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Gene expression of insulin-like growth factor-1 and epidermal growth factor is downregulated in the heart of rats with nitrofen-induced diaphragmatic hernia

Abstract Newborns with congenital diaphragmatic hernia (CDH) still have high mortality. Recently, a possible role of cardiac maldevelopment has been suggested. Human and experimental studies have demonstrated that heart weight is significantly reduced in the presence of CDH. Recent studies have suggested an important role for insulin-like growth factor-I (IGF-I) in the regulation of cardiac growth, structure, and function. Administration of IGF-I to normal rats has been shown to cause cardiac hypertrophy. Epidermal growth factor (EGF) plays an important role in cardiac differentiation and development. The aim of this study was to determine the gene-level expression of IGF-I and EGF in the hearts of rats with nitrofen-induced CDH using the reverse-transcription polymerase chain reaction technique (RT-PCR). CDH was induced in pregnant rats following administration of 100 mg nitrofen on day 9.5 of gestation (term 22 days). In control animals, the same dose of olive oil was given without nitrofen. Cesarean section was performed on day 21 of gestation. The fetuses were divided into three groups: normal controls (n = 8), nitrofen without CDH (n = 8), and nitrofen-induced CDH (n = 8). Total RNA was extracted from the hearts in each group and measured. mRNA was extracted from total RNA. RT-PCR was performed to evaluate mRNA expressions of IGF-I and EGF. Levels of mRNA were expressed as a ratio of band density divided by that of beta-actin, a housekeeping gene known to be expressed at a constant level. IGF-I mRNA expression was significantly decreased in CDH hearts (0.177 ± 0.109) compared to controls (0.393 ± 0.138) (P < 0.01) and nitrofen hearts without CDH (0.321 ± 0.088) (P < 0.05). EGF mRNA expression was significantly decreased in CDH hearts (0.218 ± 0.118) compared to controls (0.534 ± 0.196) (P < 0.01) and nitrofen hearts without CDH (0.383 ± 0.136) (P < 0.05). Decreased cardiac gene expression of IGF-I and EGF in the hypoplastic heart suggests that cardiac hypoplasia in nitrofen-induced rat CDH may be due to reduced synthesis of IGF-I and EGF by myocytes in the developing heart.

Keywords Nitrofen · Congenital diaphragmatic hernia · Hypoplastic heart · Insulin-like growth factor-I · Epidermal growth factor

Introduction

Despite advances in neonatal critical care, the mortality for congenital diaphragmatic hernia (CDH) remains high. This has been attributed to pulmonary hypoplasia and associated persistent pulmonary hypertension. Recent evidence suggests a possible role of cardiac maldevelopment as a “missing link” in the high mortality of these babies. Human and animal studies have demonstrated that heart weight is significantly reduced in the presence of CDH [9,14,22]. Autopsy reports have shown that in human newborns with CDH, the left ventricle, interventricular septum, and left atrium are smaller than those in age-matched controls. Recent studies report that in CDH cases there is a high incidence of cardiac malformations in humans and rat models. Underdevelopment of left-sided cardiac structures as detected by prenatal ultrasonography (US) has recently been identified as a poor prognostic factor in CDH [23]. However, little is known about the morphogenesis and pathologic mechanisms for hypoplasia and maldevelopment of the CDH heart.

Recent studies have suggested that peptide growth factors (GFs) may direct cardiac organogenesis and neonatal cardiac adaptation [7, 20]. Insulin-like growth factor-I (IGF-I) and epidermal growth factor (EGF) have an important role in early and late fetal cardiac development. IGF-I is a single-chain polypeptide that has structural homology with proinsulin. Accumulating evidence has indicated that IGF-I plays a specific role in...
the intricate cascade of events of cardiovascular function in addition to its well-established growth-promoting and metabolic effects. IGF-I also acts directly on both adult and neonatal cardiomyocytes in culture to augment the synthesis and accumulation of contractile and associated proteins [3]. It has been reported that IGF-I increases myocardial DNA and protein synthesis in isolated cardiomyocytes [10, 24].

