INSULIN-LIKE GROWTH FACTOR I RECEPTORS IN HUMAN CARDIAC MYOCYTES AND THEIR RELATION TO MYOCARDIAL HYPERTROPHY

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Specific binding sites for insulin-like growth factor I (IGF-I) and their expression during cardiac myocyte hypertrophy were studied by autoradiographic analysis of right ventricular biopsy specimens from patients with hypertrophic cardiomyopathy (9 cases), dilated cardiomyopathy (8 cases), and sick sinus syndrome (5 cases). Frozen specimens were cut into 5 μ m-thick sections and thaw-mounted on albumin-coated slides. After incubation with [¹²⁵I]IGF-I, with or without excess cold IGF-I, autoradiography was performed, and grains over myocytes were counted microscopically. Binding of [¹²⁵I]IGF-I was inhibited in a dose-dependent manner by unlabeled IGF-I and competed for by IGF-I > IGF-II > insulin. The maximal grain density was higher in hypertrophic cardiomyopathy ($186 \pm 47/1.8 \times 10^{-2} \text{ mm}^2$) than in dilated cardiomyopathy ($124 \pm 13/1.8 \times 10^{-2} \text{ mm}^2$) or sick sinus syndrome ($98 \pm 18/1.8 \times 10^{-2} \text{ mm}^2$) ($p < 0.01$). There was a strong correlation between the maximal grain density and the diameter of right ventricular myocytes in hypertrophic cardiomyopathy ($r = 0.83$), but no similar correlation was observed in dilated cardiomyopathy or sick sinus syndrome. These data suggest that IGF-I receptors are present in adult human cardiac myocytes, and that IGF-I receptors are related to the development of myocyte hypertrophy in hypertrophic cardiomyopathy.

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THE insulin-like growth factors (IGF) exercise their growth promoting activities through specific membrane receptors expressed by a variety of cells¹. IGF-I receptors have been found in several tissues, including

chick cartilage², human placental membrane³, and rat brain⁴. Recently, Engelmann et al⁵ have reported that the IGF-I receptor concentration in the heart is significantly elevated during the first week after birth in spontaneously hypertensive rats, which are known to exhibit growth abnormalities. However, little information is available regarding IGF-I receptors in normal and hypertrophic human cardiac myocytes. In this

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Fig.1. Photomicrograms of autoradiographs of hypertrophic cardiomyopathy. A, Tissue section incubated with 10^{-7} M 125 I-labeled insulin-like growth factor I (IGF-I). B, Tissue section incubated with 10^{-7} M 125 I]IGF-I in the presence of 10^{-6} M unlabeled IGF-I. Bar: 10 μ m

TABLE I ECHOCARDIOGRAPHIC MEASUREMENTS

	VST (mm)	PWT (mm)	LVDd (mm)	LVDs (mm)
HCM	18.4 ± 5.0	12.8 ± 4.0	44.2 ± 4.1	27.3 ± 3.1
DCM	9.1 ± 0.8	9.5 ± 0.9	61.2 ± 6.3	53.5 ± 6.0
SSS	8.2 ± 0.4	7.0 ± 0.2	49.4 ± 1.7	37.6 ± 1.1

Abbreviations: DCM=dilated cardiomyopathy, HCM=hypertrophic cardiomyopathy, LVDd=left ventricular dimension, end diastole, LVDs=left ventricular dimension, end systole, PWT=posterior wall thickness, SSS=sick sinus syndrome, VST=ventricular septal thickness.

study, we used autoradiography to examine whether IGF-I receptors are evident in adult human myocytes and to investigate changes in their concentration with myocyte hypertrophy in patients with cardiomyopathy.

METHODS

Patient Selection

Right ventricular (RV) endomyocardial

biopsies were performed on three groups of subjects. The first group included 9 cases of hypertrophic cardiomyopathy (HCM), 5 male and 4 female, ranging in age from 34–73 years. The second group included 8 cases of dilated cardiomyopathy (DCM), 7 male and 1 female, ranging in age from 15–69 years. The third group included 5 cases of sick sinus syndrome (SSS), 3 male and 2 female, ranging in age from 56–72 years. Diagnosis of the various cardiomyopathies was made according to the WHO/ISFC task force criteria⁶ Patients suffering from coronary artery disease, valvular heart disease, congenital heart disease, hypertension, diabetes mellitus, liver cirrhosis, or alcoholic heart disease were excluded. Diagnosis of SSS was based on a routine electrocardiogram, an ambulatory electrocardiogram, and electrophysiological study. HCM was sporadic in all cases. M-mode echocardiographic measurements (Table I) were made according to the leading edge methods recommended by the American Society of Echocardiography⁷ Cavities and walls were measured at the level of the

