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Expression of the peptide hormone hepcidin increases in cardiomyocytes under myocarditis and myocardial infarction☆

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Abstract

The micronutrient iron is an essential component that plays a role in many crucial metabolic reactions. The peptide hormone hepcidin is thought to play a central role in iron homeostasis and its expression is induced by iron overloading and inflammation. Recently, hepcidin has been reported to be expressed also in the heart; however, the kinetics of altered hepcidin expression in diseases of the heart remain unknown. In this study, we examined cardiac expression of hepcidin in rat experimental autoimmune myocarditis (EAM), human myocarditis and rat acute myocardial infarction (AMI). In rat EAM and AMI hearts, hepcidin was expressed in cardiomyocytes; ferroportin, which is a cellular iron exporter bound by hepcidin, was also expressed in various cells. Analysis of the time course of the hepcidin to cytochrome oxidase subunit 6a (Cox6a)2 expression ratio showed that it abruptly increased more than 100-fold in hearts in the very early phase of EAM and in infarcted areas 1 day after MI. The hepcidin/Cox6a2 expression ratio correlated significantly with that of interleukin-6/γ-actin in both EAM and AMI hearts (r=0.781, P<.0001 and r=0.563, P=.0003). In human hearts with histological myocarditis, the ratio was significantly higher than in those without myocarditis (0.0400±0.0195 versus 0.0032±0.0017, P=.0045). Hepcidin is strongly induced in cardiomyocytes under myocarditis and MI, conditions in which inflammatory cytokine levels increase and may play an important role in iron homeostasis and free radical generation.

Keywords: Hepcidin; Myocarditis; Myocardial infarction; Iron homeostatis; Reactive oxygen species; Cytokine

1. Introduction

Hepcidin, which was recently discovered to be a peptide hormone, encodes a protein of 84 amino acid residues containing a 24-residue N-terminal signal sequence and a pentaarginyl proteolysis site linked to the active C-terminal 25-amino acid peptide [1]. The active peptide contains a unique 17-residue stretch with eight cysteines forming four disulfide bridges. Hepcidin is primarily expressed in the liver [2] and is thought to play a central role in iron homeostasis [3]. It regulates cellular iron efflux through ferroportin [4], and it is increased in response to iron loading and decreased by anemia [5,6]. Hepcidin expression is increased in infection and inflammation [6], and the inflammatory cytokine interleukin (IL)-6, but not IL-1α or tumor necrosis factor (TNF)-α, strongly induces expression of hepcidin [3,7]. Reduction of iron in the extracellular space by hepcidin suppresses the iron usage of microorganisms and it is thought to be important for host defense [1]. In living cells, iron functions as a double-edged sword [8]. Iron is an essential component of many proteins and enzymes including hemoglobin and myoglobin, the cytochromes, NADH dehydrogenase, etc. [9,10]. On the other hand, excessive iron is also involved in injury of various tissues and organs due to its ability to catalyze the production of reactive oxygen species [11]. In particular, the cardiomyocyte, which contains myoglobin, is a primary cellular source of large amounts of iron [12,13]. Therefore, destruction of cardiac tissue is thought to release large amounts of iron into the extracellular space. Moreover, previous studies have demonstrated that iron plays a pathophysiological role in various heart diseases, for example, cardiac hemochromatosis and ischemic reperfusion injury [14,15]. In such situations, hepcidin has recently been reported to be

Abbreviations: AMI, acute myocardial infarction; Cox6a, cytochrome oxidase subunit 6a; EAM, experimental autoimmune myocarditis; *ferritin-H*, ferritin heavy chain; *ferritin-L*, ferritin light chain; NC, non-cardiomyocytic; NCNI, non-cardiomyocytic non-inflammatory.

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expressed not only in liver but also in other organs; its expression is reportedly also increased in the hearts of rats exposed to hypoxia or injected with turpentine oil [16]. In this way, hepcidin is speculated to play an important role in diseases of the heart; however, detailed studies of hepcidin expression in cardiac tissues have not been reported and remain to be undertaken.

Rat experimental autoimmune myocarditis (EAM) is similar to human fulminant myocarditis [17] in which many lymphocytes and macrophages infiltrate the heart [18]. In EAM heart, various cytokines including IL-6, which is thought to induce hepcidin, are abruptly expressed [19,20]. Correspondingly, acute myocardial infarction (AMI) triggers an inflammatory cascade and various cytokines are produced [21]. In this study, we investigated changes of hepcidin expression in rat EAM and AMI and human myocarditis.

2. Materials and methods

2.1. Animals

Male Lewis rats were obtained from Charles River, Japan (Atsugi, Kanagawa, Japan), and were maintained in our animal facilities until they reached 7 weeks of age for EAM experiments. Male Fisher rats were similarly obtained and maintained until they reached 8 weeks of age for AMI experiments. Throughout the studies, all animal experiments in our institute followed the guidelines for the care and use of laboratory animals published by the US National Institutes of Health.

