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Study on Changes of Heme Oxygenase-1 Expression in Patients with Coronary Heart Disease

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Summary

Background: Heme oxygenase (HO) is a rate-limiting enzyme of endogenetic carbon monoxide (CO) that degrades heme into carbon monoxide, bilirubin, and iron. These products have important physiologic effects: bilirubin is a potent antioxidant that can act against ischemia/reperfusion injury; there is a negative correlation between the content of HO-1 and the incidence of coronary heart disease (CHD).

Hypothesis: This study was undertaken to investigate the changes of HO-1 in patients with CHD.

Methods: Thirty-five patients with acute myocardial infarction (AMI), 40 patients with unstable angina pectoris (UAP, diagnosed by coronary angiography), and 30 patients with stable angina pectoris (AP, diagnosed by coronary angiography) were selected for the study; another 30 patients with normal coronary artery (diagnosed by coronary angiography) were selected as controls. The levels of HO-1 protein expression in monocyte and lymphocyte in the subjects were tested by immunohistochemistry and western blot. Computer picture analyzing systems were also used to measure the levels of HO-1 protein expression.

Results: Heme oxygenase-1 protein is located in cell plasma. The levels of HO-1 protein expression in patients with CHD were significantly higher than in those without CHD ($p < 0.01$). There were significant differences of HO-1 expression among the three groups of patients with CHD. The group with AMI was the highest, followed by the group with UAP and finally by the group with AP.

Conclusions: There is a higher expression of HO-1 in patients with CHD. The levels of HO-1 protein are associated with the severity of CHD.

Key words: heme oxygenase-1, coronary heart disease, expression

Introduction

Heme oxygenase (HO) is a rate-limiting enzyme of endogenetic carbon monoxide (CO) that degrades heme into carbon monoxide, bilirubin, and iron. These products have important physiologic effects: bilirubin is a potent antioxidant that can act against ischemia/reperfusion injury; there is a negative correlation between the content of HO-1 and the incidence of coronary heart disease (CHD).[1, 2] Carbon monoxide was previously regarded as a poisonous gas; however, recent studies have shown carbon monoxide to be a factor acting as a potent vasodilator within its physiologic concentration; CO activates soluble guanylate cyclase and elevates the level of cyclic guanosine monophosphate (cGMP) in target tissues, which dilates blood vessels, and also does this by directly activating potassium channels in vascular smooth muscle cells. In addition, CO inhibits platelet aggregation and proliferation of vascular smooth muscle cells, inhibits apoptosis, and stimulates angiogenesis for regulation of cardiovascular function. We investigated the levels of HO-1 protein expression in monocyte and lymphocyte isolated from patients in different stages of CHD to demonstrate the relationship between HO-1 protein and the severity of CHD and to establish a base of application of HO-1 in therapeutic strategies.

Materials and Methods

Sample Collection

Patients were selected from Department of Cardiology, the First Affiliated Hospital, Shantou University Medical College between April 2002 and December 2003. Of the selected 105

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patients with CHD (diagnosed by coronary angiography), 35 were diagnosed as acute myocardial infarction (AMI group: clinical presentations including sharp chest pain exceeding 30 min; ST-segment elevation and abnormal Q wave in the electrocardiogram [ECG] showing kinetic changes; serum creatine kinase [CK]-MB, lactate dehydrogenase [LDH]-1, and troponin I mounting obviously), 40 as unstable angina pectoris (UAP group: including other types of AP except stable AP), and 30 as stable AP (AP group: substernal chest discomfort with a characteristic quality and duration within 30 min, provoked by exertion or emotional stress and relieved by rest or nitroglycerin). Another 30 patients with normal coronary arteries were selected as controls. No subject had confirmed infection within 4 weeks. The history of hypertension, diabetes mellitus, hyperlipidemia, and smoking was recorded simultaneously.

