Targeted Disruption of Calcium/NFAT Signaling Reveals a Left-Right Determination Disorder in the Pharyngeal Arch Artery

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An immunosuppressant was injected into pregnant mice in order to investigate whether the immune response is involved in differentiation during embryonic development. Injection of 3 mg/kg of FK506, an inhibitor of calcineurin, early in the organogenesis period increased the penetrance of right aortic arch formation by 32% compared with saline injection. Immunosuppressants such as FTY720 and rapamycin did not affect left/right (L/R) determination. FK506 is known to work by restricting NFAT (nuclear factor activated T-cell) dephosphorylation. An L/R determination disorder in cardiac outflows appeared when an NFATc4 siRNA was directly injected into the amniotic fluid. As for the mechanism, Pitx2, which is normally expressed on the left-hand side, was found to be expressed also on the right-hand side. Furthermore, it turned out that administration of FK506 also prevented the dephosphorylation of NSFL1 cofactor p47. When an siRNA targeting p47 was introduced into the amniotic fluid of FK506-treated fetuses, both of the dorsal arteries—which should normally become one—remained. These findings indicate that the mother’s immune system contributes not only to self-defense, but also to remodeling processes in fetal morphogenesis.

1. Introduction

Recently there have been reports that the immune response controls vertebrate morphogenesis. Mukai et al. [1] indicated a role for the immune response in metamorphosis, based on the finding that syngeneic grafts of tadpole tail skin into adult Xenopus animals are rejected by T cells. I reported that lens regeneration in newt eyes was induced by antigen-presenting cells that had engulfed the remains of the destroyed lens [2]. Amphibians are the most commonly used models in this field, and there is little experimental data for the role of the immune response in morphogenesis of mammals.

The morphogenetic processes underlying pharyngeal arch artery (PAA) remodeling from the symmetrical configuration toward the unilateral left-sided aortic arch have not been fully unraveled [3]. The early embryonic mammalian system consists of five paired arch arteries, numbered I to VI from cranial to caudal. The fifth artery is considered to be rudimentary or absent. At day 11.5 of pregnancy, the PAA system consists of a left and right third (III), fourth (IV), and sixth (VI) arch artery, connecting two continuous dorsal aortas with the ventrally located aortic sac. Around day 12 of pregnancy, the arterial system develops toward the mature left-sided configuration, due to regression of the right-sided sixth arch artery, the right dorsal aorta (α-segment), and both the left and right carotid ducts. It is known that a congenital disorder of the cardiac outflow tract results from abnormal PAA remodeling. I speculated that the immune response in mothers might act in the remodeling process of fetuses, as shown for amphibian development. Therefore, to evaluate the possible causes of abnormalities in the cardiac outflow tract, fetuses taken from mothers injected with immunosuppressants were examined.

Calcium/NFAT (nuclear factor activated T-cell) signaling regulates a range of cellular processes and plays essential roles during embryonic development of the heart, skull, and brain. A rise in Ca²⁺ levels activates calcineurin, which
dephosphorylates NFAT transcription factors, facilitating their translocation to the nucleus and subsequent transcriptional activation of target genes [4]. Five mammalian NFAT transcription factors have been identified. In the present study, FK506, an inhibitor of calcineurin/NFAT signaling, was used to examine the relationship between the immune response and the developing vascular system.

2. Methods

2.1. Mice and Histochemistry. C57BL/6 were reared in poly-carbonate cages in an environmentally controlled room (water temperature: 22 ± 1°C), with a standard 12-hour light/dark cycle. Injection of mother mice with the immunosuppressants, FK506, FTY760, and rapamycin, was performed three times: on days 6.5, 7.5, and 8.5 of pregnancy. Experiments in which the drugs were introduced into the amniotic fluid were also performed. Laparotomy was performed on days 6.5 or 8.5 of pregnancy, and FK506 or saline was injected directly into the amniotic fluid using a microsyringe of 10 µL volume. After the operation, the mice were returned to their original cages and observed. To evaluate abnormalities in cardiovascular development, fetuses were removed from the uterus on days 13.5 or 18.5 of pregnancy and examined histologically. The mating of mice was performed at midnight, and pregnancy day 0.5 was the morning when a vaginal plug was detected.