Fig. 2. Effect of incubation time on ^{125}I -labeled insulin-like growth factor I (IGF-I) binding to cardiac myocytes. Tissues were incubated at 20°C . Total binding (●-●) approached a plateau at 2 h, while non-specific binding (○-○) remained constant at all times.

chordae below the mitral valve. End diastole was defined as the onset of the QRS, and end systole was determined by the peak of the posterior wall motion. The ratio of the thickness of the ventricular septum to that of the posterior left ventricular free wall was 1.3 or greater in 8 of the patients with HCM. In one patient, the ratio was less than 1.3 (septum=21 mm, posterior wall=22 mm) in the absence of other acquired or congenital heart disease. Anterior motion of the anterior leaflet of the mitral valve in systole was noted in 4 of these patients. A significant pressure gradient, of 30 mm Hg or more, was present in 2 patients with HCM. All of the patients with DCM had a heart to thorax width ratio of more than 0.50 on chest radiography. The ejection fraction (EF) in the DCM cases was $26.3 \pm 9.0\%$. In all cases, the NYHA Functional Classification at the time of biopsy was class II. The EF in the patients with HCM ($EF = 74.9 \pm 11.9\%$) and SSS ($EF = 55.8 \pm 3.1\%$) was normal. The SSS cases were used as controls, because they showed normal cardiac function and had no significant myocardial pathology. All patients provided their informed consent.

Endomyocardial Biopsy

Four biopsy specimens were obtained from each patient using a Konno-Sakakibara biotome. For light microscopy, two specimens were fixed in 10% buffered formalin,

Fig. 3. Effect of exposure time. Grains were counted over tissues exposed for periods of up to 14 days. The ratio of the grain density to the grain density over tissue exposed for 8 days is plotted on the Y axis. Grain density reached a plateau on the eighth day.

embedded in paraffin, cut into $3\text{ }\mu\text{m}$ -thick sections, and stained with hematoxylin-eosin (H-E), Mallory, elastica-van-Gieson, or Periodic-acid-Schiff reaction stain. For autoradiography, two specimens were fixed in periodate-lysine-paraformaldehyde fixative (0.01 M sodium metaperiodic acid, 0.075 M lysine monohydrochloride, 2% paraformaldehyde, 0.0375 M phosphate buffer) for 6 h at 4°C and then quick-frozen in OCT compound (Miles Scientific, Illinois, U.S.A.) which had been cooled in liquid nitrogen. Ten consecutive cryostat sections ($5\text{ }\mu\text{m}$ thick) were thaw-mounted on albumin-coated slides.

Peptides

Recombinant human IGF-I, rat IGF-II and porcine insulin were provided by Fujisawa Pharmaceutical Co. (Osaka, Japan). IGF-I was radio-iodinated by the chloramine-T method⁸ to a specific activity of 2000 Ci/mmol.

Receptor Binding

Samples were preincubated for 10 min at 20°C in 0.1 M acetic acid to exclude endogenous IGF-I binding to IGF-I receptors. This was followed by washing the samples for three 10 min periods in Tris-HCl incubation buffer (0.2 M Tris-HCl buffer, pH 7.5, with 0.04 M MgCl_2). The samples were then in-

Fig. 4. Saturation curves of specific ^{125}I -labeled insulin-like growth factor I (IGF-I) binding to cardiac myocytes. Specific binding curves were saturable. ●—●: hypertrophic cardiomyopathy, ▲—▲: dilated cardiomyopathy, ■—■: sick sinus syndrome

cubated with [^{125}I]IGF-I in the presence or absence of unlabeled IGF-I (10^{-11} – 10^{-7} M), IGF-II (10^{-10} – 10^{-7} M) or porcine insulin (10^{-9} – 10^{-5} M). Incubations were performed in 0.2 M Tris-HCl incubation buffer at 20 °C for various times. After incubation, the samples were washed three in 0.2 M Tris buffer for 10 min periods, quickly dipped in distilled water, and dried in a stream of cold air.

Autoradiography

Samples were dipped in Konika NR-M2 emulsion (Konika Corporation, Japan) diluted 1:1.5 with water at 43 °C, air dried for 30 min, and then stored with desiccant at 4 °C for different time periods. After exposure, samples were developed in Konidol X (Konika Corporation, Japan) for 5 min and rinsed in 0.1 M acetic acid, fixed in Konifix (Konika Corporation, Japan) for 5 min and rinsed in distilled water for 20 min. The tissues were fixed in Carnoy's solution⁹ and stained with H-E.