2.2. Induction of EAM and purification of cells

Cardiac myosin was prepared from the ventricular muscle of porcine hearts as previously described [17]. To produce EAM, each rat was immunized on Day 0 with 0.2-ml emulsion containing cardiac myosin with an equal volume of complete Freund's adjuvant supplemented with 10 mg/ml of Mycobacterium tuberculosis H37RA (Difco, Detroit, MI, USA). EAM rats were killed on Day 18 and, for analysis of hepcidin-, cytochrome oxidase subunit 6a (Cox6a)2-, brain natriuretic peptide (BNP)-, ferroportin-, *ferritin heavy chain* (*ferritin-H*) *and ferritin light chain* (*ferritin-L*) gene-expressing cells, fractions of cardiomyocytes, αβT cells, $CD11bc^+$ cells and non-cardiomyocytic noninflammatory (NCNI) cells (mainly fibroblasts, smooth muscle cells, and endothelial cells) were isolated and purified as follows. Cox6a2 was selected as a candidate gene expressed only in cardiomyocytes, and not in non-cardiomyocytic (NC) cells [22]. Briefly, various cells in both ventricles were isolated by collagenase perfusion treatment for 20 min using a Langendorff apparatus [23]. The isolated cells were serially separated through 38-μm stainless steel sieves to yield cardiomyocytes and then through 20-μm sieves to yield NC cells. Because almost all inflammatory cells in EAM are αβT cells and $CD11bc^+$ cells, the NC cells were further separated into αβT cells, $CD11bc^+$ cells and NCNI cells such as fibroblasts, smooth muscle cells or endothelial cells with anti-phycoerythrin (PE) micro beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and a MACS magnetic cell sorting system (Miltenyi Biotech) using appropriate monoclonal antibodies, namely PE-conjugated TCRαβ (R73) and CD11bc (OX-42) (Pharmingen, San Diego, CA, USA) [23]. Normal rats were killed and EAM rats were killed on Days 6, 9, 12, 15, 18, 30 and 60, and their cardiomyocytes were similarly purified for time course analysis of hepcidin, Cox6a2 *and hypoxia inducible factor (HIF)-1-α* gene expression. For analysis of the relationship between hepcidin/Cox6a2 and IL-6/γ-actin gene expression or between BNP/Cox6a2 and IL-6/γ-actin gene expression, parts of both ventricles from normal rats and EAM rats killed on Days 6, 9, 12, 15, 18, 30 and 60 and adjuvant control injected only with adjuvant without cardiac myosin were collected. To examine hepcidin-expressing organs in EAM, parts of hearts, livers, kidneys and spleens from normal rats and adjuvant control and EAM rats on Day13 were collected.

2.3. AMI model and purification of cells

AMI was induced by ligation of the left anterior descending coronary artery. Fisher rats were anesthetized with sodium pentobarbital (30 mg/kg i.p.), the thorax was opened, the heart was exteriorized and a ligature was placed around the proximal left anterior descending coronary artery. The heart was then returned to its normal position, and the thorax was closed. Mortality was 40% within the first 24 h. The same surgical procedure was performed in a sham group of rats except that the suture around the coronary artery was not tied. AMI rats were killed on Day 3 for analysis of hepcidin-, Cox6a2- and BNP-gene expressing cells; after cutting the thread used for ligation of the coronary artery, fractions of cardiomyocytes and NC cells in both ventricles were similarly isolated and purified by collagenase perfusion treatment and stainless steel sieves as described above. Myocardial infarcts extended over approximately 30% of the left ventricle. Normal rats were killed; AMI rats were killed on Days 1, 3 and 7 and sham group rats were killed on Day 1. Small segments of collagenase-untreated left ventricles were then collected for time course analysis of hepcidin, IL-6 and *HIF-1α* gene expression and the relationship between hepcidin/Cox6a2 and IL-6/γ-actin gene expression or between BNP/Cox6a2 and IL-6/γ-actin gene expression.

2.4. Heart samples from heart failure patients

Twenty-six patients with heart failure, 19 patients without myocarditis and seven patients with myocarditis were categorized by pathological diagnosis of cardiac biopsy or autopsy specimens (Table 1). A biopsy sample was divided into two

Table 1
Clinical characteristic of patient

Patient no.	Age/gender	diagnosis	Sample	Concomitant therapy	NYHA functional class	Result	Cause of death
		Myocarditis confirmed by histology					
1	64/M	Chronic myocarditis	Autopsy	Catecholamine	IV	Death	Heart failure
2	61/M	Fulminant myocarditis	Autopsy	Catecholamine IABP	IV	Death	Heart failure
3	69/M	Fulminant myocarditis	Autopsy	Catecholamine IABP PCPS	IV	Death	Heart failure
4	47/M	Fulminant myocarditis	Autopsy	Catecholamine IABP PCPS	IV	Death	Heart failure
5	66/M	Fulminant myocarditis	Autopsy	Catecholamine IABP PCPS	IV	Death	Heart failure
6	36/M	Acute myocarditis	Biopsy	Catecholamine diuretics	IV	Alive	
7	57/M	Fulminant myocarditis	Autopsy	Catecholamine IABP PCPS	IV	Death	Heart failure
		Non-myocarditis confirmed by histology					
1	61/F	Dilated cardiomyopathy	Autopsy	Catecholamine	IV	Death	Heart failure
2	53/F	Valvular disease	Autopsy	Catecholamine diuretics	IV	Death	Heart failure
3	22/M	Dilated cardiomyopathy	Autopsy	Catecholamine	IV	Death	Heart failure
4	20/F	Primary pulmonary hypertension	Autopsy	Catecholamine	IV	Death	Heart failure
5	54/M	Dilated cardiomyopathy	Biopsy	ARB diuretics	II	Alive	
6	50/F	Cardiac sarcoidosis (healing stage)	Biopsy	(-)	I	Alive	
7	59/M	Dilated cardiomyopathy	Biopsy	ACEI digitals diuretics	I	Alive	
8	25/M	Eosinophilic myocarditis (healing stage)	Biopsy	(-)	I	Alive	
9	59/F	Infective endocarditis	Autopsy	Catecholamine IABP PCPS	IV	Death	Heart failure
10	32/M	Dilated cardiomyopathy	Biopsy	ACEI digitalis diuretics	I	Alive	
11	58/M	Mitochondrial encephalomyopathy	Biopsy	ARB digitalis diuretics β blocker	III	Alive	
12	54/M	Hypertensive cardiomyopathy	Biopsy	ARB diuretics	II	Alive	
13	33/M	Dilated cardiomyopathy	Biopsy	ARB diuretics	III	Alive	
14	31/F	Tachycardia induced cardiomyopathy	Biopsy	Catecholamine	IV	Death	Multiple organ failure
15	69/M	Tachycardia induced cardiomyopathy	Biopsy	ARB diuretics β blocker	III	Alive	
16	73/M	Restrictive cardiomyopathy	Biopsy	ARB digitalis diuretics β blocker	II	Alive	
17	44/F	Cardiac amyloidosis	Biopsy	ARB diuretics	II	Alive	
18	49/M	Hypertrophic cardiomyopathy	Biopsy	Diuretics	III	Death	Sepsis
19	46/M	Dilated cardiomyopathy	Biopsy	ACEI digitalis diuretics	III	Alive	

IABP, intra-aortic balloon pump. PCPS, percutaneous cardiopulmonary support. ARB, angiotensin receptor blockers. ACEI, angiotensin-converting enzyme inhibitors.