Cell Separation

Lymphocytes and monocytes were isolated by lymphocyte separation medium named PAA, LABORA, TORIES (biological engineering institution of the Chinese Academy of Medical Science, Tianjing). One portion of fresh anticoagulating blood was diluted by portion of Hank's fluid, and one portion of separation medium was carefully added to the mixture. At centrifugation at 1300 r/min for 15 min, the layer between plasma and ficoll enriches the lymphocytes and monocytes. After diffusion through the ficoll layer, the granulocytes and erythrocytes produce a pellet. The interphase can then be carefully removed with a Pasteur pipette. The cell population should be washed twice in the culture medium to separate lymphocytes and monocytes.

Immunohistochemistry

Sections to distilled water, endogenous peroxidase activity was blocked by incubation in 3% H_2O_2 for 15 min, followed by overnight incubation with the primary antibody for rabbit antihuman HO-1 polyclonal antibody (Alexis Biochemicals, Rab, #ALX-210-116-R100) at a 1:400 dilution at 4˚C. Sections are incubated with biotinylated goat antirabbit IgG diluted in secondary antibody dilution buffer for 30 min at 37˚C. The detection step was performed using LSAB+ kit and 3,3'-diaminobenzidine (Dako) as chromogen. Slides were counterstained with hematoxylin, rinsed in tap water, dehydrated, placed in xylene, and mounted. Negative control was designed using phosphate-buffered saline (PBS) instead of primary antiserum. The positive immunohistochemical staining of HO-1 proteins was shown as brown signals in the cytoplasm. Digital images were acquired with a Leica imaging microscope (Leica, Germany) using Leica software with standard adjustments in image brightness and contrast. An image analyzer (HPIAS-1000) was used to analyze the gray scale scan sequantity for the expression intensity of HO-1 protein.

Western Blot

Protein was homogenized, whole-cell lysates were boiled and sonicated, and SDS-PAGE was performed with 50 µg protein per tissue. The separated blots were transferred to nitrocellulose membranes, the nitrocellulose masked by incubating it overnight in 5% nonfat dry milk, and incubated with an anti-HO-1 polyclonal antibody (1:500; Stressgen Biotechnologies Corp.). Membranes were incubated with antirabbit horseradish peroxidase-conjugated secondary antibody (1:500) followed by treatment with enhanced chemiluminescence reagents (Amersham Corp.). Densitometric analysis of gel films was performed with an image analyzer (Sigma pro, USA).

Statistical Analysis

Data were analyzed using professional statistical computer software (Statistical Package for Social Sciences version 11.0, SPSS Inc., Chicago, Ill., USA). All values are expressed as the mean ± standard error (SE). Comparisons were made using one-way analysis of variance (ANOVA) followed by Dunnet's test and chi-square test. $P \leq 0.05$ was taken as significant. All reported p values are two-sided.

Results

Clinical Data

General conditions and risk factors for CHD are shown in Table I.

TABLE I Between-group comparison of general conditions and risk factors of coronary heart disease

Team	No. of patients	Age (years)	Sex F/ M	Hypertension (n)	Diabetes mellitus (n)	Hyperlidpemia (n)	Smoking (n)
Control	30	64.4 ± 6.9 [a]	7/23 [b]	15 [b]	6 [b]	11 [b]	20 [b]
AP	30	63.5 ± 7.7	8/22	16	7	10	18
UAP	40	63.8 ± 6.8	10/30	19	8	14	26
AMI	35	62.4 ± 8.2	9/26	17	7	12	19

[a] $p > 0.05$ (intergroup comparison, one-way ANOVA followed by Dunnet's test).

[b] $p > 0.05$ (intergroup comparison, chi-square test).

Abbreviations: F = female, M = male, AP = angina pectoris, UAP = unstable angina pectoris, AMI = acute myocardial infarction.

