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ORIGINAL ARTICLE

UPREGULATION OF INSULIN-LIKE GROWTH FACTOR-1 GENE EXPRESSION IN CARDIAC MYOCYTES BY HYPOXIA : MECHANISM FOR ITS CARDIOPROTECTIVE EFFECT

Chisato Katoh, Tomohiro Osanai, Shuji Shibutani,
Kenji Hanada, and Ken Okumura

Abstract Insulin-like growth factor (IGF)-1 is known to exert beneficial effects on the heart, but its source and function under hypoxia are unknown. We investigated the effect of hypoxia on IGF-1 expression and its role in the regeneration in the heart. Cardiac myocytes and fibroblasts obtained from neonate mice heart were cultured and exposed to hypoxia. mRNA of IGF-1, IGF-binding protein 3 (IGFBP3), and vascular endothelial growth factor-A (VEGF-A) was measured by real-time PCR. In cardiac myocytes, IGF-1 mRNA was increased by 3.5±1.1 fold at 3 hours after hypoxia concomitantly with the increase in IGFBP3 and VEGF-A mRNA, and returned to the baseline at 24 hours. In contrast, IGF-1 mRNA in cardiac fibroblasts was unchanged by hypoxia, although VEGF-A mRNA was increased. To investigate the role of IGF-1 in the heart regeneration, we measured the gene expressions of stromal cell-derived factor-1 (SDF-1), its receptor CXCR4, and matrix metalloproteinase (MMP)-14 related to cell homing. In cardiac myocytes, SDF-1 and MMP-14 mRNA were increased at 3 hours after hypoxia and tended to be positively correlated with IGF-1 mRNA. These suggest that hypoxia increases IGF-1 expression in cardiac myocytes, and this endogenous IGF-1 may exert beneficial effects on regeneration in the heart.

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Key words: cardiac myocytes; cardiac fibroblasts; insulin-like growth factor-1; hypoxia.

原　著

低酸素による心筋細胞 Insulin-like growth factor-1 遺伝子発現亢進の役割：心保護作用の機序について

加藤千里　長内智宏　渋谷修司
花田賢二　奥村　謙

抄録 Insulin-like growth factor (IGF)-1 は心臓に有益な効果を及ぼす．低酸素刺激により増加する IGF-1 の起源と機能は不明である．生後 1〜2 日齢マウスの心臓から心筋細胞と線維芽細胞を単離培養した．心筋細胞では，IGF-1 mRNA 発現は低酸素条件下で 3 時間後に約 3.5 倍亢進し，それに伴い IGF-binding protein 3，vascular endothelial growth factor-A の発現も亢進した．線維芽細胞の IGF-1 mRNA は低酸素で変化しなかった．心筋細胞では，心筋再生に関連する stromal cell-derived factor-1 mRNA は低酸素条件で 3 時間後に亢進し，IGF-1 mRNA と相関する傾向が認められた．以上より，低酸素条件下において，心筋細胞では IGF-1 発現が亢進し，心筋保護的に作用する可能性が示唆された．

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キーワード：心筋細胞；線維芽細胞；insulin-like growth factor-1; 低酸素条件．

Department of Cardiology, Hirosaki University
Graduate School of Medicine, Hirosaki, Japan
Correspondence: T. Osanai
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INTRODUCTION

Insulin-like growth factor-1 (IGF-1), a 70-amino acid basic peptide, is released from the liver in response to growth hormone secreted from the pituitary gland[1]. Circulating IGF-1 is transported to target organs after binding to IGF-1 binding protein (IGFBP), and mediates the actions of growth hormone[2]. On the other hand, local IGF-1 system which is regulated differently from the circulating IGF-1 system is present and regulates tissue development and condition[2-5]. IGF-1 exerts beneficial effects on the heart and improves cardiac function of the failing heart mouse[6]. IGF-1 is reported to slow the progression of heart failure in a mouse model of dilated cardiomyopathy[7]. It is also reported that when stimulated by IGF-1, the failing heart in evolving myocardial infarction shows additional hypertrophy, resulting in the improved function[8]. IGF-1 induces hypertrophy in the isolated cardiomyocytes[9], and transgenic mice with cardiac overexpression of IGF-1 develop physiological-type hypertrophy[10]. These findings suggest that IGF-1 plays an important role in cardiac hypertrophy and remodeling in some pathological conditions. The regulatory mechanism for the production of endogenous IGF-1 in cardiac cells and its role in the heart, however, are poorly understood.

