Ventricular expression and circulating levels of insulin-like growth factor I in heart transplant recipients

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(Received 7 June 1994/1 February 1995; accepted 21 March 1995)

INTRODUCTION

Insulin-like growth factor I (IGF-I) is a circulating polypeptide hormone predominantly produced in the liver and has a high degree of structural homology with insulin [1]. IGF-I is a major mediator of growth hormone effects and is expressed in various tissues in animals [2, 3]. IGF-I may also have local autocrine or paracrine actions, in addition to its role as a circulating hormone [3]. IGF-I mRNA has been detected in cardiac myocytes from neonatal rats [4] and is up-regulated in the rat myocardium by pressure overload [5]. In humans, IGF-I has only been investigated in the fetal myocardium [6]. Recently, we reported the expression of IGF-I by human cardiac explants in culture [7]. However, the expression of IGF-I in the human heart, and in particular after orthotopic cardiac transplantation, has not been previously studied. We therefore used endomyocardial biopsies and plasma samples taken from cardiac transplant recipients to assess the expression of IGF-I gene at the mRNA and peptide levels and determined the circulating levels of IGF-I following orthotopic cardiac transplantation.

MATERIALS AND METHODS

Characteristics of patients

The study was approved by the local hospital ethics committees and informed consent was obtained. We studied 14 cardiac transplant children aged 13 ± 2 years (range 10–17 years) and 10 adult cardiac transplant recipients aged 38 ± 8 years (range 28–50 years), immunosuppressed with cyclosporin A, azathioprine and prednisolone, with no clinical features of cardiac failure. The mean age of donors for the transplant children was 18 ± 3 years (range 6–41 years), while the mean donor organ age for adult patients was 31 ± 5 (23–43) years. The time from transplantation to sampling in all the patients was between 1 month and 1 year.

Table 1 shows the characteristics of the cardiac transplant children studied. Duplicate endomyocardial biopsies for the measurements of mRNA and tissue IGF-I levels were obtained from the right ventricle at the time of elective biopsy as part of routine assessment for the presence of rejection. The biopsy specimens were rapidly collected then stored at −70°C before extraction. Whole-blood (10 ml) samples were taken from each patient via the biopsy sheath into EDTA tubes, centrifuged (2000 g, 15 min, 4°C) and plasma was collected and stored at −70°C before extraction and assay. Right ventricular tissues were obtained from 10 patients (age range 21–56 years) without any known cardiac

Key words: autocrine, cardiac transplantation, growth factor, paracrine.

Abbreviations: ANOVA, analysis of variance; bFGF, basic fibroblast growth factor; IGF-I, insulin-like growth factor I; PDGF, platelet-derived growth factor.

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complications during urgent necropsy (less than 24 h from time of death) to serve as controls. A 10-ml sample of whole blood was taken from 10 normal healthy children (age range 12-15 years) and 10 normally healthy adults (age range 23-54 years) into EDTA tubes to serve as controls. Plasma was immediately separated by centrifugation (2000g, 1 h, 4°C) and stored at −70°C before extraction and assay.

Plasma extraction of IGF-I

Plasma samples were initially extracted to dissociate and remove IGF-I-binding proteins by using the acid–ethanol–cryoprecipitation method reported by Brier et al. [8]. Briefly, 100µl of plasma was mixed with 400 µl of 87.5% (v/v) ethanol and 12.5% (v/v) 2 mol/l HCl at room temperature for 1 h. The extracted plasma was centrifuged (2000g, 1 h, 4°C) and the supernatant was neutralized with 0.86 mol/l Tris base at a ratio of 5:2. The supernatant was stored at −20°C for 1 h then underwent centrifugation (2000g, 1 h, 4°C). The supernatant was collected and filtered, then reconstituted in the radioimmunoassay buffer (0.1 mol/l phosphate-buffered saline, pH 7.2, containing 0.5% bovine serum albumin). The recovery of IGF-I from this extraction procedure was 85% (n=5).

Tissue extraction of IGF-I

Endomyocardial right ventricular biopsy tissues from cardiac transplant recipients and the native hearts (controls) were extracted to remove IGF-I-binding proteins as already described in detail [3, 7]. Briefly, snap-frozen tissue was pulverized with a mortar and pestle and extracted in 1 mmol/l acetic acid to remove IGF-I-binding proteins [3]. The homogenate was centrifuged (2000g, 1 h, 4°C) and an aliquot of the supernatant was determined for protein [9], while the rest was freeze dried, then reconstituted in radioimmunoassay buffer. The recovery of IGF-I in the tissue extraction procedure was 88% (n=5). The presence of residual IGF-I-binding protein after extraction could not be ruled out because of the microscopic size of the biopsies, precluding further analysis.

