ENDOTHELIN EXPRESSION DURING RAT HEART DEVELOPMENT: AN IMMUNOHISTOCHEMICAL AND *IN SITU* HYBRIDIZATION STUDY

TAMAKI NISHIMURA^{1,2}, HISAO YAMADA², MASAHICO KINOSHITA¹ and JUNZO OCHI²

¹The First Department of Internal Medicine, and ²Department of Anatomy, Shiga University of Medical Science, Seta, Otsu 520-21, Japan

ABSTRACT

To investigate the mitogenetic role of endothelins (ETs) in the heart, an ontogenetic analysis using immunohistochemistry and *in situ* hybridization was made in the Wistar rat embryonic heart from early embryonic day (E) 9 to E20. Mature ET peptides were detected by immunohistochemistry from E10 to E18 in almost all the atrial and ventricular cardiac myocytes and disappeared after E19. ET-1 mRNA was detected in atrial and ventricular cardiac myocytes by *in situ* hybridization from E12 to E18 and disappeared after E19. ET-3 mRNA was detected in atrial and ventricular cardiac myocytes by *in situ* hybridization from E10 to E18 and disappeared after E19. *In situ* hybridization and immunohistochemistry allowed visualization of ET-1 and ET-3 synthesis and secretion, respectively: ET-3 was first synthesized and secreted at E10, followed after 2 days by ET-1 expression. Both ET-1 and ET-3 were present throughout the period of heart development and the relation between the ET expression and the completion of embryonic circulation was suggested.

In 1988, a strong vasoconstrictive peptide was initially identified from cultured porcine aortic endothelial cells (33) and was named endothelin (ET). Subsequently, three distinct peptides corresponding to ET were discovered and were classified into ET-1, ET-2 and ET-3 (3, 8). These three ETs have different biological activities on distinct receptor subtypes; ET-A receptor and ET-B receptor (1, 23). ET-A receptor has higher affinity for ET-1 and ET-2 than for ET-3, while ET-B receptor has almost equivalent affinity for all three peptides. Hence, ET-1 and ET-3 have different receptor affinities. Moreover, it was found that vascular endothelial cells did not produce ET-2 and ET-3, and only ET-1 could be detected in these cells at both peptide and mRNA levels (3). Thus, ET-1 and ET-3 may have diverse functions and it is important to analyze them in comparison with each other.

ET was first discovered in vascular endothelial cells, but ET isopeptide expression has since been demonstrated in many tissues contrary to initial expectations. However, no ET polypeptide could be detected in the adult heart, although ET mRNA

expression has been reported in the human heart by Northern blot hybridization (3). Immunohistological methods are useful to distinguish cell types but ET has been hardly detected in adult tissues at the translation level, since the ET content in various tissues is very low. Previously, we reported the existence of ET peptide in fetal lung and salivary glands by immunohistochemistry and showed the presence of its mRNA by RT-PCR (11). By analogy with high expression of vasoactive peptides such as atrial natriuretic peptide (ANP) (28), ET may also be expressed at a high level during the developmental period. In the present study, we analyzed the distribution of the mature type (secretion type) of ET in embryonic rat hearts by immunohistochemistry using an anti-ET antibody that recognizes the C-terminus of all ET isopeptides. To distinguish ET-1 and ET-3, we analyzed the gene expression of these two isopeptides by *in situ* hybridization. By these methods, we addressed ourselves to questions concerning the roles of ET in the physiology of the heart and heart development.

MATERIALS AND METHODS

Tissue Preparation

Fifteen female Wistar rats at each stage of pregnancy were used throughout the present study. Ten to 15 embryos were collected from each dam. The age of the embryos was determined as follows; the next day of mating when vaginal plugs were found was taken as embryonic day zero (E0). Embryos were collected at E9, E10, E11, E12, E13, E14, E15, E18, E19 and E20 from dams anesthetized by intraperitoneal injection of 40 mg/kg pentobarbital and immediately frozen with CO₂ gas. Sagittal sections of embryos (16 μ m thick) were cut using a cryostat and thaw-mounted on gamma-aminopropyltriethoxysilane-coated glass slides. The sections were washed with 100 mM sodium phosphate buffer (pH 7.4) containing 154 mM NaCl (PBS) at room temperature for 5 min, fixed with 4% formaldehyde in PBS at room temperature for 10 min and then washed twice (5 min each) with PBS again at room temperature.