Previous studies showed that the ventricular content of IGF-1 and its gene expression are increased in a rat model of ventricular hypertrophy[11, 12]. In humans, IGF-1 mRNA is upregulated in the compensated cardiac hypertrophy[13, 14]. IGF-1 production in the heart, which was estimated by the difference in the plasma concentrations between the aorta and coronary sinus, is increased in patients with valvular heart disease[13]. We recently showed that the pericardial IGF-1 level is increased in patients with left ventricular (LV) dysfunction and congestive heart failure (CHF)[15]. Taken together, the endogenous cardiac IGF-1 system is upregulated in various states and is likely to serve as a compensatory mechanism for LV dysfunction. Hypoxia is closely related to hypertrophy, which contributes to the genesis of CHF. To clarify the mechanism for the increase in the pericardial IGF-1 level observed in patients with CHF, we investigated the effect of hypoxia on the endogenous IGF-1 expression in the heart-constructing cells and its possible role using cultured neonate mouse cardiac myocytes and fibroblasts.

METHODS

Cell culture

Primary cultures of neonatal ventricular myocytes and nonmyocytes were prepared as described previously[16]. Primary cardiac myocytes and fibroblasts were obtained from C57BL/6J neonatal mice at 1-2 days of age. Mice were sacrificed, and hearts were extracted and chopped into small fragments (3-4mm each). These fragments were subjected to enzymatic digestion in a solution of calcium and bicarbonate free Hanks with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (NaCl 136.9 mM, KCl 5.36 mM, $MgSO_4 \cdot 7H_2O$ 0.81 mM, glucose 5.5 mM, KH_2PO_4 0.44 mM, $Na_2HPO_4 \cdot 7H_2O$ 0.31 mM, HEPES (pH 7.4) 20 mM) and collagenase clostridium histolyticum type 2 (Sigma-Aldrich, USA). Serial cycles of agitation at 37℃ for 10 min were performed. After each cycle, the cells were resuspended in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) with 10 % fetal bovine serum (FBS) and plated equally into 6-cm culture dishes for 1 hour at 37℃ to obtain cardiac myocytes (nonattached cells) and cardiac fibroblasts (attached cells). The nonattached viable cells (myocytes) were plated on other culture dishes, and the cardiac myocytes and fibroblasts were separately cultured in DMEM supplemented with 10 % FBS at 37℃ in humidified air with 5% CO_2.

Hypoxic stimulation

After 48 hours, the culture media were replaced with the fresh serum-free media. The subconfluent cardiac myocytes and fibroblasts on culture dishes were then placed in the hypoxic chamber with the Anaeropack, a disposable oxygen-absorbing and CO_2-generating agent, and incubated at 37℃ for 3, 6, 12, and 24 hours. The control cells were incubated at 37℃ in an atmosphere of 21% O_2 and 5% CO_2 for the same duration as the hypoxic cells were. The Anaeropack started to absorb oxygen within 1 minute; oxygen tension inside the box droped to 1 mmHg within 1 hour (O_2<1% , CO_2 around 5%), and it was continued for 24 hours[17].

Real-time quantitative reverse transcriptase (RT)-polymerase chain reaction (PCR)

Total RNA was extracted from the cells using QIA shredder and RNeasy Protect Mini Kit. RNA quality and quantity were assessed by the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The RNA was transcribed into the cDNA by two-step RT reaction protocol with the TaqMan Reverse Transcription Reagent according to the manufacturer's instructions. Quantitative RT-PCR was performed with TaqMan Universal PCR Master Mix in duplicate using an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA). The specific primers and the probe were purchased from Applied Biosystems for detecting IGF-1 (Assay ID: Mm00439561_m1), IGFBP3 (Assay ID: Mm00515156_m1), vascular endothelial growth factor-A (VEGF-A) (Assay ID: Mm00437304_m1), chemokine (C-X-C motif) ligand 12 (CXCL12) (Assay ID: Mm00445552_m1), chemokine (C-X-C motif) receptor 4 (CXCR4) (Assay ID: Mm99999055_m1), matrix metallopeptidase 14 (MMP-14) (Assay ID: Mm01318969_g1) and GAPDH (Assay ID: Mm99999915_g1). For quantitative analysis, the relative amounts of mRNA were normalized to GAPDH as the housekeeping gene.