Table 2
List of primers for quantitative RT-PCR

	Sense primer	Antisense primer
Rat hepcidin	5′-ctgtctcctgcttctcctcct-3′	5′-ctatgttatgcaacagagaccaca-3′
Rat Cox6a2	5′-cgcatccaaaggagaccac-3′	5′-aaaggattgacgtggggatt-3′
Rat ferroportin	5′-atgctaccattagaaggattgacc-3′	5′-cattcaagttcacggatgttagag-3′
Rat ferritin-heavy chain	5′-actttgccaaatactttctccatc-3′	5′-gttctttaatggatttcacctgct-3′
Rat ferritin-light chain	5′-cctcttacacctacctctctctgg-3′	5′-aagaagtcacagaagtgagggtct-3′
Rat HIF-1 α	5′-ctttagacagcaagacatttctca-3′	5′-gtggctgagagttcttcgtattat-3′
Human hepcidin	5′-atggcactgagctcccagat-3′	5′-cagcacatcccacactttgat-3′
Human BNP	5′-ctcctgctcttcttgcatctg-3′	5′-ggtccatcttcctcccaaag-3′
Human Cox6a2	5′-gtctgctgaccttcgtgctg-3′	5′-gtggctattgtggaacagagtgt-3′

parts, one for histological examination and the other for gene expression analysis by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Autopsy samples were collected within 12 h after death, and all collected samples were immediately immersed and homogenized in Trizol and then stored at −80°C. Hepcidin and Cox6a2 gene expression was subsequently examined. The local ethics committee approved the study, and all patients signed an informed consent form in relation to the risk of biopsy and diagnosis by histological examination including gene expression analyses.

2.5. RNA extraction and real time RT-PCR

Total RNA was isolated from the materials described above using Trizol (Invitrogen, Tokyo, Japan). The scar tissue and ventricle in the left ventricular anterior wall of AMI lesions were used together, and the left ventricular posterior wall was used to represent undamaged tissue in AMI hearts. cDNA was synthesized from 2 to 5 μg of total RNA with random primers and murine Moloney leukemia virus reverse transcriptase. To create the plasmids used for the standard, rat hepcidin, Cox6a2, ferroportin, γ-actin, BNP, *HIF-1α*, *ferritin-H*, *ferritin-L* and IL-6 mRNA were amplified from an EAM heart-derived cDNA library using the primer pairs shown in Table 2 and the primers as reported previously [19,20,23]. The standard plasmids for human hepcidin, BNP and Cox6a2 were similarly created from myocarditis autopsy specimens using the primer pairs shown in Table 2. PCR-amplified cDNA inserts were directly inserted into the pGEM-T easy vector, and recombinant plasmids were isolated following transformation into *Escherichia coli* JM109-competent cells using a MagExtractor plasmid kit (Toyobo, Osaka, Japan). Absolute copy numbers of their mRNA were also measured by quantitative real-time RT-PCR using a LightCycler instrument (Roche Diagnostics, Tokyo, Japan) together with the same primers and SYBR Premix Ex Taq (Takara, Otsu, Japan). After an initial denaturation step of 10 min at 95°C, a three-step cycling procedure (denaturation at 95°C for 10 s, annealing at 62°C for 10 s and extension at 72°C for 13 s) was used for 45 cycles. The absolute copy numbers of particular transcripts were calculated by LightCycler software using a standard curve approach.

2.6. Cell staining

Cytospin preparations of the purified cell fraction of NC cells from AMI hearts were made and slides were stained with May-Giemsa stain. Purified cell fractions of cardiomyocytes without staining were observed under an inverted microscope.

2.7. Statistical analysis

Statistical assessment was performed by one-way analysis of variance (ANOVA) and Bonferroni's multiple comparison test or nonpaired *t* test. Differences were considered significant at $P<.05$. Data obtained from quantitative RT-PCR were expressed as the mean±S.E.M. Correlations between log-transformed IL-6/γ-actin mRNA copy

