IGF-II and MMP9 as Surgical Repair Indicators of Ventricular Septal Defects

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ABSTRACT

Objective: The insulin-like growth factor -I (IGF-I), insulin-like growth factor binding protein-3 (IGFBP-3) and human growth hormone (hGH) have been recognized as therapeutic targets for the heart disease therapy. The bioavailability and actions of insulin-like growth factors-II (IGF-II) and matrix metalloproteinase-9 (MMP9) are important for embryonic development and cardiomyocyte differentiation as well. However, the clinical manifestations following the change in the serum IGF-II and MMP9 in infants with isolated ventricular septal defect (VSD) undergoing surgical repair have not been clearly defined.

Study design: Serum samples were collected from 72 infants: Twenty normal infants (group I) and 51 consecutive infants with echocardiography established isolated VSD (aged from 3 months to 1 year) were investigated. Among the 51 infants with VSD, 28 with shunt fraction, Qp/Qs < or = 1.5 were free of congestive heart failure symptoms (group II); 23 with shunt fraction, Qp/Qs > or = 2.0 were in congestive heart failure (group IIIa); and 23 of these 23 infants had undergone VSD repair 6 months before their second study (group IIIb). All insulin-like growth factors-II (IGF-II) and human growth hormone (hGH), insulin like growth factor binding protein-3 (IGFBP-3) and its specific serum protease-MMP9 concentration were analyzed using ELISA and zymography, respectively.

Results: Serum IGF-II and MMP9 exhibited significant decreasing trends among the three groups and significantly lower concentrations of IGF-II, IGF-II/IGFBP-3 ratio and MMP9, were found only in the severe group whereas h-GH/IGF-II ratio became significantly higher in this group. Moreover, there were no significant differences in these parameters between the infants after surgical correction and the normal ones.

Conclusions: The improvement in IGF-II and MMP9 serum concentration were identified in infants with VSD after surgical repair. These findings also indicate a significant relationship between IGF-II, MMP9 and VSD which might be used as diagnosis and prognosis indicators for this defect. Slight reductions in IGF-II /IGFBP3 ratio and slight increase in the h-GH/IGF-II ratio indicate mild VSD. The reductions in the MMP9, IGF-II, and IGF-II/IGFBP3 ratio plus high increase in the h-GH/IGF-II ratio indicate severe VSD.

KEYWORDS: insulin-like growth factor-II; matrix metalloproteinase-9; ventricular septal defect infants; surgical repair

1. Introduction

The insulin-like growth factors (IGFs) have a regulatory function in fetal cardiac development [1]. These factors belong to the insulin-related peptide family. IGF-I and IGF-II are important members [2]. In postnatal animals, IGF serum concentration are growth hormone (GH) dependent and they have an endocrine function mediated through the IGF-I receptor on the surface of their corresponding responsive cells [3,4]. Moreover, IGF gene expression has been demonstrated in various tissues including the myocardium [3-5].

IGF-II regulates cell proliferation and differentiation by their insulin-like metabolic effects through the endocrine, autocrine and/or paracrine mechanisms [6]. IGF-II has a significantly higher level of transcripts than IGF-I in the liver, kidney and heart of the fetus [7] and can be detected in heart muscles in early adulthood [8]. It is important for fetal growth [9]. Disruption of the interaction between this factor and its receptor may result in embryonic growth deficiency [10]. In fetal rats, IGF gene expression is abundant in most tissues. Although IGF-II mRNA and circulating IGF-II decrease dramatically at birth, the IGF gene expression in the brain and heart remains relatively constant in the postnatal stage [9]. All of these findings suggest the importance of IGF-II in cardiac development.

IGF are regulated by a family of six IGF binding proteins (IGFBPs) through proteolysis using matrix metalloproteinases (MMPs) [11, 12]. MMP have been reported to produce IGFBP fragments with a low affinity to IGF, which in turn increases the bioavailability of IGF for IGF receptors for smooth muscle cell proliferation induction [11]. IGFBP-3 modulates the proliferation of the prostate adenocarcinoma cell line by

reducing signaling through the IGF-I receptor. This binding protein is regulated by its specific protease-MMP9 [12]. It is critical for circulating IGF bioactivity and the increase in the molar ratio between IGF-II and IGFBP-3 reflects an increase in free, biologically active IGF-II [13]. Moreover, the IGF-II and IGFBP-3 serum concentration may also reflect endogenous GH secretion in a normal child [14], although the regulation of the autocrine/paracrine IGF and the functions of the locally produced GH are not well defined [15].