Statistical analysis

Results are expressed as mean±one standard error. Data were analyzed using one-way or two-way analysis of variance (ANOVA). The Bonferroni's test was used for comparison of multiple datasets as the post hoc testing. All tests were considered to be statistically significant at $p<0.05$.

RESULTS

Effect of hypoxia on IGF-1 gene expression

To determine whether hypoxia enhances the gene expressions of IGF-1, IGFBP3, and VEGF-A in the cardiac myocytes and fibroblasts, we measured the amount of IGF-1, IGFBP3, VEGF-A, and GAPDH mRNA in the cells exposed to hypoxia for each indicated time. The gene expression of IGF-1 in cardiac myocytes was increased by 3.5±1.1 fold at 3-hour hypoxia compared with 3-hour normoxia (n=4, p=0.0075) and returned to the baseline at 24 hours (Figure 1a). This IGF-1 upregulation was concomitant with the upregulation of IGFBP3 (n=4, p=0.0070) and VEGF-A mRNA (n=4, p<0.0001) (Figure 1b, 1c).

In the cardiac fibroblasts, the gene expression of IGF-1 at baseline was higher than that in the cardiac myocytes (p=0.0003) (Figure 2). When the fibroblasts were exposed to hypoxia, VEGF-A mRNA expression was increased by 3 fold at 24 hours. The gene expressions of IGF-1, IGFBP3 were increased at 24 hours under normoxia and hypoxia, but there was no significant difference between normoxia and hypoxia (Figure 3a-c).

Effect of hypoxia on regeneration-related genes

We measured the gene expression of CXCL12, which is also known as stromal cell-derived factor-1 (SDF-1), its receptor CXCR4, and MMP-14, all of which are related to stem cell adhesion. In the cardiac myocytes, SDF-1 mRNA expression was increased by 1.7±0.3 fold at 3