Immunohistochemistry

The sections were treated with 0.3% H₂O₂ in PBS at room temperature for 1 h to inactivate endogenous peroxidase. Following a 10-min rinse in PBS, they were immunostained with the avidin-biotin-peroxidase complex (ABC) method using a polyclonal anti-ET antibody produced in our laboratory as described previously (11). The primary antibody for ET was raised in rabbits by inoculation with the C-terminus (9 amino acids) of ET-1, which has the biological activity and is well preserved among the ET-family. This antibody recognizes only the mature type (secretion type) of ETs but not precursor type big-ETs (31); and can be used as a neutralization antibody (12). The sections were incubated with the primary antibody diluted 1:1,000 with PBS for 48–72 h at room temperature, then washed in PBS for 10 min at room temperature. Subsequently, they were incubated in biotinylated anti-rabbit IgG goat serum (Vector, Burlingame, U.S.A.) for 3 h, followed by rinsing with PBS, and then incubated with the ABC solution (Vector, Burlingame, U.S.A.) for 80 min at room temperature. After rinsing with PBS, sections were reacted with the 3,3'-diaminobenzidine solution (20 mg in 100 ml of 50 mM Tris-HCl buffer, pH 7.6) containing 20 μ l of 30% H₂O₂ for 15 min at room temperature, rinsed with PBS, counterstained with hematoxylin, dehydrated in alcohol and mounted with

Entellan (Merck, Darmstadt, Germany).

In Situ Hybridization

The sequence corresponding to bases 50–483 of the rat preproET-1 cDNA was subcloned following insertion into plasmid pSPT 18 upstream of the T7 promoter to allow transcription of an anti-sense probe. This probe was synthesized by linearizing the plasmid with *Hind*III, followed by transcription with T7 RNA polymerase. The human ET-3 cDNA from nucleotide 917–1,457, which has no homology with ET-1, was subcloned following insertion into the plasmid pBluescript II KS(+) upstream of the T3 promoter to facilitate transcription of an anti-sense probe. This probe was synthesized by linearizing the plasmid with *Pst*I followed by transcription with T3 RNA polymerase. Transcription to produce both ET-1 and ET-3 riboprobes was carried out with a digoxigenin-RNA labeling kit, according to the manufacturer's instructions (Boehringer, Mannheim, Germany). Briefly, 2 μ g of linearized plasmid DNA was incubated for 2 h at 37°C in 40 μ l of transcription buffer containing GTP, ATP, CTP (10 mM each), unlabeled UTP (6.5 mM), 3.5 mM digoxigenin-labeled UTP (Boehringer), 40 units of T7 or T3 RNA polymerase, and 20 units of RNase inhibitor (human placenta). Subsequently, 20 units of DNaseI (RNase-free) was added and incubated at 37°C for 15 min to digest the DNA template. We stopped the reaction with 2 μ l 0.2M EDTA (pH 8.0) and 2 μ l 10% SDS and the cRNA probe was ethanol-precipitated and dried up. This pellet was then stored at –20°C until use for *in situ* hybridization.

Sections were incubated in 0.2N HCl at room temperature for 20 min, washed with PBS at room temperature for 5 min and air-dried. They were then covered with 200 μ l of prehybridization buffer consisting of 4 $\times$ saline-sodium citrate (SSC; 1 $\times$ SSC = 150 mM sodium chloride, 15 mM sodium citrate), 50% formamide, 5 $\times$ Denhardt's reagent (1 $\times$ Denhardt's reagent = 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% RNase-free bovine serum albumin) and 0.2% Tween 20 at 50°C for 24 h. After prehybridization, sections were covered with 100 μ l of hybridization solution consisting of 4 $\times$ SSC, 50% deionized formamide, 1 $\times$ Denhardt's reagent, 0.2% Tween 20, 250 μ g/ml tRNA, 500 μ g/ml fragmented salmon sperm DNA and digoxigenin-labeled RNA probe, and incubated at 50°C for 72 h. The slides were then rinsed sequentially with 2 $\times$ SSC twice (5 min each) at

room temperature, $2 \times$ SSC containing RNaseA ($50 \mu\text{g/ml}$) and RNaseT₁ (20 units/ml) at 37°C for 30 min, $2 \times$ SSC at room temperature for 1 min, $0.2 \times$ SSC at room temperature for 10 min, $0.2 \times$ SSC twice (30 min each) at 50°C , and PBS at room temperature for 10 min.