Radioimmunoassay for IGF-I

The radioimmunoassay was a non-equilibrium procedure described in detail previously [7, 8]. Samples were assayed in triplicate at 4°C. Briefly, 100µl of IGF-I standard (1.1–1000 ng/ml) [courtesy of Dr Adrian Bristow, National Institute for Biological standards (NIBS), Mmms, U.K.], reconstituted acid extracts of tissue, plasma and quality controls (kindly provided by Dr David Holt, Analytical Unit, St George’s Hospital Medical School, London, U.K.) were incubated with 100 µl of antihuman IGF-I serum (NIBS) for 24 h. Then 100 µl of iodinated human IGF-I (Amersham, U.K.) diluted in radioimmunoassay buffer (7000 p.c.m.) was added to each tube and incubated for a further period of 48 h. Bound and unbound IGF-I were separated by the addition of 200 µl of a secondary antibody in 8% polyethlene glycol 6000. The assay tubes were centrifuged (2000g, 4°C, 1 h) and the pellets were determined for radioactivity. The sensitivity of the assay was 5 pg/tube. The inter- and intra-assay coefficients of variation were 4.9% (n=10) and 3.6% (n=10) respectively.

Extraction of total RNA

Each endomyocardial biopsy tissue was homogenized with RNazol (Biotex, Friendswood, TX, U.S.A.) in a mixer and total RNA extracted by the method of Chomczynski and Sacchi [10]. The total RNA concentration was measured spectrophotometrically at an absorbance of 260 nm. The integrity of the RNA samples was initially verified by electrophoresis of an aliquot of each sample in a denaturing agarose–glyoxal gel followed by ethidium bromide staining. The 28S to 18S ratio was 2:1 for all total RNAs. Hybridization of the IGF-I probe was evaluated by Northern blotting in preliminary experiments.

Slot-blot hybridization

Total IGF-I mRNA levels were assessed by slot-blot hybridization as already reported in detail [11, 12]. Briefly, aliquots (14 µg) of the extracted total RNA were denatured in a mixture of 6.15 mmol/l formaldehyde/20× SSC (sodium chloride, sodium citrate) and incubated at 65°C for

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Table I. Characteristics of the cardiac children. Abbreviations: MRAP, mean right atrial pressure; SRVP, mean systolic right ventricular pressure; RVEDP, mean right ventricular end-diastolic pressure.
Insulin-like growth factor I expression after heart transplant

15 min, then loaded directly on to a pre-wet Hybond-N membrane (Amersham, U.K.) with a slot-blot apparatus (Schleicher & Schull, Germany). A serial dilution of human placental RNA (0.09–5.5μg) was denatured likewise and slotted with ventricular RNA to serve as internal standard. After slotting, the RNA was irreversibly 'fixed' on to the membrane by baking at 80°C for 2 h.

The slot-blot membrane was initially prehybridized overnight at 65°C in 0.5 mmol/l phosphate buffer (pH 7) containing 0.1% SDS and subsequently hybridized overnight to a [32P]CTP (Amersham, UK)-labelled rat IGF-I cDNA probe (courtesy of Dr Liam Murphy, Department of Physiology, University of Manitoba, Canada) according to a previously reported method. The membrane was washed at low stringency (1×SSC/0.1% SDS, 65°C, 10 min) and high stringency (0.1× SSC 0.1% SDS, 65°C, 10 min), then autoradiographed at −70°C using intensifying screens (Amersham, U.K.) for 24–48 h. After the detection of IGF-I mRNA by densitometric scanning using a Joyce-Loebl chromoscan 3 densitometer, the membrane was stripped and rehybridized to a β-actin probe to normalize RNA loading [11]. Results were expressed as the ratio IGF-I mRNA to β-actin mRNA (arbitrary units).

Statistical analysis

Data are presented as means±SEM and range where appropriate. Correlations were tested by Spearman's test or least-squares method. Multiple data sets were compared by one-way analysis of variance (ANOVA) after normalization by log transformation where appropriate. Data sets were then compared by unpaired tests using the variance derived from the ANOVA. A P-value of less than 0.05 was considered significant.

RESULTS

Plasma IGF-I levels

Fig. 1 shows plasma IGF-I levels against age of subjects studied. Mean plasma IGF-I levels were 164±10 (range 135–192) ng/ml (n=14) in the cardiac transplant children and 176±15 (range 146–244) ng/ml (n=10) in cardiac transplant adults. The corresponding levels in normal healthy children and adults were 168±12 (range 150–200) ng/ml (n=10) and 182±16 (range 160–220) ng/ml (n=10) respectively. In effect, there was no significant difference in plasma IGF-I levels between cardiac transplant recipients and normal healthy controls. There was no relationship between plasma IGF-I and time after transplant in the cardiac transplant recipients.

Ventricular concentration of IGF-I

Fig. 2 shows the myocardial ventricular IGF-I levels against age of subjects. The content of ventricular IGF-I in the cardiac transplant children [174±15 (range 39–950) pg/mg soluble protein] was significantly higher (one-way ANOVA after log transformation) than in cardiac transplant adults [39±2 (range 14–92) pg/mg soluble protein] and normal native hearts (post mortem) obtained during urgent necropsy [30±1 (range 11–90) pg/mg soluble protein, P<0.001]. However, there was no significant difference in ventricular IGF-I levels between cardiac transplant adults and normal native hearts (P<0.13) (see Fig. 2). In the cardiac transplant recipients, we could not demonstrate any significant relationship between either time after transplant or plasma IGF-I and levels of ventricular IGF-I.