Ventricular septal defect (VSD) is the most common congenital heart disease in infants. This disease may cause significant hemodynamic volume overloading, resulting in heart failure. Although spontaneous closure may occur in mild cases, early surgical correction is required for the severe ones. Diagnosis of this disease is based only on ultrasonography results. As we know that IGF are both important for embryonic heart development [16], we have reported the improvement of serum IGF-I in VSD infants after surgical repair [17]. However, there is still no available information concerning IGF-II and MMP9 with ventricular septum formation at the embryonic stage. In this study, we determined the changes in IGF-II serum concentration and the factors related to the IGF axis in infants with different VSD severity after surgical correction. The results indicate an association between the IGF-II axis and VSD and the changes in IGF-II axis may be helpful criteria for the diagnosis and prognosis of VSD.

2. Methods

2.1 Subjects

The subjects were 72 infants (40 males and 32 females, aged 3 to 12 months) participating a screening program for congenital heart diseases at the Division of

131 Pediatric Cardiology, Department of Pediatrics, China Medical University Hospital.
132 These infants were divided into three groups according to clinical manifestations and
133 the ratio of pulmonary to systemic blood flow (Qp/Qs). The overload volume was
134 determined using echocardiography and catheterization analysis. The normal group
135 (group I) included infants without congestive heart failure symptoms and Qp/Qs < 1.5.
136 The mild group (group II) included those without congestive heart failure symptoms but
137 Qp/Qs = 1.5-2.0. The severe group (group IIIa) included those with intractable heart
138 failure (Qp/Qs ≥ 2.0) and required surgical correction. The post-surgery group (group
139 IIIb) was comprised of infants with surgical corrections examined 6 months after
140 operation. Whole blood samples were collected from these infants. Each sample (4 mL)
141 was centrifuged at 3000 *g* for 5 min. The serum was then aspirated and stored at -20°C
142 for further analysis.
143
144 *2.2 ELISA for IGF-II, IGFBP-3, hGH and tPA*
145 Serum IGF-II and IGFBP-3 were determined using a commercially available non-
146 extraction enzyme-linked immunosorbent assay (ELISA) kit (Diagnostic Systems
147 Laboratories, Inc., Webster, TX, USA) with a monoclonal antibody according to the
148 manufacturer's instructions (DSL-10-2600, DSL-10-6600, Texas, USA). The target
149 proteins were immunosorbed using enzyme-conjugated antibody before adding the
150 corresponding substrates for color development. Protein contents were determined by
151 comparing the relative absorbency of the samples to that of known amounts of standard
152 using a microplate reader (Model: RS01, Kansin instruments. CO, LTD). Serum human
153 growth hormone (hGH) and tissue plasminogen activator (tPA) were determined using
154 another ELISA kit (Biosource, Europe, SA and American Diagnostica, Inc., Greenwich,

155 CT) according to the manufacturer's instructions (IBL-MG-59121, IMUBIND®tPA
156 ELISA KIT Product NO. 860).
157
158 *2.3 Gelatin Zymography Protease Assay*
159 Gelatin zymography analysis was carried out by loading 10-µl sample of serum on
160 0.1% gelatin - 8% SDS-PAGE. Electrophoresis was run at 150 V for 2.5 h. Enzymes on
161 the gels were renatured by washing twice in a 2.5% Triton X-100 solution with shaking
162 for 30 min. The gels were then incubated with a reaction buffer (50 mL) containing 40
163 mM Tris-HCl (pH 8.0), 10 mM $CaCl_2$, and 0.01% NaN_3 at 37°C for 16 h before staining
164 with 0.25% Coomassie brilliant blue R-250 for 30 min. Quantitative analysis was
165 carried out after discoloring the stain in a discoloring solution (875 mL H_2O, 50 mL
166 methanol, and 75 mL acetic acid). A randomly chosen human breast cancer biopsy
167 extract was used as the marker. Expression of 92 kDa (proMMP-9) and 72 kDa
168 (proMMP-2) gelatinase in the serum were determined using the Kodak Scientific
169 Imaging Systems SP700 (Eastman Kodak Company, Rochester, NY).
170
171 *2.4 Determination of Protein Contents*
172 Total protein and albumin in the serum samples were determined by the Lowry
173 protein assay [18]. A commercial available bovine serum albumin (Sigma Chemical, St.
174 Louis, MO) was employed as a standard.
175
176 *2.5 Statistical analysis*
177 Data were expressed as mean ± SEM. Differences among the groups were
178 determined by one-way analysis of variance (ANOVA). Fisher's least significant