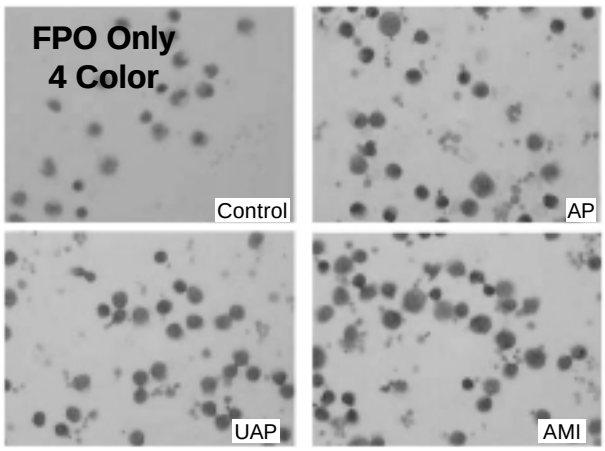

FIG. 1 Expression of heme oxygenase-1 protein in the different groups with immunohistochemical staining (1:400). Control: few positive signals in the cytoplasm; AP: a few brown signals in the cytoplasm; UAP: evident brown signals in the cytoplasm; AMI: many more brown signals in the cytoplasm than UAP group. Abbreviations as in Table I.

Histopathology

The positive immunohistochemical staining of HO-1 proteins was shown as brown signals in the cytoplasm; there was little expression in the cytoplasm in the control group. The levels of HO-1 protein expression in patients with CHD were significantly higher than in those without AMI ($p < 0.01$). There was a maximum expression of HO-1 in patients with AMI and a minimum expression in the group with AP. Results are shown in Figure 1 and Table II.

Western Blot

We measured expression of HO-1 by immunohistochemistry and western blot, and compared the results (Figs. 2 and 3, Table III). Expression of HO-1 increased significantly as it progressed from the AP to the UAP and to the AMI team. The expression of HO-1was low in the controls, slightly increased in group with AP, markedly increased in group with UAP, and further increased in group with AMI. This pattern of change was similar to that of immunohistochemistry.

Discussion

Among the three reported HO isoforms (HO-1, -2, -3), HO-1 is inducible and ubiquitously expressed, particularly after induction by oxidative stress, but HO-2 and HO-3 are constitutively expressed in cells; HO-1 is a microsomal enzyme, easily found in both spleen and liver tissue in which reticuloendothelial cells are abundant; HO-1 expression varies in other tissues induced by oxidative stress. In this research, we investigated the expression of HO-1 in monocyte and lymphocyte coming from patients with CHD. Heme oxygenase-1 plays an important role in the conservancy of the cardiovascular system:[3] protecting cardiac muscle cells and endothelial cell by antioxidant, blocking the growth and proliferation of vascular smooth muscle cells (VSMC), maintaining tissue homeostasis and in-

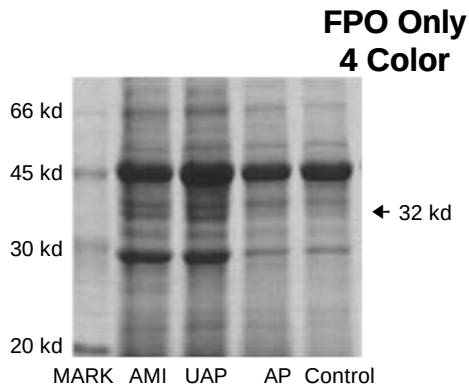

FIG. 2 Results of electrophoresis: at right is the marker; the 30 kD protein is in the middle. Next to the marker are AMI, UAP, AP, and Control groups. A protein band, with molecular weight of about 32 kD, is just above 30 kD in each group. Among the four groups, the intensity of 32 kD protein in the groups with AMI and UAP is heavier than that in the group with AP and the control group. Abbreviations as in Table I.

FIG. 3 Western blot analyses of heme oxygenase (HO)-1 in the four groups. Among them, the intensity of HO-1 (32 kD) is heaviest in the group with AMI, less heavy in the group with UAP, and least heavy in the group with AP. There is little HO-1 expression in control group.

TABLE II Between-group comparison of the expression of heme oxygenase (HO)-1 (mean ± standard deviation)

Team	No. of patients	Mean area	Mean gray intensity
Control	30	12.8 ± 2.5	24.7 ± 9.5
AP	30	34.7 ± 5.4	65.4 ± 13.2
UAP	40	42.8 ± 5.8	78.8 ± 10.5
AMI	35	62.5 ± 7.6	96.7 ± 11.4

Abbreviations as in Table I.