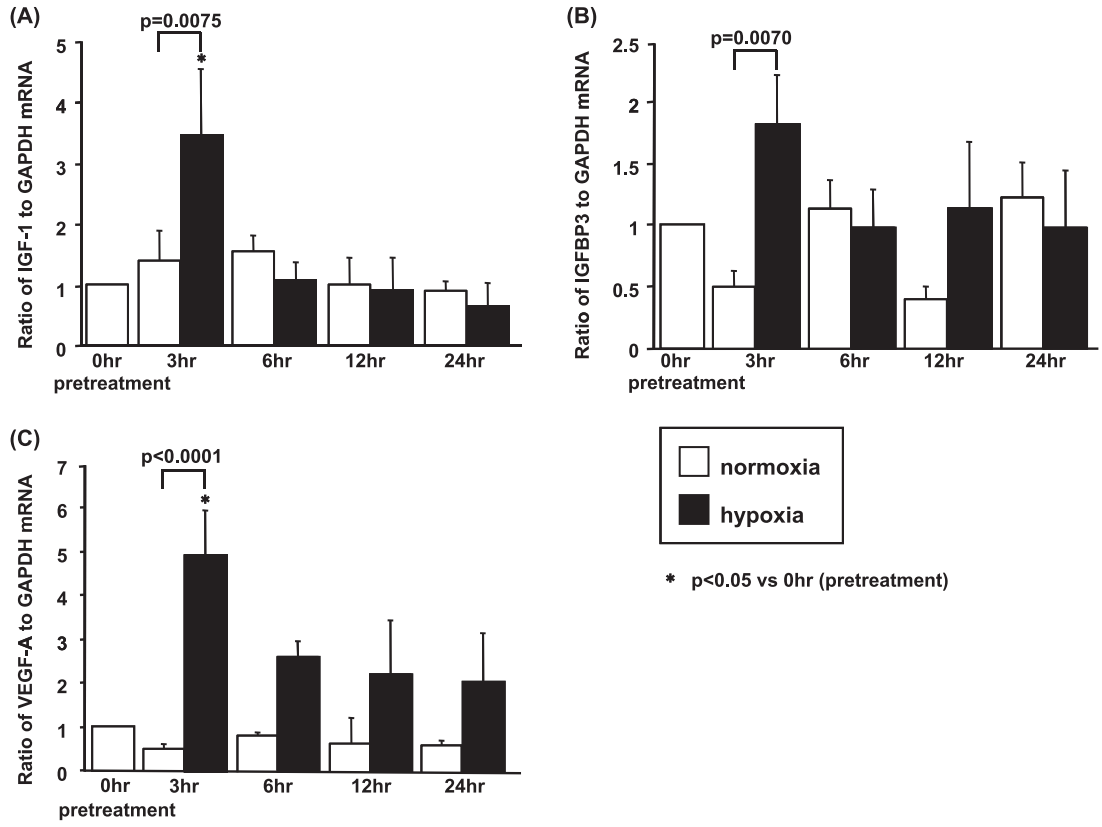

Figure 1 Upregulation of insulin-like growth factor-1 (IGF-1) gene expression, IGF-binding protein 3 (IGFBP3) gene expression, and vascular endothelial growth factor-A (VEGF-A) gene expression by hypoxia in cardiac myocytes. Bar graphs show the ratio of IGF-1 gene expression (A), IGFBP3 gene expression (B), VEGF-A gene expression (C) to glyceraldehyde-3-phosphatase-dehydrogenase (GAPDH) expression.

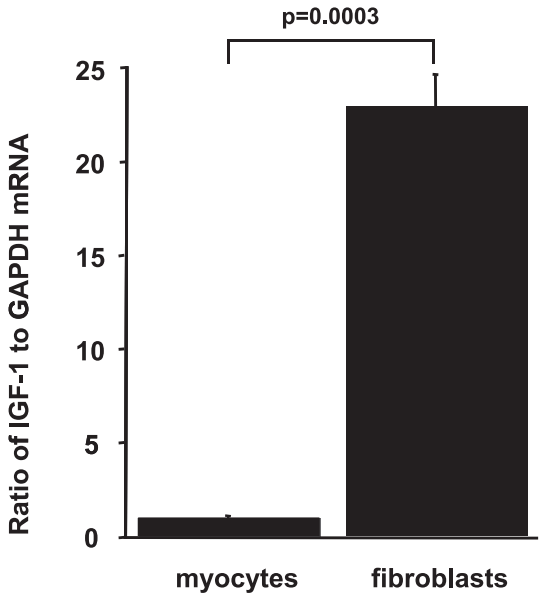

Figure 2 Comparison of the gene expression of insulin-like growth factor-1 (IGF-1) in cardiac fibroblasts and cardiac myocytes at baseline.

hours after hypoxia (n=4, P=0.049) (Figure 4a). There was a tendency of positive correlation of SDF-1 mRNA expression with IGF-1 mRNA expression (r=0.69, p=0.08) (Figure 5a). CXCR4 was unchanged (Figure 4b), but MMP-14 was increased by 1.9±0.21 fold at 3 hours after hypoxia (n=4, p=0.011) (Figure 4c). There was a tendency of positive correlation between MMP-14 and IGF1 mRNA expression (r=0.64, p=0.08) (Figure 5b). In cardiac fibroblasts, none of SDF-1, CXCR4 and MMP-14 mRNA changed after hypoxia. These suggest that hypoxia increases IGF-1 expression in cardiac myocytes, and endogenous IGF-1 might exert beneficial effects such as stem cell recruitment and adhesion in the heart.