EGF, a 53-amino-acid peptide derived from a 1, 217-residue precursor protein [4], is thought to be a normal fetal growth hormone and stimulates epithelial growth in several tissues [2, 24, 26]. EGF acts on many cell types, including the cardiomyocyte element of the heart. EGF is localized to the perinuclear region of cardiomyocytes; when secreted from the cells it acts as a growth signal for other cardiomyocytes [10, 11, 15]. Recently, Rabkin and Rebsamen et al. demonstrated that EGF increases protein synthesis and early-response gene expression in cardiomyocytes, responses considered markers of hypertrophy in these cells [15, 16].

We hypothesized that heart hypoplasia in CDH may result from decreased synthesis of cardiac GFs such as IGF-I and EGF during early fetal life and designed this study to determine the gene-level expression of IGF-I and EGF in the hearts of rats with nitrofen-induced CDH.

Positive controls for each specimen consisted of the consistently expressed housekeeping gene, β-actin. The specific primer sets used in this study, cycle numbers of PCR, and estimated size of the PCR products are listed in Table 1.

The PCR conditions were preliminarily confirmed to be within the exponential phase. PCR products were electrophoresed on 1.5% agarose gel and stained with ethidium bromide. Semiquantitative analysis was similar to the method described previously. The intensity of each band was analyzed using the GDS 8000 Gel Documentation System (UVP Inc, Upland, CA) with image-analysis software for quantitation. Relative IGF-I and EGF mRNA levels were expressed as a ratio of the band intensity divided by that of β-actin.

### Results

The PCR conditions described above successfully amplified fragments of IGF-I, EGF, and β-actin in each sample (Fig. 1). The intensity of the bands corresponding to β-actin mRNA was similar in all the samples. IGF-I mRNA expression was significantly decreased in CDH hearts (0.177 ± 0.109) compared to controls (0.393 ± 0.138) (P < 0.01) and nitrofen hearts without CDH (0.321 ± 0.088) (P < 0.05). EGF mRNA expression was also significantly decreased in CDH hearts (0.218 ± 0.118) compared to controls (0.534 ± 0.196) (P < 0.01) and nitrofen hearts without CDH (0.383 ± 0.136) (P < 0.05) (Fig. 2).

### Discussion

Several investigators have shown that in human neonates dying of CDH, total heart weight is significantly decreased compared with age-matched controls [22]. In addition, associated cardiac anomalies are frequent in high-risk newborns with CDH and account for high mortality. Of the cardiac defects, hypoplasia has been reported to be the most common [5]. Underdevelopment of left-sided cardiac structures as detected by prenatal US has recently been identified as a poor-risk prognostic factor in CDH [23].

The first experimental evidence of cardiac maldevelopment in CDH came from the lamb model of CDH, in which a hypoplastic heart is consistently found at term. Karamanoukian et al. demonstrated that animals with CDH have a significant reduction in total-heart weight and weight of left cardiac structures with identical

### Table 1 Reverse transcription polymerase chain reaction (PCR) primer sequences for Insulin-like growth factor-I (IGF-I), epidermal growth factor (EGF), and β-actin

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5′ → 3′</th>
<th>PCR cycle</th>
<th>Tm (°C)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I</td>
<td>Sence Antisence</td>
<td>AAG CCT ACA AAG TCA GCT CG GGT CTT GTT TCC TGC ACT TC</td>
<td>28</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GAC AAC TCC CCT AAG GCT TA CAT GCA CAC GCC ACC ATT GAG GCA GTA CCC ATC GTA CGA</td>
<td>35</td>
<td>55</td>
</tr>
<tr>
<td>EGF</td>
<td>Sence Antisence</td>
<td>ATG TGG CAC CAC ACC TTC TAC CGT CAT ACT CCT GCT TGC TGA</td>
<td>26</td>
<td>68</td>
</tr>
<tr>
<td>β-actin</td>
<td>Sence Antisence</td>
<td>ATG TGG CAC CAC ACC TTC TAC CGT CAT ACT CCT GCT TGC TGA</td>
<td>26</td>
<td>68</td>
</tr>
</tbody>
</table>