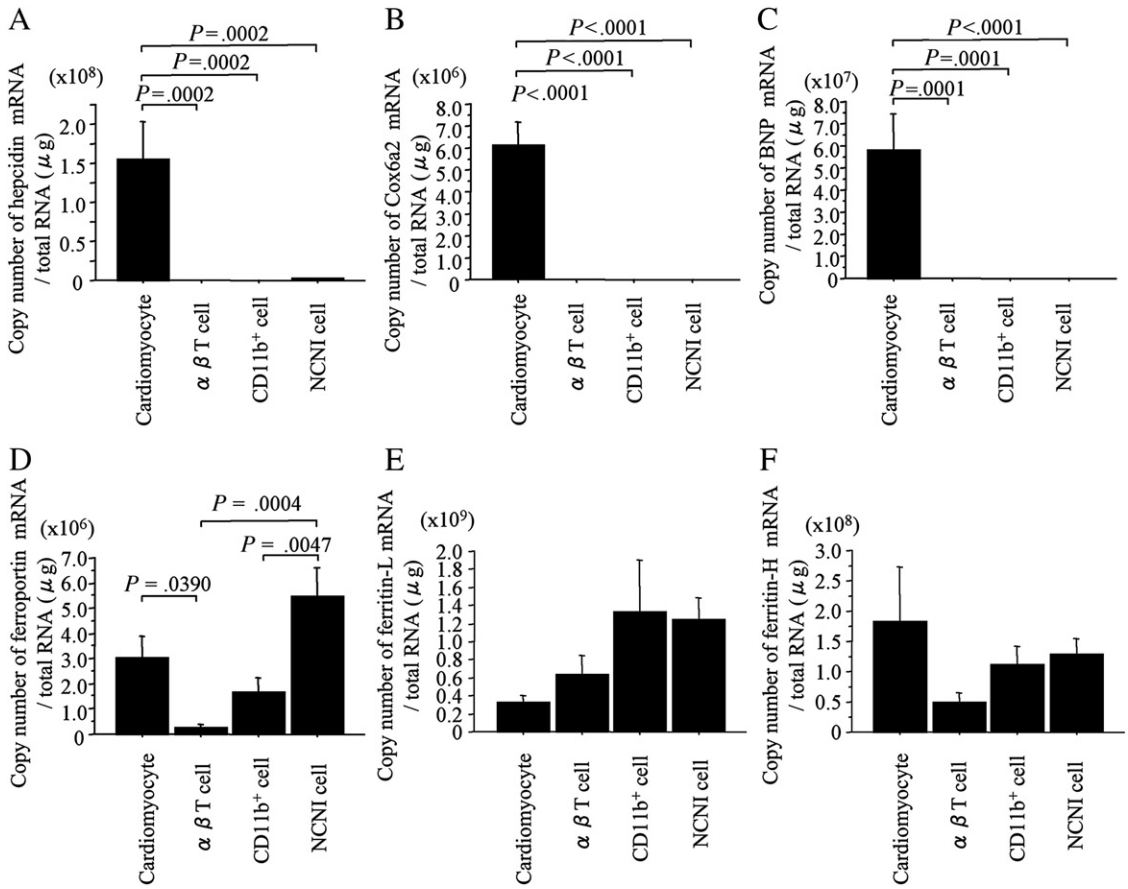

Fig. 1. Gene expression in fractions from EAM hearts. EAM rats were killed on Day 18 and fractions of cardiomyocytes ($n=5$), αβT cells ($n=5$), CD11bc$^+$ cells ($n=5$) and NCNI cells ($n=6$) were isolated and purified. (A) Copy number of hepcidin mRNA/total RNA (μg). (B) Copy number of Cox6a2 mRNA/total RNA (μg). (C) Copy number of BNP mRNA/total RNA (μg). (D) Copy number of ferroportin mRNA/total RNA (μg). (E) Copy number of ferritin-L mRNA/total RNA (μg). (F) Copy number of ferritin-H mRNA/total RNA (μg). Error bars represent S.E.M. Statistical assessment was performed by one-way ANOVA and Bonferroni's multiple comparison test.

number and log-transformed hepcidin or BNP/Cox6a2 mRNA copy number were analyzed by linear regression analysis. Data were analyzed using Pearson's correlation coefficient and Fisher's Z-transformation test.

3. Results

3.1. Analysis of hepcidin-expressing cells

Hepcidin gene-expressing cells in EAM hearts on Day 18 were in the cardiomyocyte fraction (Fig. 1A). Cox6a2 and BNP, which are thought to be expressed only in cardiomyocytes [24,25], were also expressed in the cardiomyocyte fraction (Fig. 1B and C). On the other hand, ferroportin, to which hepcidin binds, was expressed in the NCNI cell fraction, in cardiomyocytes and in CD11bc^{+} cells (Fig. 1D) and *ferritin-H* and *ferritin-L* were highly expressed in all fractions (Fig. 1E, F). Hepcidin-expressing cells in AMI hearts on Day 3 were also in the cardiomyocyte fraction (Fig. 2A and D). A small amount of hepcidin mRNA detected in the NC cell fraction was suspected to have occurred due to contamination by disrupted cardiomyocytes, since it was identified in cytospin preparations; low levels of Cox6a2 and BNP expression were also detected in the NC cell fraction (Fig. 2B, C, E).

3.2. Time course of hepcidin expression in cardiomyocytes

Because hepcidin and Cox6a in hearts are expressed only in cardiomyocytes, we purified cardiomyocytes from EAM hearts and examined hepcidin and Cox6a mRNA levels in these cells. In time course analysis of hepcidin expression, hepcidin mRNA levels abruptly increased from Day 9, peaked on Day 12 and then declined gradually (Fig. 3A). On the other hand, Cox6a2 mRNA levels remained approximately constant (Fig. 3B). For these reasons, we concluded that amounts of hepcidin mRNA per unit RNA of cardiomyocytes in EAM were proportional to the levels of hepcidin/Cox6a2 mRNA calculated from collagenase-untreated EAM hearts, since infiltration or proliferation of various cell types may result in altered ratios of cardiomyocytes to non-cardiomyocytes in different heart samples. In fact, the level of hepcidin/Cox6a2 mRNA in small samples of collagenase-untreated EAM cardiac tissue and hepcidin mRNA/total RNA in cardiomyocytes purified from EAM hearts changed similarly over time (Figs. 3A, 4A). In the meantime, hepcidin/Cox6a2 mRNA levels in rat hearts on Day 18 after injection with adjuvant alone were lower than those in the hearts of EAM rats (Fig. 4A).

Also in the case of myocardial infarction, the ratio of cardiomyocytes to non-cardiomyocytes differed in several cardiac tissue samples. However, amounts of Cox6a2 mRNA in cardiomyocytes purified from AMI hearts were similar to those in cardiomyocytes purified from normal hearts (Fig. 5A), and Cox6a2 and hepcidin in AMI hearts are thought to be expressed only in cardiomyocytes (Fig. 2A, B). Therefore, we measured levels of hepcidin/Cox6a2 mRNA to evaluate amounts of hepcidin mRNA per unit of cardiomyocyte RNA at various sites in AMI. The level of hepcidin/Cox6a2 mRNA in AMI lesions on Day 1 was highest and declined gradually thereafter. The level at intact sites and in sham-operated hearts on Day 1 was suspected to be slightly higher than in normal hearts; however, the