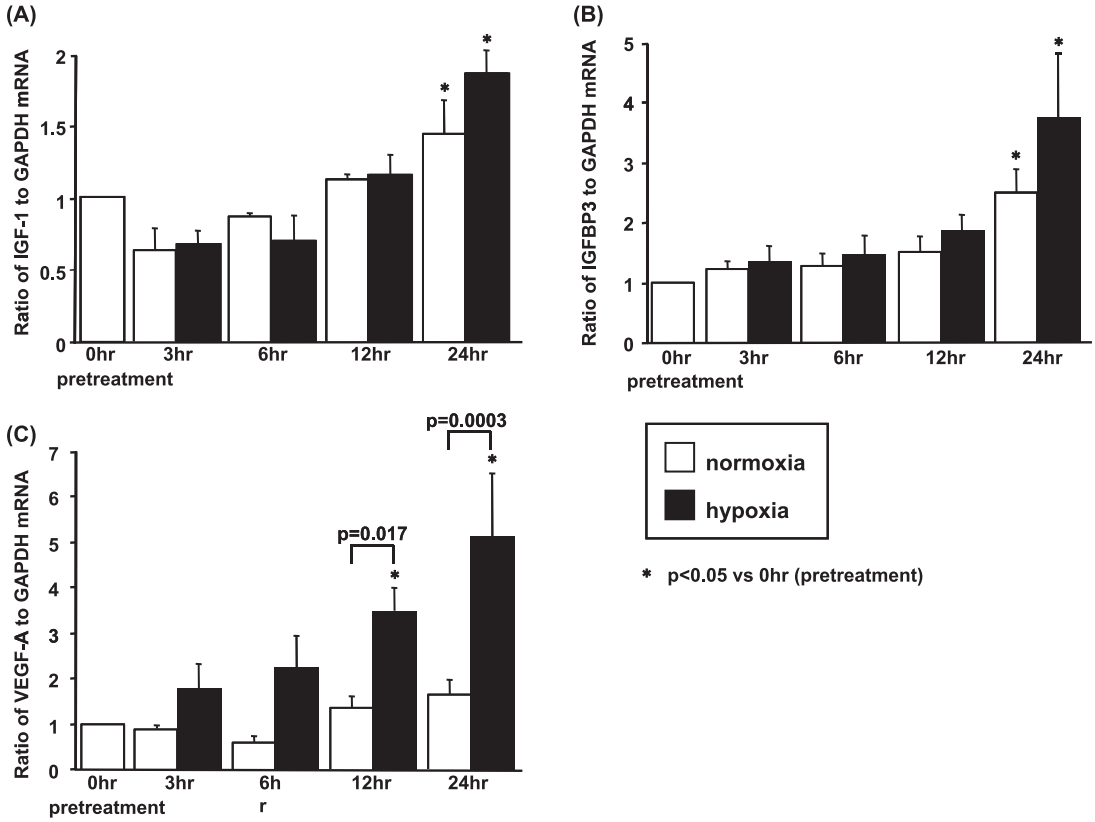

Figure 3 Changes in insulin-like growth factor-1 (IGF-1) gene expression, IGF-binding protein 3 (IGFBP3) gene expression, and vascular endothelial growth factor-A (VEGF-A) gene expression by hypoxia in cardiac fibroblasts. Bar graphs show the ratio of IGF-1 gene expression (A), IGFBP3 gene expression (B), VEGF-A gene expression (C) to glyceraldehyde-3-phosphatase-dehydrogenase (GAPDH) expression.

DISCUSSION

The major finding of this study was that the gene expression of IGF-1 in cardiac myocytes was increased after hypoxia concomitantly with the increase in IGFBP3, VEGF-A, SDF-1, and MMP-14 expression. In cardiac fibroblasts, the gene expression of IGF-1 at baseline was higher than that in cardiac myocytes, but was unchanged after exposure to hypoxia.

Hypoxia increases IGF-1 gene expression in cardiac myocytes

Cardiac IGF-1 formation was increased in patients with increased left ventricular wall stress and athletes with physiological hypertrophy[13, 14]. In an experimental myocardial infarction model, the myocardial IGF-1 mRNA level was shown to be increased by in situ hybridization and RT-PCR[18, 19]. To our knowledge, the myocardial IGF-1 protein level has not been reported in any experimental ischemic or myocardial infarct models. In the rat heart Langendorff model which is not affected by circulating IGF-1, the mRNA and protein expressions of IGF-1 are increased by graded mechanical stress[20]. These studies support the presence of a local tissue IGF-1 system in the heart by protein or mRNA analysis. Additionally, we recently showed that the pericardial IGF-1 level is increased in patients with LV dysfunction and CHF. However, it remains unclear whether LV dysfunction leads to an increased level of pericardial IGF-1 or vice versa, although many studies have reported that

