of myocardium throughout the experimental period. Recently, Ide *et al*. reported that mitochondrial DNA damage and dysfunction were associated with oxidative stress in the failing hearts after MI by showing decreased mitochondrial DNA copy number and a parallel decrease in the mitochondrial DNA-encoded gene transcripts.[32] Consistent with their report, we observed cytoplasmic granular positivity of 8-OHdG in the acute phase of MI (Fig. 3A). Immunohistochemistry at the electron-microscopic level might be necessary to prove that mitochondria are damaged.

In conclusion, we observed for the first time that the oxidative stress after MI has two successive peaks, as shown by assessment of 8-OHdG. Our results strongly suggest that supportive antioxidative therapy may be useful after MI, as has been suggested by other investigators.[33,34]

Acknowledgements

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