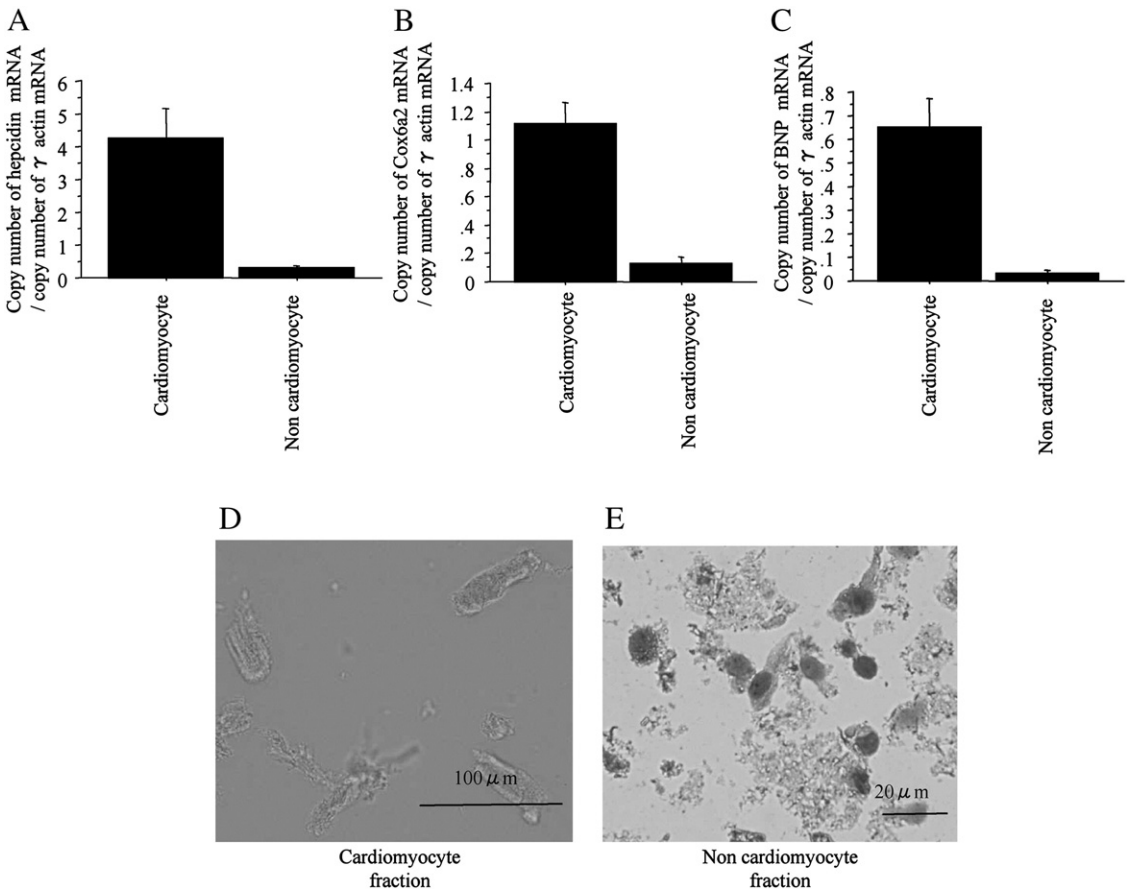

Fig. 2. Gene expression in of mRNA in fractions and microscopic finding from AMI hearts. AMI rats were killed on Day 3 and fractions of cardiomyocytes (n=5) and NC cells (n=5) were isolated and purified. (A) Copy number of hepcidin mRNA/copy number of γ-actin mRNA. (B) Copy number of Cox6a2 mRNA/copy number of γ-actin mRNA. (C) Copy number of BNP mRNA/copy number of γ-actin mRNA. (D) Cardiomyocyte fractions from AMI hearts on Day 3 were observed under inverted microscope. (E) Cytospin preparations of NC cells fraction purified from AMI hearts on Day 3 were stained with May-Giemsa stain. Contamination by disrupted cardiomyocytes was observed. Error bars represent S.E.M.

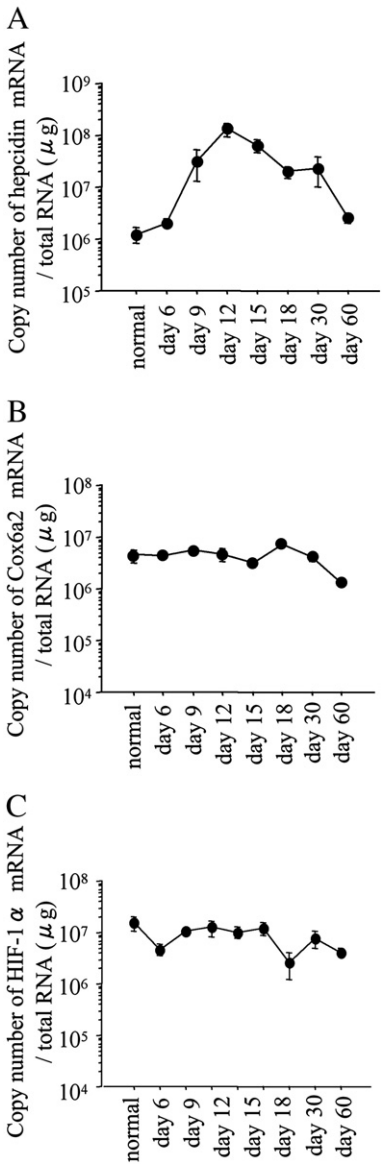

Fig. 3. Gene expression in cardiomyocytes from EAM hearts. Normal rats (n=4) were killed and EAM rats were killed on Day 6 (n=4), Day 9 (n=5), Day 12 (n=4), Day 15 (n=5), Day 18 (n=5), Day 30 (n=4), Day 60 (n=3) and cardiomyocytes purified using stainless steel sieves after collagenase perfusion treatment with a Langendorff apparatus. (A) Copy number of hepcidin mRNA/total RNA (μg). (B) Copy number of Cox6a2 mRNA/total RNA (μg). (C) Copy number of HIF-1α mRNA/total RNA (μg). Error bars represent S.E.M.

increase was not statistically significant (Fig. 5B). We speculated that this slight increase in sham-operated hearts probably resulted from surgical wound inflammation after thoracotomy.