Ventricular IGF-I mRNA

Fig. 3 shows a typical slot-blot analysis for IGF-I mRNA in total RNA from the right ventricular biopsy of the transplanted hearts. Levels of IGF-I mRNA in the serial dilution of human placental RNA correlated linearly with the concentration of total RNA slotted (Fig. 3, lane A). IGF-I mRNA [160±23 (range 128–283) arbitrary units] was detectable in all the ventricular biopsy tissues from cardiac transplant children (lane B) but below the limit of detection in all the adult ventricular samples.
Fig. 3. Slot-blot analysis of IGF-I mRNA in total RNA from the right ventricular biopsy of transplanted hearts. Lane A, serial dilution of placental RNA (see the Methods section). Lane B, RNA from 10 typical biopsies from children.

at similar loading and autoradiography. Prolonged autoradiography did not produce significant signals in the adult RNA extracts (results not shown). There was a positive correlation between IGF-I mRNA and ventricular IGF-I (r=0.65, P<0.01, n=14). However, there was no significant relationship between either plasma IGF-I or time after transplant and levels of ventricular IGF-I mRNA expression, nor was there a significant relationship between IGF-I mRNA and any of the haemodynamic variables or age of donors (Table 1).

Limitations of study

We acknowledge the possibility of blood contamination of the biopsy (for example, 1 μl of plasma contains about 150 pg of IGF-I). However, plasma levels of the peptide were not significantly different in adults as compared with children.

DISCUSSION

It is now established that the heart is an endocrine organ with the synthesis by cardiac myocytes of many polypeptide growth factors with autocrine, paracrine and endocrine functions [13]. Evidence from in vitro studies suggests that the proliferation of cells at the site of vascular injury is modulated by a number of locally produced growth factors such as basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) and insulin-like growth factor I (IGF-I).

We examined ventricular IGF-I gene expression at the mRNA and peptide levels and determined the circulating levels of IGF-I after orthotopic cardiac transplantation in children and adults. Slot-blot hybridization of total RNA from ventricular biopsies with IGF-I cDNA probe indicated the presence of IGF-I mRNA in cardiac transplant children but not in any of the cardiac transplant adults. This demonstration of ventricular IGF-I mRNA provides supportive evidence that the human heart may be a site for IGF-I production, thereby extending previous studies of human cardiac explant in culture [7] and human fetal myocardium [6] and the rat heart [5]. The finding that IGF-I mRNA was not expressed in the myocardial ventricular biopsies from the adults apparently reflects mRNA levels below the limit of detection by slot-blot hybridization. It is clear that the ventricular IGF-I gene can be expressed in adults as it was detectable in children who had received adult hearts, but there is no published information on the stability, turnover or translation of IGF-I peptide or mRNA.

Ventricular IGF-I levels were significantly higher in cardiac transplant children than in cardiac transplant adults and the native hearts (Fig. 2). Our data also showed a positive correlation between ventricular IGF-I and IGF-I mRNA in the cardiac transplant children. The apparently low ventricular IGF-I levels in the cardiac transplant adults and the native hearts may represent part of regenerative and repair processes in the heart, as is the case with skeletal muscle [14].

It has been suggested that bFGF in conjunction with IGF-I may be involved in the stimulation of DNA synthesis and proliferation of cardiac myocytes [15]. Recently, we demonstrated increased ventricular bFGF gene expression at the mRNA level after orthotopic cardiac transplantation [16]. Our present data, together with these observations, suggest that IGF-I may have a pathophysiological role in the regulation of cardiac function, possibly in the growth and regenerative process after myocardial injury.

IGF-I is a known major mediator of post-natal growth in many tissues [4] and is important in growth hormone-stimulated skeletal muscle growth [14]. It is now established that IGF-I gene expression is directly regulated by growth hormone. By the end of 1993, 1668 heart and 186 heart-lung transplants had been performed in children [17]. It is therefore surprising how little is known of the subsequent growth of the transplanted heart. Data from a small study in 13 subjects published in 1992 suggested that both the right and left ventricles increase in volume and remain hypertrophied for at least 3 years after transplant [18]. However, a separate study has suggested that the cardiac
enlargement may in part result from the response to rejection [19], at least in very young heart transplant recipients.

In the present study, we found no difference in levels of plasma IGF-I between the cardiac transplant recipients and the normal healthy controls. In effect, the circulating levels of IGF-I in the cardiac transplant recipients reflect the documented trend of IGF-I in normal children and adults [20, 21]. However the significance of the level is uncertain as it appears largely to represent hepatic production [22].

In conclusion, these findings provide support for an autocrine role for IGF-I in the ventricle, despite cardiac denervation. The greater expression of ventricular IGF-I in the cardiac transplant children is consistent with an increased ventricular growth stimulus during adolescence, even after transplantation.

ACKNOWLEDGMENTS

We gratefully acknowledge grant support from the British Heart Foundation. We are also grateful to Dr Liam Murphy (Department of Physiology, University of Manitoba, Canada) for the IGF-I cDNA probe. Thanks are expressed to Drs S. H. Jennison and P. Keeling for help with collection of samples.

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