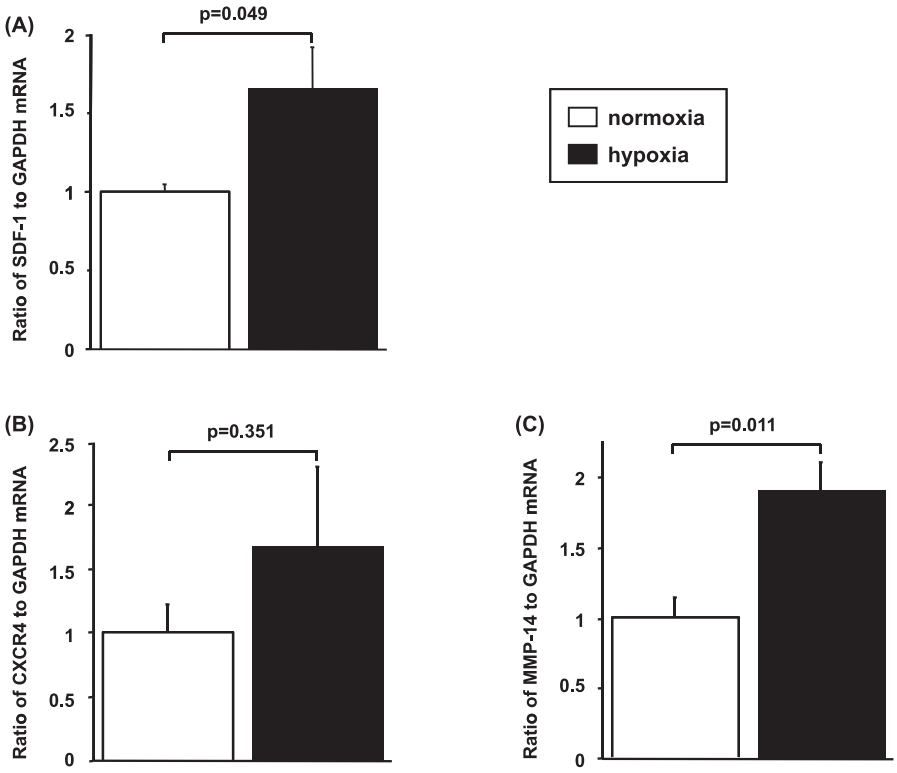

Figure 4 Comparison of the gene expressions of stromal cell-derived factor-1 (SDF-1) (A), its receptor CXCR4 (B), and matrix metalloproteinase (MMP)-14 (C) by hypoxia for 3 hours in cardiac myocytes between normoxic and hypoxic conditions.

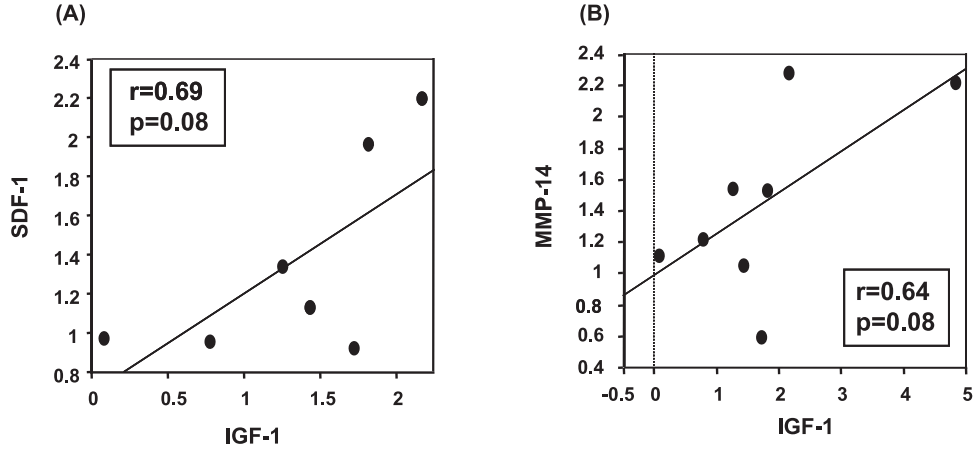

Figure 5 Relationship between IGF-1 and stromal cell-derived factor-1 (SDF-1) or matrix metalloproteinase (MMP)-14 gene expression in cardiac myocytes.

IGF-1 has a protective effect on the heart[7, 8, 21–24].