3.3. Relationship between IL-6, HIF-1α and hepcidin

Hepcidin expression is thought to be induced by IL-6 but not IL-1α or TNF-α [7,26]. IL-6 is produced mainly by NCNI cells in EAM [20]; therefore, IL-6 in EAM hearts seems to act as a paracrine factor for hepcidin expression in cardiomyocytes. For these reasons, we postulated that the amount of IL-6 in heart was represented by IL-6 mRNA copy number/γ-actin mRNA copy number and the amount of hepcidin expressed in cardiomyocytes was represented by hepcidin mRNA copy number/Cox6a2 mRNA copy number, and we analyzed the results statistically. The correlation between log-transformed IL-6/γ-actin mRNA and log-transformed hepcidin/Cox6a2 mRNA analyzed using the EAM data shown in Fig. 4A and B was good and statistically significant (r=0.781, P<.0001) (Fig. 4C). Similarly, their correlation analyzed using the AMI data shown in Fig. 5B and C was also good and statistically significant (r=0.563, P=.0003) (Fig. 5D). The correlation between log-transformed IL-6/γ-actin mRNA and log-transformed BNP/Cox6a2 mRNA was fairly good and also statistically significant (EAM, r=0.516, P=.0052; AMI, r=0.387, P=.019) (Figs. 4D, 5E). On the other hand, we couldn't demonstrate that HIF-1α is critically involved in hepcidin production in rat EAM or AMI hearts. HIF-1α has been reported to decrease hepcidin production [27]; however, the expression of HIF-1α mRNA of cardiomyocytes in EAM hearts was largely unchanged (Fig. 3C) and did not correlate with that of hepcidin. Expression levels of HIF-1α mRNA in all samples from AMI hearts were also largely unchanged (Fig. 5D) and did not correlate with that of hepcidin.

3.4. Hepcidin expression in organs in EAM

As reported previously [2], hepcidin was highly expressed in the liver of normal and adjuvant control rats. Hepcidin mRNA in the heart of normal and control rats was moderately expressed and that in the kidney and spleen was poorly expressed (Fig. 6). Compared with normal and control rats, expression of hepcidin mRNA in the heart of EAM rats on Day 13 markedly increased and that in the liver slightly

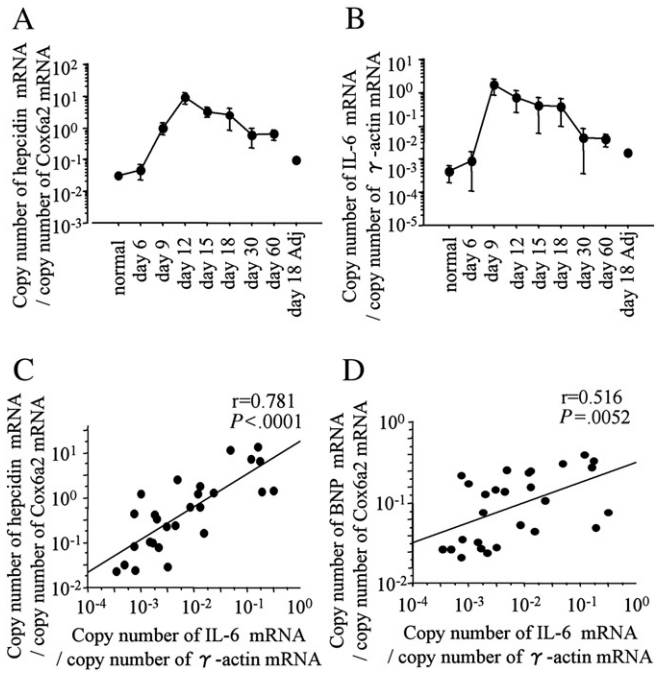

Fig. 4. Gene expression of hepcidin and IL-6 and correlation between hepcidin and IL-6 or BNP and IL-6 in control rat and EAM hearts. Normal rats (n=3) were killed and EAM rats were killed on Day 6 (n=4), Day 9 (n=3), Day 12 (n=3), Day 15 (n=5), Day 18 (n=4), Day 30 (n=3), Day 60 (n=4); control rats were killed on 18 day (n=4) after injection with adjuvant alone and portions of their hearts were collected. RNA was extracted with Trizol and hepcidin, Cox6a2, γ-actin, IL-6 and BNP mRNA were examined by real-time RT-PCR. (A) Ratio of hepcidin to Cox6a2 mRNA copy number. (B) Ratio of IL-6 to γ-actin mRNA copy number. Error bars represent S.E.M. Adj, rat injected with adjuvant alone. (C) Correlations between log-transformed IL-6/γ-actin mRNA copy number and log-transformed hepcidin/Cox6a2 mRNA copy number, demonstrating strong positive correlation between two parameters. (D) Correlations between log-transformed IL-6/γ-actin mRNA copy number and log-transformed BNP/Cox6a2 mRNA copy number, demonstrating moderate positive correlation between the two parameters.