Sections were then incubated in 5% sheep serum in PBS containing 0.4% Triton X-100 at room temperature for 3 h. Subsequently, they were incubated with the alkaline phosphatase-labeled anti-digoxigenin antibody diluted 1:1,000 in the same solution for 24 h. After incubation, they were washed three times (60 min each) in 100 mM Tris HCl buffer (pH 7.6) containing 150 mM NaCl and 0.2% Tween 20 at room temperature, and then incubated in 100 mM Tris-HCl (pH 9.5) buffer containing 100 mM NaCl, 50 mM MgCl_2 , 0.4% Triton X-100 and 1 mM levamisole (reaction buffer) for 5 min. For detection of the signals, slides were incubated in $45 \mu\text{l}$ of nitro blue tetrazolium ($75 \text{ mg}/70\%$ N,N-dimethylformamide) solution and $35 \mu\text{l}$ of 5-bromo-4-chloro-3-indolylphosphate-*p*-toluidine salt ($50 \text{ mg}/100\%$ N,N-dimethylformamide)/10 ml reaction buffer in the dark at room temperature for 0.5–24 h. Then reaction was stopped by immersion in 100 mM EDTA (pH 8.0) for 1 min, and sections were dehydrated in alcohol and mounted with Entellan.

RESULTS

Immunohistochemical Staining

At E9, the cardiogenic primordium is located in the mesoderm in front of the oral plate but is difficult to discriminate. There was no positive immunostaining in this mesodermal mass (data not shown). ET-immunoreactivity in the embryonic heart was first detected weakly at E10 and was more apparent at E11. At this stage, the heart is tubular and consists of endocardium and epi-myocardium, the latter of which differentiates into myocardium and epicardium. ET-immunoreactivity was observed as diffuse fine granular dark-brown deposits in the cytoplasm, and was stronger in ventricular than in atrial areas. Almost all the undifferentiated or differentiated myocardial cells were immunopositive, but the immunoreactivity was stronger on the endocardial than on the epicardial side (Fig. 1, a and b).

In the hearts at E15, the atrioventricular valves were completed and atria and ventricles could be distinguished from each other. ET-immunoreactivity was observed in almost all myocardial cells in

atria and ventricles, but was stronger in ventricles, especially on the endocardial side (Fig. 1c).

ET-immunoreactivity gradually increased up to E15 and remained up to E18. At E19, ET-immunoreactivity disappeared throughout the heart (Fig. 1d).

In Situ Hybridization Analysis

By *in situ* hybridization, positive staining for ET-1 and ET-3 mRNA was observed as fine granular light blue deposits. At E9, the mesodermal mass where the cardiogenic primordium is located was not stained (data not shown). Positive staining for ET-3 but not ET-1 was first detected weakly in the E10 and was apparent in the E11 embryo. At this stage, the heart is tubular as mentioned above, and the cytoplasm was diffusely stained in almost all the undifferentiated and differentiated myocardium (Fig. 3, a and b). Before E12, however, the probe for ET-1 mRNA showed no hybridization and no positive signal was observed (Fig. 2a).

During the developmental period from E12 to E15, ET-1 expression was strongly detected in almost all cardiac myocytes, but the reaction was stronger in ventricular than in atrial areas (Fig. 2b). ET-3 was also expressed in a similar appearance. After E15, the reaction of both ET-1 and ET-3 was stronger in ventricles than in atria (Figs. 2c and 3c). The intensity of reaction increased until E15 and remained up to E18, but no positive signal was detected in the E19 heart (Figs. 2d and 3d).

DISCUSSION

In the present study 1) ET peptide was detected in embryonic cardiac myocytes by immunohistochemical study, 2) by *in situ* hybridization ET mRNA expression was demonstrated in the embryonic heart of rats, 3) there was a time lag between the first expression of ET-1 and ET-3, and 4) both ET-1 and ET-3 gene expression disappeared in the heart before birth.