Since hypoxia is closely related to ventricular hypertrophy that is one of the main causes of CHF, we investigated the effect of hypoxia on the endogenous IGF-1 expression in the heart-constructing cells. The results clearly showed that the gene expression of IGF-1 in cardiac myocytes was increased after exposure to hypoxia, whereas that in cardiac fibroblasts was similar between normoxia and hypoxia. Additionally, the level of

IGF-1 gene expression at baseline was higher in cardiac fibroblasts than in cardiac myocytes. This result is consistent with the recent evidence that IGF-1 is produced and released from the cultured cardiac fibroblasts to a greater degree compared with cardiac myocytes in rats[25].

Although the level of IGF-1 gene expression was higher in cardiac fibroblasts compared with myocytes, the cells responded to hypoxia were cardiac myocytes rather than cardiac fibroblasts. Therefore, it is likely that the increased pericardial IGF-1 in CHF is mainly derived from cardiac myocytes. Since IGF-1 exerts beneficial effects on the heart such as physiological-type hypertrophy[10], the increase in local myocyte IGF-1 expression may improve cardiac function in the failing heart. In the present study, the expression of IGF-1 protein was not measured in the cells. Therefore, it is unclear whether the endogenous IGF-1 produced from cardiac myocytes functions *in vivo*. Apart from cardiac myocytes, the endogenous IGF-1 produced from rat cardiac fibroblasts was shown to promote collagen synthesis in cardiac fibroblasts and hypertrophy in myocytes in a fashion of autocrine or paracrine[25].

Relationship between IGF-1, SDF-1, and MMP-14 in cardiac myocytes

Recent studies have shown that local IGF-1 production may itself act as a stem cell recruitment factor in skeletal muscle[26] and cardiac over-expression of IGF-1 in heart elevates markers of cell division and stem cell recruitment[7, 27]. It is therefore possible that elevated IGF-1 acts as a survival and regeneration factor in the tissue recovery. Barton PJ. et al showed that SDF-1 and IGF-1 mRNA levels correlate with each other in the patients treated with LV assist device[28]. It is of interest that SDF-1 was reported to play a role in the myocardial response to damage[29] and MMP-14 contributes to the adhesion of stem cells in the damaged tissues. We clearly showed that in cardiac myocytes, SDF-1 and MMP-14 mRNA expressions were increased at 3 hours after hypoxia but CXCR4 was unchanged. In contrast, in cardiac fibroblasts, none of SDF-1, CXCR4 and MMP-14 changed after hypoxia. The response of these genes related to regeneration was similar to that of IGF-1, which was increased at 3 hours after hypoxia in cardiac myocytes. These raise one possibility that hypoxia increases IGF-1 expression in cardiac myocytes, and endogenous IGF-1 exerts beneficial effects such as stem cell recruitment and adhesion in the heart. Further studies are needed.

Study limitations

There are three limitations in the present study. First, our biochemical and cell culture data shows the gene expression, but not their protein expression. It still remains unclear that hypoxia directly increases the protein expressions of those in cardiac myocytes. We recently showed that the pericardial IGF-1 level is increased in patients with LV dysfunction and CHF. That suggested that cardiac IGF-1 expression was upregulated. Second, we didn't have any direct evidence that IGFBP-3, VEGF-A, SDF-1 and MMP14 gene expression were directly upregulated in connection with IGF-1. IGF-1 bioactivity is determined by the interplay among IGF-1, IGFBPs, and IGFBP proteases[30]. At the tissue level, binding of IGFBP-3 to cells reduces affinity of IGFBP-3 for IGF-1, permitting interactions between IGF-1 and its receptors[31]. We suggest that the intersection among the IGF-1 and IGFBP3, VEGF-A, SDF-1, and MMP-14 networks may be potentially important biological consequences. Third, the IGF-1 gene expression was investigated in neonatal cardiac myocytes. Therefore, the present data may not directly apply to the adult cardiac myocytes. Further investigation is necessary to clarify the physiological and pathophysiological significance of IGF-1 in the heart.

In conclusion, this study first showed that the

gene expression of IGF-1 in cardiac myocytes was upregulated after exposure to hypoxia concomitantly with the increases in IGFBP3, VEGF-A, SDF-1, and MMP-14 expression. In light of the current findings, it appears that endogenous IGF-1 plays an important role in the protection of the heart from the progression of CHF.

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