TABLE III Among-group comparison of the expression of heme oxygenase (HO)-1

Team	No. of patients	Gray intensity
Control	30	10.5 ± 1.4
AP	30	27.6 ± 1.8
UAP	40	42.5 ± 2.7
AMI	35	80.6 ± 9.5

Abbreviations as in Table I.

hibiting endothelial cell apoptosis, lightening vascular bed reconstruction after injury, and restraining directly cardiac muscle cells hypertrophy. Its product CO can dilate blood vessels directly. Juan *et al.*[4] injected recombinant adenovirus (containing the HO-1 gene) into 14-week-old ApoE-deficiency mice and found that HO-1 cannot only choke back the development of early atherosclerosis, but also control the progress of later atherosclerosis. Tulis *et al.*[5] injected recombinant adenovirus containing the HO-1 gene into the rat with balloon angioplasty in the carotid artery. Two weeks later, elevated HO-1 protein was observed in the Ad-HO-1 arteries compared with those exposed to empty adenovirus (Ad-E). The arteries exposed to Ad-HO-1 exhibited a significantly reduced neointimal area, medial wall area, neointimal area/medial wall area ratio, and neointimal thickness compared with arteries exposed to Ad-E. Wang *et al.*[6] found that there was overexpression of HO-1 in atherosclerotic lesion. Li Volti *et al.*[7] investigated the effect of HO-1 expression on cell cycle progression in endothelial cells (EC) and smooth muscle cells (SMC) and found that HO-1 regulated the cell cycle in a cell-specific manner; it increased EC but decreased the SMC cycle progression. Mayer[1] and Santiago *et al.*[2] found that an inverse relationship exists between circulating bilirubin concentrations and risk of CAD, but studies on the relationship between HO-1 and CHD were rarely conducted.

While the majority of evidence supports a cytoprotective role for HO-1, this is not a universal finding. Paradoxically, studies found that a high-level HO-1 expression following adenoviral-mediated gene transfer induces SMC apoptosis both in vitro and in vivo.[5, 8] Under these conditions, the apoptotic action of HO-1 appears to be mediated via the release of the bile pigments, biliverdin, and bilirubin, which at high concentrations are known inducers of apoptosis.[8] These findings clearly demonstrate that HO-1 and its products can exert divergent effects on cell survival depending on dose and cell type.

In this study, we directly observed the expression of HO-1 in the patients with CHD, and found that the levels of HO-1 protein expression in these patients were significantly higher than those in normal persons. It may suggest that HO-1 be expressed at high levels under nonphysiologic conditions. In addition, among the patients with CHD, we found that the expression of HO-1 was highest in the patients with AMI, lowest in those with stable AP, and intermediate in those with UAP. This indicates that there is a relationship between expression of HO-1 and the severity degree of CHD. The fact that the expression of HO-1 was increased may be an auto-protective reaction to injury. Yachie *et al.*[9] presented not only the first human case of HO-1 deficiency, but may also provide clues to the key roles played by this important enzyme in vivo.

Studies of the heart suggest that HO-1 may modulate cellular hypertrophy. Heme oxygenase-1 knockout mice exposed to hypoxia exhibit significantly greater ventricular hypertrophy than do wild type controls, suggesting that the induction of HO-1 may function in an autocrine manner to limit cardiac hypertrophy.[10] It may therefore be useful to induce HO-1 expression to prevent cardiac hypertrophy, thereby improving heart dilating function.

Substantial evidence indicates that HO-1 is a negative regulator of growth in vascular SMCs. The induction of HO-1 expression by hemin administration or by gene transfer blocks growth in porcine and rat aortic SMCs.[11, 12] Vascular SMCs obtained from HO-1 knockout mice exhibit enhanced SMC proliferation and DNA synthesis compared with SMCs from wild type animals.[13] The inhibition of vascular SMC proliferation by HO-1 appears to be mediated via the release of CO. The CO scavenger, hemoglobin, potentiates the proliferative response of vascular SMC to several growth factors.[14, 15] In addition, the administration of physiologically relevant concentrations of CO (100–200 ppm) inhibits SMC proliferation and DNA synthesis in response to several growth factors.[12, 15]

Based on the above findings, we may treat some occlusive vascular disease via enhancing expression of or inhaling CO within its physiologic concentration. Furthermore, we may add some HO-1 revulsant or CO releasant to coronary stents to reduce the restenosis rate of stentings.