ET expression has been studied by many investigators and was detected in many tissues such as brain, colon, lung and others. In the circulatory system, ET-1 mRNA has been detected by Northern blot analysis in human heart and vascular endothelium (3), and in rat heart (22). By the same method, ET-3 mRNA was not detected in human heart or vascular endothelium (3). Using the RT-PCR method, the presence of ET-1 mRNA was demonstrated in porcine and human heart (20).

Fig. 1 Photomicrographs showing rat hearts at E10, E11, E15 and E19 immunostained with anti-ET antibody. (a) E10 heart. Immunopositivity in ventricular area (V) is somewhat stronger than in atrial area (A). $\times 84$. (b) E11 heart. Immunopositivity becomes apparent on the endocardial side of ventricular area (V). The atrial area (A) is weakly stained. $\times 42$. (c) E15 heart. Almost all the myocytes of atrium (A) and ventricle (V) are immunopositive. Immunoreactivity is strong especially on the endocardial side of ventricle. $\times 34$. (d) E19 heart. No positive staining is seen throughout the heart. $\times 17$

These studies indicated that ET-1 but not ET-3 mRNA was detectable in human, porcine and rat heart by Northern blotting or RT-PCR analyses. ET-1 gene expression was demonstrated in the heart but its source was unclear. Suzuki *et al.* (25) demonstrated the existence of ET-1 peptide by immunohistochemistry and ET-1 mRNA by RT-PCR in cultured neonatal ventricular cardiac myocytes. They then concluded that cardiac myocytes were capable of synthesizing ET-1.

There have been few previous reports regarding ET expression in embryonic organs. MacCumber *et al.* (17) investigated ET-1 gene expression in 19-day rat fetuses by *in situ* hybridization. In their study, positive signals were detected in lung, intestine and meninx, but the heart was negative. In the present study, we showed the distribution of ET peptide in the developing embryonic heart tissue and gene expression of ET-1 and ET-3 in temporal

sequence for the first time. By means of immunohistochemistry and *in situ* hybridization the existence of the mature peptides and transcripts of ET-1 and ET-3 can be detected, respectively. Thus, we can reliably monitor both biosynthesis and secretion of ET. In the early developmental period (E10–E12), ET protein was detected first immunohistochemically at E10 and ET-3 mRNA was first observed on the same day. At E12, ET peptide, ET-1 and ET-3 mRNA were all detected in the heart. ET-1 mRNA was not seen before this day. Thus, the immunoreactivity observed in the cardiac myocytes of embryonic heart at E10 and E11 is considered to indicate ET-3 secretion. At this stage of development, the heart is tubular and consists of endocardium and epi-myocardium, the latter of which differentiates into epicardium and myocardium (2, 19, 30). It is assumed that ET-3 might participate in this initial myogenic differentiation

Fig. 2 *In situ* hybridization analysis showing ET-1 mRNA expression at E11, E12, E15 and E19 rat heart. (a) E11 heart. No positive staining is seen in the tubular heart. $\times 85$. (b) E12 heart. Positive staining is detected in the ventricular area (V). The reactivity in atrial area (A) is weak. $\times 51$. (c) E15 heart. The positive staining is stronger in ventricle (V) than in atrium (A). $\times 34$. (d) E19 heart. No positive staining is seen throughout the heart. $\times 17$

of cardiac myocytes. During the late embryonic period (E13 to E20), ET-immunoreactivity gradually increased up to E15 and remained until E18. This sequential pattern was equivalent to that observed by *in situ* hybridization analysis of ET-1 and ET-3. Thus, ET-immunoreactivity in the cardiac myocytes during E12 to E18 appears to represent both ET-1 and ET-3 secretion. By E16, the heart is partitioned and the embryonic circulatory system is complete (organogenesis is completed). Following this day, the maturation period starts and the heart undergoes growth until birth. As ET peptide and mRNA both disappeared shortly after E16 and before birth, it is possible that both ET-1 and ET-3 might play significant roles mainly in the differentiation of the heart.