In order to investigate the participation of NFAT in aortic arch formation, siRNAs targeting NFATc1 (Santa Cruz Biotechnology, sc-36054) or NFATc4 (Santa Cruz Biotechnology, sc-38116) were administrated into the amniotic fluid on day 6.5 or 8.5 of pregnancy.

Embryos were fixed for 24 hours in a 4% paraformaldehyde solution buffered with 0.15 M sodium phosphate at a pH of 7.3. They were then washed in several changes of the same buffer and embedded in paraffin. Serial sections of 2 µm were prepared and stained with Mayer’s hematoxylin and eosin.

The protocol for immunohistochemistry used to detect CD31 was similar to that described by Cursiefen et al. [5]. Briefly, the whole embryos were frozen on dry ice in Tissue-Tek and sectioned into 8 µm thick slices. The sections were collected onto microscope slides, dried at room temperature, fixed in acetone, rinsed in PBS, blocked in 2% BSA, and stained with Alexa Fluor 488-conjugated CD31 antibody overnight. All staining procedures were performed at room temperature. Staining with secondary antibody alone, or with an isotype control instead of with CD31 primary antibody, was negative. A similar procedure was adopted for CD34 and CD117 (c-kit) immunohistochemistry. All experiments were approved by the animal ethics committee of the National Cerebral and Cardiovascular Center.

2.2. Two-Dimensional Electrophoresis and Western Blotting. Whole embryos were first washed in cold saline and then homogenized in the presence of 5 M urea, 2 M thiourea, 2% CHAPS, 2% SB3-10, and 1% DTT. Supernatants were collected after centrifugation at 20,000 g for 30 min. The total protein concentrations of the samples were determined using a protein assay kit (Pierce). First-dimensional separation of the proteins was performed on an IPGphor IEF system using immobiline DryStrips pH 4–7 (GE Healthcare Bio-sciences) or Pharmalyte broad range, pH 3-10 (GE Healthcare Life Sciences). The extracts were loaded onto rehydrated immobiline strips and electrophoresed with internal protein markers (Promega). Running conditions were 3,500 volts maximum for 8 hours. Vertical SDS-PAGE was used for the second dimension, using 9–18% acrylamide gradient gels.

After 2DE, gels were dyed using a fluorescence staining reagent for the detection of phosphorylated proteins (Pro-Q Diamond phosphoprotein gel stain, Molecular Probes) or all proteins (SYPRO Ruby protein gel stain). Gel images were obtained using a fluorescence scanner, and images were evaluated using ImageMaster Platinum (GE). The signal intensity of all spots was computed using this software. Signal intensity was shown with the value (%volume value) which was divided by the total of the signal intensity of all spots on gel.

The phosphorylation index (i.e., the number of phosphate groups per molecule of protein) was calculated from the two %volume values as follows: phosphorylation index = (%Vol. value of the Pro-Q Diamond dye spot)/(%Vol. value of the SYPRO Ruby dye spot). Spots satisfying the following two conditions were listed as protein identification candidates. (1) The sum of the % values from two 2DE gels dyed with SYPRO Ruby was 0.1 or more. (2) The relative change in phosphorylation index for a spot in the FK506-injected groups was 1.5 or more relative to the saline-injected groups.

The same samples used for 2DE were used for western blotting. First, equal quantities of each sample were mixed with SDS sample buffer (125 mM Tris–HCl, 4% SDS, 10% sucrose, 2% DTT) and heated for 5 minutes at 95°C. Each sample was then run on 4% SDS-PAGE gels using standard procedures. Subsequently, the proteins were transferred onto a PVDF membrane (GE Healthcare Bio-Sciences) using a blotting unit (GE Healthcare Bio-Sciences) with blotting buffer (25 mM Tris–HCl, 200 mM glycin, 10% methanol, 0.02% SDS). Blots were incubated with primary antibodies against Lefty2 (PTG 13991-1-AP) and Pitx2 (NOV NBPI-70363) for 4 hours at 4°C and with the corresponding secondary antibody (GE NA934, anti-rabbit IgG, HRP-linked whole Ab from donkey) for 1 hour at RT. Finally, the PVDF film was used to expose the X-ray film.