179 difference test was used to determine differences. $P < 0.05$ was considered to be
180 statistically significant.
181
182 **3. Results**
183 *3.1 Clinical Characteristics*
184 There was no significant difference in the sex ratio, birth weight, gestation, heart rate,
185 age, and respiratory rate among the four groups ($p > 0.05$). However, the body weight in
186 the severe group IIIa was lower than that of the remaining groups ($p < 0.05$) (Table 1).
187 Total protein albumin serum concentration in each group were not significant (Data not
188 show).
189
190 *3.2 Serum IGF-II, IGF-II/IGFBP-3 ratio, and h-GH/IGF-II ratio*
191 Figure 1 shows the serum concentration of (A) IGF-II, (B) IGF-II/IGFBP-3 ratio and
192 (C) h-GH/IGF-II ratio. Normal infants had the highest concentration. The IGF-II and
193 IGF-II/IGFBP-3 ratio showed a significant decreasing trend for the group II and group
194 IIIa subjects ($p < 0.05$). Although the post-surgery group had lower concentration than
195 their normal counterpart, there were no significant differences between groups IIIb and
196 I ($p > 0.05$), whereas the concentration in group IIIa returned to normal range after VSD
197 repair in group IIIb ($p < 0.01$). However, the h-GH/IGF-II ratio became significantly
198 higher in group IIIa and returned to normal range after VSD repair in group IIIb ($p <$
199 0.01). There were no significant differences in these parameters between the infants
200 after surgical correction and the normal ones.
201
202 *3.3 Serum matrix-metalloproteinases 2 ,9 and tissue plasminogen activator*

Although no significant differences were observed in the serum concentration of MMP-2 and tissue-type plasminogen activator (tPA) among the normal and remaining groups, MMP-9 was found significantly lower in the serum samples of group IIIa ($p < 0.05$). However, no significant differences were observed in the concentration between the infants after surgical correction and the normal infants ($p > 0.05$) (Figure 2 A, B).

3.4 Serum total protein, albumin, lactate, and pH

In order to determine if the reduction in IGF does not result from the change in nutritional status, we measured other parameters. There were no significant differences in the serum total protein, albumin and creatine kinase among the four groups (Data not show).

4. Discussion

Insulin-like growth factors (IGFs) are growth hormone (hGH)-dependent peptides, which play important roles as autocrine or paracrine growth factors and also exert growth-promoting actions in an endocrine manner to the myocardium [19, 20]. IGFBP-3 combines with a glycoprotein, the acid-labile subunit, to form a ternary complex with IGF-I or IGF-II [21, 22]. This complex determines the release of IGF. It has been demonstrated that the serum IGF-I, II concentration and weight gain may become lower in new born lambs with experimental cyanotic heart disease [23]. Similar findings were observed in humans with congenital heart diseases, suggesting that nutritional deficiencies may contribute to the reduction in the IGF concentration [24]. In our series, serum IGF-II and the IGF-II/IGFBP-3 ratio (free form IGF-II) had a significantly decreasing trend with the severity of patients with VSD. Because IGFBP-3 is critical for

227 the circulating IGF bioactivity, the decrease in the molar ratio between IGF-II and
228 IGFBP-3 may reflect a decrease in the concentration of free and biologically active
229 IGF-II. However, there were no significant differences in the serum total protein and
230 albumin concentration between normal and VSD infants [17]. These findings indicated
231 that factors other than malnutrition are important in determining the circulating IGF-II
232 concentration.
233 The release of IGF is facilitated by the reduction in IGFBP-3 through the proteolysis
234 mechanism during pregnancy [25]. The collagen proteases, MMP-2 and MMP-9
235 produced mainly by connective tissue, have been considered to contribute to collagen
236 turnover for tissue remodeling in development [26]. The reduced expression of MMP-9
237 is associated with a decrease in IGFBP-3 proteolysis and reduced signaling through the
238 IGF-IR [12]. Moreover, since the MMP serum concentration may decrease significantly
239 in essential hypertension patients with left ventricular hypertrophy, this change not only
240 depresses collagen degradation but also accelerates organ fibrosis [27]. In our series, we
241 observed that the concentration of MMP-9 was significantly reduced in the group IIIa
242 severe VSD patients, indicating that this change may reflect a reduction in the
243 concentration of circulating IGF-II bioactivity and in ventricle septum remodeling.
244 GH treatment has also been demonstrated to be effective in improving congenital
245 heart diseases and growth retardation in children [28]. In our series, the ratio of h-
246 GH/IGF-II was found to be significantly elevated in the group IIIa severe VSD patients
247 and returned to a concentration comparable to the normal group I in the group IIIb post-
248 surgery patients. These findings indicate that the reduction in IGF-II in infants with
249 VSD may not only be caused by disturbance in the GH/IGF-II axis.