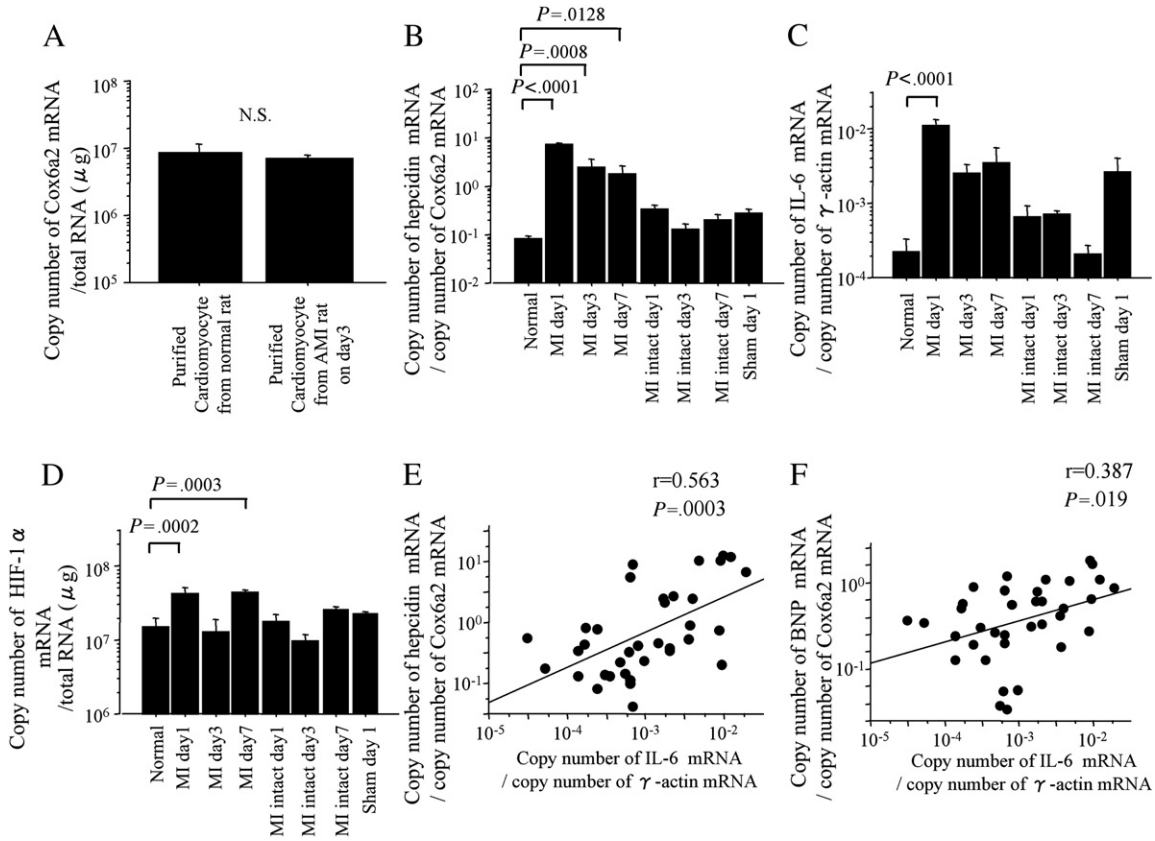

Fig. 5. Gene expression of Cox6a2, hepcidin, IL-6 and HIF-1α and correlation between hepcidin and IL-6 or BNP and IL-6 in AMI hearts. Normal rats (n=4) were killed and AMI rats (n=5) were killed on Day 3 and cardiomyocyte fractions isolated and purified. Normal rats (n=7) and AMI rats were killed on Day 1 (n=6), Day 3 (n=4) and Day 7 (n=4) and sham-operated rats (n=6) were killed; portions of their hearts were then collected. RNA was extracted with Trizol and hepcidin, Cox6a2, γ-actin, IL-6 and BNP mRNA were examined by real-time RT-PCR. (A) Copy number of Cox6a2 mRNA/total RNA (μg) in cardiomyocytes purified from normal rats and AMI rats on Day 3. (B) Ratio of hepcidin to Cox6a2 mRNA copy number in collagenase-untreated left ventricles. (C) Ratio of IL-6 to γ-actin mRNA copy number in collagenase-untreated left ventricles. (D) Copy number of HIF-1α mRNA/total RNA (μg) in collagenase-untreated left ventricles. MI, lesion site of AMI heart: left ventricular anterior wall identified as white-colored region; MI intact, intact site of AMI heart: left ventricular posterior wall identified as red-colored region; Sham, sham-operated heart. Error bars represent S.E.M. Statistical assessment was performed by one-way ANOVA and Bonferroni's multiple comparison test. Significant differences between normal and other groups are shown. (E) Correlations between log-transformed IL-6/γ-actin mRNA copy number and log-transformed hepcidin/Cox6a2 mRNA copy number, demonstrating strong positive correlation between the two parameters. (F) Correlations between log-transformed IL-6/γ-actin mRNA copy number and log-transformed BNP/Cox6a2 mRNA copy number, demonstrating moderate positive correlation between the two parameters.

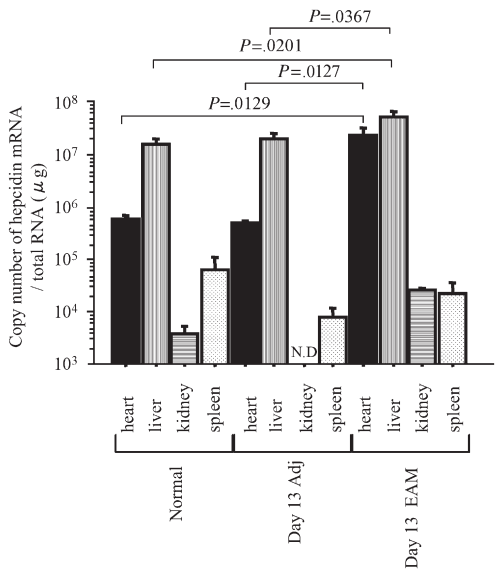

Fig. 6. Gene expression of hepcidin mRNA in organs of normal, control and EAM rats. Normal rats (n=4), control rats (n=4) and EAM rats (n=4) on Day 13 were killed and portions of their hearts, livers, kidneys and spleens were collected. RNA was extracted with Trizol and hepcidin mRNA were examined by real-time RT-PCR.

increased. Hepcidin mRNA in EAM rats on Day 13 was detected mainly in the liver and heart.

3.5. Hepcidin expression in human myocarditis hearts

Because there are a number of different cell types in autopsy and biopsy samples obtained from human hearts, the ratio of cardiomyocytes to non-cardiomyocytes varies between samples. However, if hepcidin and Cox6a2 are expressed only in cardiomyocytes in human hearts as in EAM hearts, levels determined from autopsy and biopsy samples would represent the cardiomyocyte content of hepcidin/Cox6a2 mRNA. Therefore, we compared expression levels in myocarditis and non-myocarditis using this ratio. The level of hepcidin/Cox6a2 mRNA in hearts with pathologically diagnosed myocarditis was significantly higher than that in hearts without myocarditis, (0.0400±0.0195 versus 0.0032±0.0017, P=.0045); however, BNP/Cox6a2 mRNA ratios were not significantly different between the two groups (58.5±24.1 versus 42.3±21.4, P=.68) (Fig. 7).