2.3. Mass Spectrometry. First, isolated gel pieces representing spots were decolorized, and then the peptide was extracted using 50% acetonitrile and 1% TFA. The peptide extraction liquid was condensed with a vacuum dryer, loaded into a Zip-Tip C18 (Millipore) column, and extracted with 50% acetonitrile again. These extracts were used for mass spectrometry (Bruker Daltonics).

3. Results

3.1. Restriction of Calcineurin/NFATc4 Signaling by FK506. My analysis demonstrated a change in aortic arch formation; results are summarized in Table 1. In saline-injected fetuses,
Figure 1: Right aortic arch formed after administration of FK506. (a) A photograph of a normal fetus at E13.5. The arrow indicates the normal aortic arch that runs toward the left-hand side. The dotted line arrow indicates the normal ductus arteriosus. The asterisk indicates the brachiocephalic trunk. (b) A photograph of an FK506-treated fetus at E13.5. The arrow indicates the right aortic arch, which runs toward the back of the right-hand side (*). The dotted line arrow indicates the ductus arteriosus running to the right. Asterisks (**) indicate the arteria subclavia sinistra. (c) An HE-stained section of dorsal artery in a normal fetus. The white dotted line indicates the positions of the pharynx and esophagus, and the asterisk indicates the oropharynx. The arrow indicates the dorsal artery located on the left-hand side of the white dotted line. (d) An HE-stained section of the dorsal artery in an FK506-treated fetus. The asterisk and the white dotted line indicate the same as that in (c). The arrow indicates the dorsal artery located on the right-hand side of the white dotted line.

The cardiac outflow tract (aorta) arose from the left ventricle, ascended for a short distance, and then curved to the left and descended through the left side of the chest (Figures 1(a) and 1(c)). Injection of 3.0 mg/kg of FK506 into the mother mouse on days 6.5, 7.5, and 8.5 of pregnancy induced an L/R determination disorder, in which the cardiac outflow tract ascended toward the right side, formed an arch, and then descended through the right side of the chest (Figures 1(b) and 1(d)). Injection of 3.0 mg/kg of FK506 increased the penetrance of the right aortic arch by 32% (ten of 31) relative to saline injection. The ductus arteriosus also ran toward the right side of the chest and connected with the right aortic arch after the right subclavian artery branch. When the dose of FK506 was increased to 6.0 mg/kg, vascular abnormalities could not be evaluated because of abortion of the fetuses before 10.5 days of pregnancy. The L/R determination disorder was observed in fetuses given a dose of 1.5 mg/kg, when FK506 was injected directly into the amniotic fluid at day 8.5 of pregnancy (Table 2). The same effects could be observed after injections at 7.5 days. However, the right aortic arch was not seen in fetuses after injections into the amniotic fluid at 10.5 days. FK506 blocks the dephosphorylation reaction of calcineurin signaling. Therefore, additional experiments were performed. A phosphate buffer (pH 7.4, TAKARA Bio T-900) was introduced into the amniotic fluid at day 8.5 of pregnancy for the purpose of raising the concentration of intracellular phosphoric acid. Also after administration of a phosphate buffer into amniotic fluid, right aortic arches appeared clearly (Table 3) although the effect was not as strong as that with FK506. These results indicated that there is a critical time during remodeling of the aortic arch between 7.5 and 8.5 days of pregnancy and that remodeling of PAA involves a phosphorylation reaction.

 Interruption of the aortic arch, ventricular septal defects, and persistent truncus arteriosus were not induced by FK506 injection. Interestingly, other immunosuppressants, such as...
### Table 1: Appearance of right aortic arch after injection to mother mouse.

| Drugs   | N of fetuses | % of right aortic arch + right ductus arteriosus | % of other abnormalities |
|---------|--------------|-------------------------------------------------|--------------------------|
| FK506   |              |                                                 |                          |
| 6.0 mg/kg | 3           | 0                                               | 33                       |
| 3.0 mg/kg | 31          | 32                                              | 0                        |
| 0.8 mg/kg | 30          | 23                                              | 3                        |
| 0.05 mg/kg | 13         | 0                                                | 0                        |
| Saline  | 34           | 0                                                | 0                        |
| FTY720  |              |                                                 |                          |
| 6.0 mg/kg | 8           | 0                                                | 0                        |
| 3.0 mg/kg | 26          | 0                                                | 0                        |
| Saline  | 32           | 0                                                | 0                        |
| Rapamycin |            |                                                 |                          |
| 1.0 mg/kg | 8           | 0                                                | 0                        |
| 0.1 mg/kg | 16          | 0                                                | 0                        |
| 0.01 mg/kg | 14         | 0                                                | 0                        |
| Saline  | 24           | 0                                                | 0                        |

Injection was performed during 6.5, 7.5, and 8.5 days after pregnancy.