In infants with VSD, the opening between the septum shunted oxygenated blood and peripheral blood becomes less oxygenated. This may induce chronic metabolic acidosis and significantly higher serum lactate and lower serum pH in the severe group IIIa VSD subjects in our series [17]. It has been reported that a significant increase in the plasma GH and lactate concentration as well as in acid-base alterations may occur in submaximal exercise on bicycle ergometer [29]. Since acidosis revealed a significant decrease in GH and IGF-I receptors [30], the IGF-II response to growth hormones may also be significantly blunted during acidosis [31]. These might explain why significantly lower IGF-II concentration and a higher ratio of GH/IGF-II occur in severe VSD patients. After surgical correction, the acidosis stress is removed and this positive feedback situation is released. Therefore, the IG-II concentration and the related parameters in the group IIIb post-surgery group returned to concentration comparable to that in the normal group I. These changes also provide a feasible strategy to improve the consequences of severe VSD by suppressing the lactate serum concentration and adjusting the serum pH. The observation that IGF-II concentration and GH/IGF-II ratio gradually return towards normal following surgical repair, which supports the theory that GH insensitivity is a secondary phenomenon associated with IGFII and MMP9 deficiencies, rather than a primary defect in VSD patients contributing to the progression of heart failure.

Based on the results of this study, slight reductions in IGF-II and MMP9 indicate mild VSD and a significant reduction in IGF-II and MMP9 indicate the severe VSD. Moreover, these parameters may return to concentration comparable to those of the normal infants after surgical correction. These changes in the IGF-II axis may be used as an indicator for severity evaluation, requirement for surgical correction, and

prognosis. Moreover, fortified milk formula with IGF-II or gene therapy may be employed to over express IGF-II and in turn recover the GH/IGF-II axis.

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Table 1. Clinical characteristics of the normal infants and those with ventricular septal defect (VSD)

Characteristics	GroupI (n = 21)	Group II (n = 28)	Group IIIa (n = 23)	Group IIIb (n = 23)
Male : Female	11 : 10	16 : 12	13: 10	13 : 10
Birth weight (kg)	3.5 ± 0.72	3.4 ± 0.8	3.3 ± 0.6	3.4 ± 0.7
Gestation (wk)	37.8 ± 2.6	37.2 ± 2.4	37.5 ± 1.8	37.0 ± 1.6
Age at study (mo)	6.8 ± 2.7	7.1 ± 3.2	6.9 ± 3.6	8.3± 2.5
Weight at study (kg)	7.1±0.7$^{\#\#}$	8.3±0.5$^{\#\#}$	5.1±0.3	7.6±0.7$^{\#\#}$
Heart rate (beats/min)	138 ± 17	139 ± 15	143 ± 16	137 ± 15
Respiratory rate(counts/min)	46.5 ± 8.3	50.6 ± 18.4	53.4 ± 16.2	50.4 ± 16.4

Data were expressed as mean ± SEM. $^{\#\#}p < 0.01$ compared with Group IIIa Severe VSD.

403 **Figure Legends**

404

405 **Figure 1.** ELISA analysis of (A) insulin-like growth factor II (IGF-II), (B) IGF-
406 II/IGFBP3 ratio in the serum samples of the subjects, (C) h-GH/IGF-II ratio in
407 the serum samples from subjects. Data were expressed as mean ± SEM.
408 Concentration of significant differences compared with normal group I: *$p <$
409 0.05, **$p < 0.01$. Concentration of significant differences compared with the
410 group IIIa severe VSD group: #$p < 0.05$, ##$p < 0.01$.

411

412 **Figure 2.** (A) Zymography analysis of matrix-metalloproteinase-2 (MMP-2) and MMP-
413 9 in the subjects. (B) Serum matrix metalloproteinase (MMP-2 and MMP-9)
414 activities and tissue plasminogen activator (tPA) concentrations of the
415 subjects. The optical density (O.D.) of MMP-2 and MMP-9 were analyzed
416 using a desnitometer and the tPA was analyzed using ELISA. Data are
417 expressed as mean ± SEM. Concentration of significant differences compared
418 with normal group I: *$p < 0.05$.

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