Microscopic Grain Counting

Grain counting was performed only over cardiac myocytes using an ocular grid on a Nikon microscope with a 100× objective, according to the method of Bar et al.¹⁰ Fields of $9.0 \times 10^{-4} \text{ mm}^2$ of cardiac myocyte were chosen randomly for grain counting, and the total area counted on each section was $1.8 \times 10^{-2} \text{ mm}^2$ (20 fields). The grains over two sections were counted to determine

Fig. 5. Competition for ^{125}I -labeled insulin-like growth factor I (IGF-I) binding. Tissue sections were incubated with [^{125}I]IGF-I in the absence or presence of unlabeled IGF-I (10^{-11} – 10^{-7} M), IGF-II (10^{-10} – 10^{-7} M) or porcine insulin (10^{-9} – 10^{-5} M) for 3 h at 20 °C. Half-maximal inhibition was observed at IGF-I concentrations of 3.6×10^{-9} M. The relative abilities to compete against [^{125}I]IGF-I, as compared to the ability of IGF-I, were IGF-II—1:81; insulin—1:2300.

mean values. The nonspecific grain density over cardiac myocytes incubated in [^{125}I]IGF-I plus 10^{-6} M unlabeled IGF-I was subtracted from total grain density over serial sections incubated only in [^{125}I]IGF-I to derive the specific grain density. Intra-observer and inter-observer variances in grain counting were less than 5% and 7%, respectively.

Measurement of Right Ventricular Myocytes

Sections were examined through a 40× objective and a 10× eyepiece with an ocular micrometer disc. The diameter of the myocytes was determined by the shortest distance across the cell at the nucleus in longitudinally-cut myocytes using the methods of Baandrup and Olsen¹¹ and Sekiguchi et al.¹² At least 30 cells from each biopsy specimen were measured, and their diameters were averaged.

Statistical Analysis

All data are expressed as the mean $\pm$ standard deviation. One-way analysis of variance (ANOVA) and the multiple com-

Fig. 6. Relationship between maximal grain density and the diameter of right ventricular myocytes in hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM) and sick sinus syndrome (SSS).

parison method of Scheffe¹³ were used to compare the grain density and the diameter of RV myocytes in HCM, DCM, and SSS. The relation between the grain density and the diameter of RV myocytes was examined by linear regression analysis. P values of less than 0.05 were considered to be significant.

RESULTS

Fig. 1 is a sample autoradiogram from this study, in which cardiac myocytes were incubated in 10^{-7} M [^{125}I]IGF-I with (nonspecific binding) and without (total binding) 10^{-6} M IGF-I. At 20°C , total binding approached a plateau at 2 h, while nonspecific binding was constant at all times. Therefore, specific binding appeared to reach a plateau at 2 h (Fig. 2). Specific binding at 3 h averaged 57% of total binding. When labeled tissues were exposed to photographic emulsion for up to 14 days, the density of silver grains reached a plateau after 8 days (Fig. 3). Therefore, we performed labeling incubations at 20°C for 3 h to characterize the specificity of the IGF-I receptors, and exposed tissues for 8 days to count grain density.

Specificity of the Receptor

Fig. 4 shows specific binding curves, which were saturable, in individual cases of HCM, DCM, and SSS. The binding of [^{125}I]IGF-I to cardiac myocytes was inhibited in a dose-dependent manner by unlabeled IGF-I, and half-maximal inhibition was observed at IGF-I concentrations of 3.6×10^{-9} M. The binding of [^{125}I]IGF-I was competed for by

IGF-I > IGF-II > insulin (Fig. 5).

Diameter of Right Ventricular Myocytes

The diameters of RV myocytes in HCM, DCM, and SSS were $20.4 \pm 3.4 \mu\text{m}$, $17.1 \pm 2.1 \mu\text{m}$ and $12.9 \pm 0.5 \mu\text{m}$, respectively. The diameter of myocytes in HCM was significantly larger than that in DCM ($p < 0.05$) or SSS ($p < 0.01$), and the diameter in DCM was larger than that in SSS ($p < 0.05$).

Correlation between IGF-I Receptors and Cell Diameter

The means of maximal grain density (the specific binding at [^{125}I]IGF-I concentrations of 10^{-7} M) in HCM, DCM, and SSS were $186 \pm 47 / 1.8 \times 10^{-2} \text{ mm}^2$, $124 \pm 13 / 1.8 \times 10^{-2} \text{ mm}^2$ and $98 \pm 18 / 1.8 \times 10^{-2} \text{ mm}^2$, respectively. Maximal grain density was significantly higher in HCM than in DCM or SSS ($p < 0.01$). There was no significant difference in maximal grain density between DCM and SSS. There was no significant correlation between patient age and maximal grain density in HCM, DCM, or SSS. The relationship between maximal grain density and the diameter of RV myocytes is shown in Fig. 6. There was a strong correlation between maximal grain density and the diameter of RV myocytes in HCM ($r = 0.83$), but no such correlation appeared in DCM or SSS. If all of the HCM, DCM, and SSS cases are taken together, then maximal grain density was directly proportional to the diameter of myocytes ($r = 0.82$, $p < 0.01$, data not shown).