Because HO-1 induction stimulates cell cycle progression and proliferation in vascular endothelium,[7, 16] and because transduction of the HO-1 gene into microvascular ECs promotes the formation of capillary-like tube structures when ECs are embedded within a matrigel matrix,[16, 17] we may use HO-1 to treat patients with CHD in their last stage to improve the development of new capillary vessels in cardiac muscle.

Because creatinine kinase, troponin, or high-sensitivity C-reactive protein was not detected in all the subjects, the relationships between levels of HO-1 and creatinine kinase, troponin, or high sensitivity C-reactive protein have not been evaluated. All these and the relationship of HO-1 and the prognosis of CHD need to be studied further.

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Images in Cardiology: Right Ventricular Rupture

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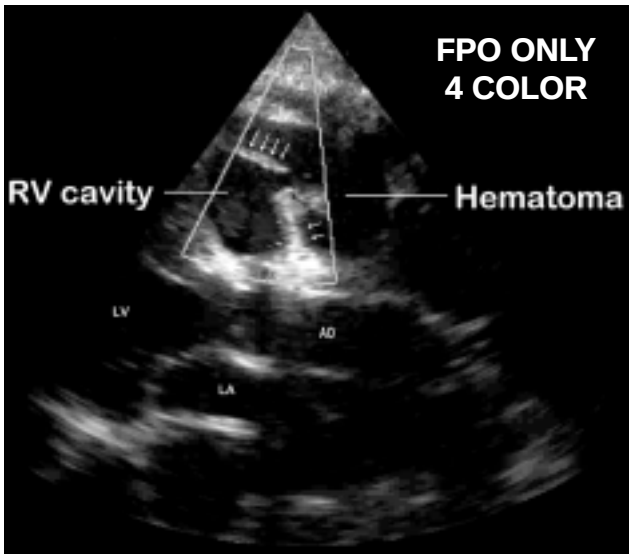

FIG. 1 Transthoracic echocardiogram demonstrating a ruptured right ventricular free wall (closed arrows).

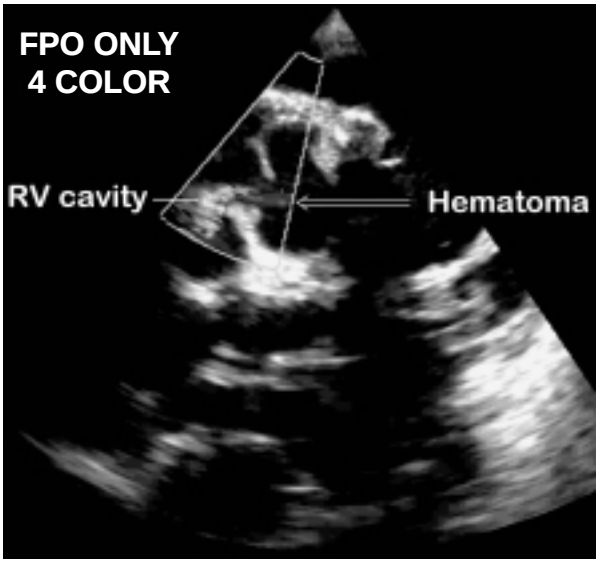

FIG. 2 Transthoracic echocardiogram demonstrating a connection between the right ventricular cavity and a massive mediastinal hematoma (open arrow).

A 64-year-old man had repair hemorrhage from the sternotomy wound late after repair of an acute aortic dissection. The right ventricle was densely adherent to the chest wall and diaphragm. The aortic graft was dehisced and surrounded by infected hematoma, which were removed and a new dacron graft inserted. On the third day the requirement for inotropic drugs increased and there was a pulsatile swelling to the left of the sternotomy wound. The transthoracic echocardiogram showed a ruptured right ventricular free wall (Fig. 1) and a communication to a massive mediastinal hematoma (Fig. 2). The patient died a few hours later.