There have been many previous reports concerning the interaction between ET and ANP. These peptides antagonize each other in several organs such as kidney and vascular smooth muscle to con-

trol the water-electrolyte balance and vascular tone. It has been reported that ET stimulates ANP secretion from cultured rat atrial myocytes mediated by ET-A receptor (9, 27). Therefore, it is thought that mainly ET-1 stimulates ANP secretion in cultured atrial cardiac myocytes. Furthermore, ET-A receptor mRNA expression has been observed in the atrium at higher levels than ET-B receptor (7, 21). This ET-A receptor expressed on the atrial wall may participate in the induction of ANP secretion. On the other hand, ANP was recently reported to inhibit ET-1 secretion stimulated by angiotensin II and thrombin in cultured endothelial cells of human umbilical vein (13), as well as of porcine (14) and rat (5) aorta through a cyclic GMP-dependent process. However, basal secretion of ET is not affected by ANP, so ANP may suppress excess secretion of ET-1 stimulated by secretagogues such as angiotensin II or thrombin. Thus, it is supposed that ET and ANP are regulated via a feedback con-

Fig. 3 *In situ* hybridization analysis showing ET-3 mRNA expression at E10, E11, E18 and E19 rat heart. (a) E10 heart. Weakly positive staining is seen in the ventricular area (V). $\times 128$. (b) E11 heart. Positive staining is seen in the ventricular area (V). $\times 42$. (c) E18 heart. Almost all the cardiac myocytes are positively stained. The staining is stronger in ventricle (V) than in atrium (A). $\times 24$. (d) E19 heart. No positive staining is seen throughout the heart. $\times 17$

trol mechanism in the circulatory system. Toshimori *et al.* (28) reported an immunohistochemical study of ANP in embryonic and fetal rat heart. They showed that weak immunoreactivity first appeared in cardiocytes of atrial and ventricular areas equally at E11. Thereafter, the immunoreactivity became intense and stronger in the atria than in the ventricles. Our present data showed that ET-1 first appeared at E12. The time of appearance must be considered approximate, but may vary by no more than plus or minus half a day. Thus, ET-1 and ANP may appear almost simultaneously. Therefore, we presume that ET-1 appears and stimulates ANP secretion from the atria, and as the serum level of ANP gradually elevates, the ET secretion is inhibited and finally reaches the limit of sensitivity of the assay or basal level at E19.

It became now obvious that ET played a role not only in vasoconstriction but also in cell growth. Previous studies have concentrated mainly on ET-

1, which was observed to stimulate DNA synthesis and proliferation of many kinds of cultured cells independently or in cooperation with growth factors such as PDGF. For example, ET-1 stimulates in a dose-dependent manner the DNA synthesis in cultured rat 'vascular smooth muscle cells' (VSMCs) (6, 15, 18), cultured swine pulmonary artery smooth muscle cells (10), cultured fibroblasts (26), and cultured vascular pericytes (32). ET-1, ET-2 $\gg$ ET-3 also induced DNA synthesis in cultured rat VSMCs (29). Furthermore, ET-1 also induced cellular hypertrophy in cultured neonatal rat myocardial cells (24). Eguchi *et al.* (4) reported that switching the ET receptor subtype from ET-A to ET-B occurred during *in vitro* culture. It is well known that cultured VSMCs undergo phenotypic modulation from the contractile to the synthetic phenotype, and they reported that this phenotypic change took place concomitantly with this change in ET receptor subtype. Thus, ET-3 as well as ET-1

appears to be effective in stimulating the proliferation of VSMCs. Our present observation in developing heart that ET-1 and ET-3 are both evenly expressed in embryonic cardiac myocytes may support this report and the hypothesis that ET plays a role as growth factor *in vivo*.

Our present results showed that ET-1 and ET-3 genes were expressed in fetal rat heart from E12 to E18, during the period of organ formation, and that very early in development at E10 and E11, ET-3 might play a major role. Recently, Kurihara *et al.* (16) generated mice deficient in ET-1 by homologous recombination of disrupted ET-1 gene in the embryonic stem cells. They found that these homozygous mice developed major abnormalities of the craniofacial organs and died of respiratory failure at birth, which indicated that ET-1 was necessary for pharyngeal arch development. From these and our present findings, it is assumed that ET acts as a kind of cytokine/growth factor during ontogenetic stage.

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