DISCUSSION

IGF-I binding to its receptor is most likely dependent on the temperature of the incubation buffer and the incubation time. Megyesi et al¹⁴ previously reported that IGF-I binding reaches a steady state within 120 min at 20°C. Since IGF-I binding approached a plateau at 2 h in our study as well, samples were incubated at 20°C for 3 h to characterize the specificity of the IGF-I receptor. In the present study, the specific binding during incubation for 3 h represented only 57% of the total binding. Vandermolen et al¹⁵ reported that specific binding represents 67% of the total binding in autoradiographic analysis of beta-adrenergic receptors in cardiac myocytes fixed in 0.1% paraformaldehyde. In our study, biopsy specimens were fixed in 2% paraformaldehyde to preserve the fine structure for microscopic grain counting and measurement of myocytes. According to Young and Kuhar,¹⁶ fixation with 2% formaldehyde caused a significant loss of specific binding, while fixation with 0.1 or 0.5% formaldehyde did not change the specific binding from that observed in unfixed tissues. Therefore, in our study, the fixation technique we used may have decreased specific binding.

It has been shown that IGF-I receptors are down-regulated in a dose-dependent manner by IGF-I,¹⁷ and that the down-regulation of IGF-I receptors results in an apparent decrease in the affinity of cultured mouse muscle cells for IGF-I.¹⁸ In this study, the maximal grain density in HCM was significantly higher than that in DCM or SSS. However, since we did not measure plasma IGF-I levels, it is unclear whether down-regulation affected maximal grain density. In addition, plasma insulin levels might affect maximal grain density. DCM may be generally complicated by the development of liver cirrhosis that affects plasma insulin levels. However, all patients with DCM showed normal liver function.

The diameters of RV myocytes in HCM, DCM, and SSS were $20.4 \pm 3.4 \mu\text{m}$, $17.1 \pm 2.1 \mu\text{m}$ and $12.9 \pm 0.5 \mu\text{m}$, respectively. Hypertrophied myocytes were present in HCM and DCM, but not in SSS. Cardiac hypertrophy results from a variety of pathological processes including pressure over-

load,¹⁹ volume overload,¹⁹ and α_1 -adrenergic effects.²⁰ In addition, abnormalities of catecholamine metabolism or sympathetic innervation²¹ may account for the development of cardiac hypertrophy in HCM. Although we have previously reported that the number of α_1 -adrenergic receptors is correlated with the degree of hypertrophy of myocytes in HCM,²² we expect that other hormonal mechanisms may also be relevant. For example, Bassas et al²³ provided evidence of distinct tissue-specific developmental regulation by IGF-I receptors in chicken embryo hearts. There is no doubt that several growth factors may influence the growth and maintenance of cardiac myocytes. Our data showed a strong correlation between maximal grain density and the diameter of RV myocytes in HCM, which suggests that IGF-I receptors may be involved in the hypertrophy of myocytes in HCM. On the other hand, while hypertrophied myocytes were also present in DCM, there was no correlation between maximal grain density and the diameter of RV myocytes in DCM, suggesting that this relationship may be specific to HCM.

The advantage of the autoradiographic method is that it can clearly reveal the distribution of binding sites in tissues. By using autoradiographic grain counting, Bar et al¹⁰ have identified IGF-I binding sites in the capillaries of a beating heart model which have specificities similar to the IGF-I binding sites of cultured capillary endothelial cells. The autoradiographic method is clearly suitable for observing receptors in specific areas of tissue, and was therefore suitable for our study of IGF-I binding to cardiac myocytes in biopsy specimens.

We performed autoradiography using right ventricular endomyocardial biopsy specimens. Several investigators have reported that there is little significant difference between the pathological findings in right and left ventricular specimens.^{24,25} Furthermore, RV biopsy and post-mortem diagnoses are usually consistent.^{26,27} Based on these reports, we presumed that the features of right ventricular biopsy specimens were representative of the entire heart in idiopathic cardiomyopathy.

Our study leaves several questions unanswered. SSS cases may not necessarily

show normal IGF-I binding, although they do have normal cardiac function and show no significant myocardial pathology. Furthermore, since the number of IGF-I receptors normally decreases during maturation;²³ the maximal grain density in DCM or SSS may be higher than that in normal hearts. An important question is whether or not the increase we found in the number of IGF-I receptors is secondary to hypertrophy and unrelated to its pathogenesis. When all of the HCM, DCM, and SSS cases were taken together, maximal grain density was well correlated with the diameter of RV myocytes. Therefore, these results suggest that the size of myocytes in various diseases may be unrelated to their pathogenesis.

In summary, we have shown that IGF-I specific receptors are present in adult human cardiac myocytes. Our data suggest the possibility that IGF-I receptors play a role in the development of myocyte hypertrophy in HCM. Further studies are needed to fully delineate the role of IGF-I in cardiac myocytes.

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