4. Discussion

Hepcidin expression has recently been reported in the hearts of rats with local skin inflammation or subject to hypoxic stress [16]. In

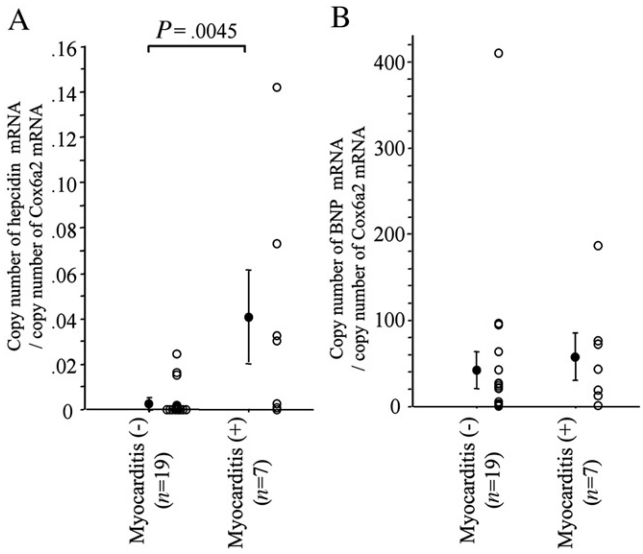

Fig. 7. Gene expression of hepcidin and BNP mRNA in human heart samples. Biopsy or autopsy heart samples were obtained from heart failure patients and RNA was extracted with Trizol; hepcidin, Cox6a2 and BNP mRNA were examined by real time RT-PCR. (A) Ratio of hepcidin to Cox6a2 mRNA copy number. (B) Ratio of BNP to Cox6a2 mRNA copy number. The ratio of hepcidin to Cox6a2 mRNA copy number of myocarditis hearts was significantly higher than that of non-myocarditis hearts. Error bars represent S.E.M. Statistical assessment was performed by nonpaired *t* test.

this study, we demonstrated that hepcidin was strongly expressed in cardiomyocytes under myocarditis and acute myocardial infarction. Hepcidin expression in hearts of adjuvant-injected rats and sham-operated rats and at intact sites of AMI hearts was lower than that at sites of myocarditis and AMI lesions. These data suggest that hepcidin expression is more strongly influenced by local environmental conditions than by systemic conditions. In fact, analysis using small samples from native hearts showed that hepcidin mRNA levels strongly correlated with those of IL-6 mRNA. Interleukin like IL-6 usually acts in an autocrine/paracrine fashion, not in an endocrine fashion [28]. Therefore, IL-6 produced in the heart may strongly induce expression of hepcidin in the heart. Cardiomyocytes have been reported to possess the IL-6 receptor and gp-130 [29], and our data also showed that they were expressed in the cardiomyocyte fraction (data not shown). We suspect that IL-6 produced by NCNI cells [20] in cardiac lesions acts on surrounding cardiomyocytes to induce hepcidin expression in the these cells.

Hepcidin-25 has not only antimicrobial activity but also iron-regulatory one, but hepcidin-22 and hepcidin-20 have only anti-microbial activity [2]. Ferroportin, which is bound by hepcidin-25 [30] then internalized and degraded, was expressed in various cells, including NCNI cells, cardiomyocytes and $CD11bc^{+}$ cells. Ferroportin contains multiple transmembrane domains [31] is involved in iron efflux and its function is inhibited by hepcidin binding. Our data suggest that cardiomyocytes in myocarditis and AMI enhance the expression of hepcidin under the influence of cytokines, and hepcidin inhibits ferroportin-mediated iron efflux from NCNI cells, cardiomyocytes and $CD11bc^{+}$ cells. Iron is closely associated not only with anemia and hemochromatosis but also various disease states. Iron is indispensable for oxygen transport, ATP production and DNA synthesis as a cofactor and all living cells need iron for crucial metabolic pathways. On the other hand, free or loosely bound iron is well known to generate free radicals and causes various types of damage [32]. It can be speculated that the large amount of iron, capable of generating reactive oxygen species, is scattered within the extracellular space of the heart in an acute cardiac injury. Therefore, excessive release of iron from cells via ferroportin, especially macrophages possessing a high level of iron in the lesion, may enhance the killing of neighboring cells. Hepcidin may inhibit the release of iron from macrophages or NCNI cells with a high expression of ferroportin in the heart. Because intracellular iron is sequestered, stored and detoxified in ferritin, which is highly expressed in all cells, hepcidin may play an important cytoprotective role against extracellular free radical formation by inhibiting an increase of extracellular iron [33]. However, further studies are needed to elucidate hepcidin function in myocardial diseases.

Anemia, with which iron is closely associated, is very common in congestive heart failure [34]. Anemia with heart failure is often resistant to erythropoietin and various mechanisms has been proposed to explain this resistance [35]. Several studies have reported increases in proinflammatory cytokine levels in patients with heart failure [36,37]. Proinflammatory cytokines antagonize the action of erythropoietin by exerting an inhibitory effect on erythroid progenitor cells and by disrupting iron metabolism [35,38]. The present study showed that hepcidin, which plays a central role in iron homeostasis [3], increased in heart and liver under myocarditis. We speculate that the increase of inflammatory cytokines in heart may induce hepcidin gene expression in cardiomyocytes and liver. Hemoglobin levels are slightly decreased during advanced stages of EAM (data not shown). Hepcidin may be involved in anemia in patients with heart failure. In any case, cardiomyocytes can produce hepcidin under certain conditions and elevated proinflammatory cytokines in failing heart may increase expression of hepcidin in liver. Future studies are needed to elucidate the role of hepcidin in failing hearts and also to investigate why anemia is common in heart failure.

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