### Table 2: Appearance of right aortic arch after infusion of FK506 into amniotic fluid.

| Days of infusion of FK506 | % of right aortic arch + right ductus arteriosus |
|---------------------------|-----------------------------------------------|
| E7.5                      |                                               |
| 0.8 mg/kg                | 23 (5/22)                                     |
| 0.08 mg/kg               | 0 (0/18)                                      |
| Saline                   | 0 (0/28)                                      |
| E8.5                      |                                               |
| 3.0 mg/kg                | 0 (0/4)                                       |
| 1.5 mg/kg                | 33 (10/30)                                    |
| 0.8 mg/kg                | 28 (9/32)                                     |
| Saline                   | 0 (0/30)                                      |
| E10.5                     |                                               |
| 0.8 mg/kg                | 0 (0/16)                                      |

FTY720 and rapamycin, did not induce an L/R determination disorder (Table 1).

Calcineurin causes the rapid dephosphorylation and nuclear import of the products of the NFATc genes. Therefore, I next looked for abnormalities in fetuses given an siRNA targeting NFATc4 (nuclear factor of activated T cells, calcineurin-dependent 4) in the amniotic fluid at day 8.5 of pregnancy. In these fetuses, there was a significant increase in the right aortic arch being accompanied by right ductus arteriosus when compared with saline-injected fetuses (Table 3). However, the NFATc1 siRNA caused embryonic lethality. These findings indicate that the reversed orientation of the cardiac outflow tract might be related to the expression of cytokine genes following the action of NFATc4.

### Table 3: Appearance of right aortic arch after infusion of NFATc4 siRNA into amniotic fluid.

| Drugs               | % of right aortic arch + right ductus arteriosus |
|---------------------|-------------------------------------------------|
| siRNA of NFATc4     | 31 (11/35)                                      |
| siRNA of NFATc1     | Embryonic lethal                                |
| Phosphate buffer (pH 7.4) | 20 (6/30)                              |
| Saline              | 0 (0/24)                                       |
| KCL                 | 0 (0/18)                                       |

It is known that haematopoietic stem cells (HSCs), which are responsible for blood production, emerge in the dorsal aorta at day 10.5 of pregnancy [6]. Therefore I investigated whether the emergence of HSCs moves to the right side when the cardiac outflow tracts of the fetuses injected with FK506 move from the left to the right using an immunohistochemical analysis of CD34+ and CD1I17+ (c-kit). In the saline-injected group, CD34+ and CD1I17+ (c-kit) cells were found to be attached to the endothelium in the left side of the dorsal artery, where HSCs might localize (Figure 2). However, when FK506 was given directly into the amniotic fluid, CD34+ and CD1I17+ cells existed clearly in the right side of the dorsal artery and could remain there during development. These findings suggest that not only is the L/R direction of the blood vessel reversed, but so is the L/R localization of specific functions.

3.2. Mechanism of the L/R Determination Disorder. The mechanisms underlying L/R determination have fascinated biologists for decades. Pitx2 is a homeobox gene that has been shown to play a central role in the late aspects of L/R asymmetric morphogenesis [7]. Since expression of Pitx2 takes place before the aortic arch is formed, all embryos treated with FK506 were investigated. After that, the difference in Pitx2 expression between FK506- and saline-treated embryos was evaluated. As shown in Figure 3, the expression of Pitx2 in the FK506-treated group shifted to the right-hand sides of embryos, crossing the median line, whereas Pitx2 expression in saline-treated embryos was restricted to the left side at the 8-somite stage. Importantly, FK506 induced ectopic expression on the right side. Western blotting revealed increased expression of Pitx2 by a factor of approximately 1.5 compared with saline-injected fetuses. In contrast, the expression of Lefty 2 (left-right determination factor 2) decreased slightly relative to the levels in saline-injected fetuses. These findings demonstrated that the expression of Pitx2 might be regulated by calcineurin signaling and that the right aortic arch may be induced by the ectopic expression of Pitx2.

3.3. Phosphorylation Analysis by Two-Dimensional Electrophoresis. To investigate how many intracellular dephosphorylation events were prevented by injection of FK506, two-dimensional electrophoresis (2DE) analysis was performed. Using a staining solution for phosphorylated proteins, spots were selected from the gels obtained after FK506 injection based on the strength of their signals. In Figure 4, spots are...
Figure 2: HSCs migration after administration of FK506. (a) Immunostaining with anti-CD31 antibody. Arrows indicate the left and right sides of the dorsal arteries. (b) Immunostaining with anti-CD34 or anti-CD117 (c-kit). Saline-treated fetuses exhibited positive staining for both CD34 and c-kit in the left dorsal artery. FK506-treated fetuses had positive staining on the right-hand side, opposite to that seen in saline-treated fetuses.

arranged according to their D/S ratio and mass spectrometry (MS) was performed on the six spots with the highest D/S ratio. MS of fetuses from mothers given FK506 early in the organogenesis period identified hyperphosphorylation of the NSFL1 cofactor p47 (p97 cofactor p47) protein. Hyperphosphorylation was also seen at a spot of poly(C) binding protein 2. Therefore I investigated whether NSFL1 cofactor p47 is related to L/R determination. FK506-treated fetuses were subjected to siRNA against p47 in the amniotic fluid. Figure 5 shows that not only the right dorsal artery but also the left dorsal artery remained: in other words, siRNA against P47 inhibited the disappearance of the left dorsal artery in FK506-treated fetuses.

4. Discussion and Conclusion

This study has clearly revealed that Pitx2, which is normally expressed at the left-hand side of embryos, is also expressed at the right-hand side and promotes the formation of a right aortic arch when calcineurin is inhibited using FK506. Furthermore, it was observed that administration of FK506 prevented dephosphorylation of NSFL1 cofactor p47.

This study also found that an siRNA targeting NFATc4 exhibits the same effects as FK506, inducing an L/R determination disorder in aortic arch remodeling. NFATc4 plays a role in the inducible expression of cytokine genes in T cells, including the induction of IL-2 and IL-4. The p38 MAP kinase phosphorylates multiple residues in the NFAT homology domain of NFATc4. The answer to the question that was the purpose of this research—whether the immune system is involved in the morphogenesis of an animal—was considered to be likely yes in the case of aortic remodeling. My result showed that the dephosphorylation reaction that is required for T-cell activation is important for embryonic development and that it is not an immune regulator, such as a cytokine, that was thought to be directly involved in early
development. The data in Table 3, in which the injection of a phosphate salt into amniotic fluid also easily induced the L/R determination disorder, has supported this result.

When a cell enters mitosis, p47 localized to the nucleus moves to the cytoplasm after nuclear envelope breakdown and forms a complex with p97. However, it has been suggested that if p47 cannot be phosphorylated by cdc2 in cytoplasm, it cannot form a complex with p97. As a result, Golgi bodies are destroyed and changed to small-granule vesicles [8]. In the present study, when p47 siRNA was introduced into the amniotic fluid of FK506-treated fetuses, both dorsal arteries remained. There is a high probability that p47 is involved in the cell death in the dorsal artery, where cells normally disappear during embryonic development.

It has been proposed that the process of LR determination commonly involves a cilia-driven leftward flow in the mammalian node and its equivalents, such as the Kupffer vesicle in zebrafish and the gastrocoel roof plate in Xenopus [9]. Asymmetric Ca$^{2+}$ signaling has been detected at the left margin of the node [10]. The asymmetric elevation of Ca$^{2+}$ and its lateral propagation have also been reported [11]. Moreover, it was reported that Ca$^{2+}$ flux regulates Kupffer’s vesicle development and is required for LR determination. Microinjection of an IP3 receptor function-blocking antibody that can inhibit IP3 calcium channel activity randomized the LR axis in terms of left-sided Pitx2 expression and organ laterality [12]. There are also some reports that L/R asymmetry depends on early differential ion flux created by H$^+$/K$^+$ ATPase transporter activity [13]. An H$^+$/K$^+$ ATPase blocker inhibited the expression of Nodal and Pitx-2, which are normally expressed in the left lateral mesoderm, and induced these genes on the right side. The result was heterotaxia of all organs. However, in this present study, after FK506 treatment, only cardiac outflow showed reversed left-right positioning: reversal of internal organs did not occur. Moreover, after FK506 treatment, hematogenous functions also appeared among the blood vessels that ran on the right-hand side. The phenomenon of how the hematopoietic stem cell, which moves from yolk sac, discerns the blood vessel that remains is interesting.

FTY720 is an immunosuppressant drug, approved for treating multiple sclerosis. It is a sphingosine-1-phosphate receptor modulator, which sequesters lymphocytes in lymph nodes, preventing them from contributing to an autoimmune reaction. Rapamycin is not a calcineurin inhibitor, but the action of rapamycin is to bind the cytosolic protein FKBPI2 in a manner similar to that of FK506. Unlike the FK506-FKBPI2 complex, which inhibits calcineurin, though, the rapamycin-FKBPI2 complex inhibits the mammalian target of rapamycin (mTOR) pathway by directly binding the mTOR complex 1 (mTORC1) [14]. In the present study, immunosuppressants such as FTY720 and rapamycin did not induce an L/R determination disorder. My results suggest that only NFATc signaling is important for L/R axis determination during embryonic development. Of additional importance was the finding that although the fetuses given an NFATc siRNA exhibited an L/R determination disorder, the infusion did not induce an abnormality in myocardial development, including any apparent defects in cardiac morphology.

The important issue that must be considered in the future is whether the immune system of the fetus is involved in

| Drugs | Value |
|-------|-------|
| Saline | 2864.5 |
| FK506 | 2369.5 |

Quantitative value

| (kDa) | (a) Lefty2 | (b) Pitx2 |
|-------|------------|-----------|
| Saline | 31         | 1893      |
| FK506  | 4481       |           |

Figure 3: Lefty2 and Pitx2 analyzed after administration of FK506. (a) Western blotting of Lefty2. Bands from FK506-treated fetuses were slightly thinner compared with those from saline-treated fetuses. Lefty2 expression was decreased by about 20% in FK506-treated fetuses relative to saline-treated fetuses. (b) Western blotting of Pitx2. The Pitx2 band from FK506-treated fetuses was strong, and quantification indicated that pitx2 protein expression was about 50% greater than that in saline-treated fetuses. (c) and (d) Pitx2 immunohistochemistry on a section from saline-treated fetuses (c) and FK506-treated fetuses (d). After injection of 1.5 mg/kg of FK506 into amniotic fluid, the expression of Pitx2 extended to the right side of the embryo. The arrow indicates the front edge of ectopic expression on the right side.
Figure 4: 2DE analyzed after administration of FK506. (a) 2DE of saline-treated fetuses. (b) 2DE of FK506-treated fetuses. Many red circles indicate hyperphosphorylation spots that were used to perform mass spectrometry analysis. (c) Results of mass spectrometry analysis.

| Sample number | D/S ratio | Protein name                        |
|---------------|-----------|-------------------------------------|
| 1424          | 3.06      | NSFL1 cofactor p47                  |
| 1382          | 2.86      | mCBP                                |
| 1734          | 1.92      | Heat shock cognate 71 kDa protein   |
| 2070          | 1.83      | HSP 84                              |
| 2127          | 1.73      | α-Actinin-4                         |
| 2175          | 1.68      | Caprin-1 isoform a                  |

Figure 5: Disappearance of the left dorsal artery was prevented by infusion of p47 siRNA into FK506-treated fetuses. (a) An HE-stained section of a control fetus. Arrow indicates the left dorsal artery. (b) An HE-stained section of a fetus given p47 siRNA. Arrows indicate both dorsal arteries.
the determination of the L/R axis. The thymus is generated from the 3rd pharyngeal pouch, and it is thought that T-lymphocyte progenitors move into the thymus around day 11.5 of pregnancy. In the present study, it was hypothesized that the determination of the L/R axis had a high possibility of having happened by day 8.5 of pregnancy (Table 2). It seemed that the appearance of the right aortic arch would be a phenomenon that occurred in collaboration with the immune system of the mother, including that associated with the placenta.

Conflict of Interests
The author declares that there is no conflict of interests.

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