

Short Communication

Immunohistochemical analyses of the kinetics and distribution of macrophages in the developing rat kidney

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Abstract: Macrophages are required during kidney development and appear in the initiation and propagation of renal injury. To establish baseline data, we analyzed the kinetics of the macrophage with different immunophenotypes in the developing rat kidney (fetus at 18 and 20 days, neonate at 1–21 days, and adult at 7-weeks old). Macrophages reacting to CD68, CD163, and MHC class II were identified in the cortex and medulla of the developing rat kidney. CD68⁺ macrophages appeared in the fetal kidney as early as fetal day 18, and the number increased gradually in the neonatal kidney, whereas MHC class II⁺ and CD163⁺ macrophages first appeared on neonatal days 4 and 8, respectively. Apoptotic bodies were seen in the fetal kidney and early stages of the neonatal kidney (days 1–4), and simultaneously CD68⁺ macrophages appeared, indicating that CD68⁺ macrophages may have roles in phagocytosis of apoptotic bodies and contribute to renal tissue maturation. Colony stimulating factor 1 and insulin growth factor 1 mRNAs were increased in the late stage of renal development (neonatal day 12 or later), and simultaneously CD163⁺ and MHC class II⁺ cells appeared, suggesting that these cells may be a source of these growth factors and participate in renal tissue modeling. Generally, the CD163⁺ and MHC class II⁺ cell number was much smaller than that of CD68⁺ cells in the developing neonatal kidney. Therefore, the obtained findings provide valuable information on the participation of macrophages in the developing rat kidney. This information may be useful for evaluation of renal toxicity when macrophages are involved in the development of renal injury. (DOI: 10.1293/tox.2018-0002; J Toxicol Pathol 2018; 31: 207–212)

Key words: developing kidney, immunohistochemistry, macrophages, rats

Macrophages play important roles in renal development as well as renal injury and are contributors to subsequent tissue regeneration¹. They also have the ability to change phenotype in response to tissue microenvironments. M1 and M2 macrophages (M1/M2 polarization) actively participate in lesion development and reparative fibrosis in renal diseases, respectively². On the other hand, macrophages are important during embryogenesis, particularly playing an important role in programmed cell death and tissue modeling during organogenesis^{3, 4}. Such tissue development may be due to growth factors such as colony stimulating factor (CSF-1) and insulin growth factor (IGF-1) produced by macrophages, in addition to phagocytic activity^{5, 6}. In fact, defective macrophage development impaired renal growth in mice⁶. Expression and functions of renal macrophages are dependent on the microenvironment or age^{7, 8}. For instance,

macrophages actively participate in the pathogenesis of cisplatin-induced renal interstitial fibrosis in neonatal rats⁸. Despite of importance of macrophages, the appearance and distribution of macrophage with different phenotypes in the developing rat kidney has not been reported in detail.

Immunohistochemical staining has been used in identification of macrophage phenotypes⁹. To identify renal macrophages, we used antibodies against CD68, CD163 and major histocompatibility complex class II (MHC class II). CD68 is located on lysosomal membranes, particularly phagosomes, and thus, the expression level implies enhanced phagocytosis^{10, 11}. CD163 is a member of the macrophage scavenger receptor family, and its increased expression may be related to production of inflammatory-related factors; CD163 antibody labels resident macrophages in normal tissues¹². MHC class II molecules are expressed on mature dendritic cells and activated macrophages¹³. In the present study, we investigated the appearance and kinetics of different macrophage populations in renal fetal tissue, neonates, and young adults using immunohistochemistry in order to provide data that may be useful for the evaluation of juvenile renal toxicity when macrophages are involved in the disease process.

Four adult F344/DuCrj male (117–140 g body weight; 7 weeks old) and sixteen pregnant F344/DuCrj female rats at day 15 of gestation were obtained from Charles River

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Laboratories Japan (Hino, Shiga, Japan). They were housed in an animal room controlled at $22 \pm 3^\circ\text{C}$ and with a 12 h light-dark cycle and being allowed free access to a standard commercial diet (DC-8, CLEA, Tokyo, Japan) and tap water. Fetal kidneys were obtained from the pregnant rats on gestational (fetus) days 18 and 20. After delivery, renal tissues were collected from neonates on days 1, 4, 8, 12, 15, and 21. At each examination point, 3–4 fetuses and neonates were euthanized by exsanguinations under deep isoflurane anesthesia. Four adult rats (7 weeks old) were also used. The animal experiments were conducted under the institutional guidelines approved by the ethical committee of Osaka Prefecture University for the Care and Use of Experimental Animals.

Renal tissues from the left and right kidneys were collected and immediately fixed in 10% neutral buffered formalin (NBF) or periodate-lysine-paraformaldehyde (PLP) solution and were processed by PLP-AMeX (acetone, methyl benzoate, and xylene). The paraffin-embedded tissues were sectioned at a thickness of 3–4 μm . Deparaffinized sections were stained with hematoxylin and eosin (HE) for morphological observations. PLP-fixed tissue sections were deparaffinized and used for immunohistochemistry. Tissue sections were immunostained with mouse monoclonal antibodies specific for CD68 (ED1, 1:500, AbD Serotec, Oxford, UK), CD163 (ED2, 1:300, AbD Serotec), and MHC class II (OX6, 1:1,000, AbD Serotec) using a Histostainer (Histofine, Nichirei Biosciences Inc, Tokyo, Japan). Briefly, sections were incubated with 5% skimmed milk for 10 min, followed by a 1 h incubation with the primary antibody. After treatment with 3% H_2O_2 for 15 min, a horseradish peroxidase-conjugated secondary antibody (Histofine Simple Stain MAX PO[®], Nichire Biosciences Inc., Tokyo, Japan) was applied for 30 min. Positive reactions were visualized with 3,3'-diaminobezidine tetrahydrochloride (DAB Substrate Kit, Vector Laboratories, Burlingame, CA, USA) and the sections were lightly counterstained with hematoxylin. For negative controls, tissue sections were treated with mouse nonimmunized serum instead of the primary antibody.

Fresh frozen sections (10 μm in thickness) from developing rat kidney on neonate day 1 were used. Double immunofluorescence was carried out using antibodies against CD68 for M1 macrophages and CD163 for M2 macrophages. Briefly, after fixation in cold acetone:methanol (1:1) for 10 min at 4°C , the sections were incubated with 10% normal goat serum for 30 min. The sections were reacted with the primary antibody against CD68 (1:500, AbD Serotec) overnight at 4°C . After rinsing with PBS, the sections were incubated for 45 min with a secondary anti-mouse IgG conjugated with Alexa 568 (Invitrogen, Carlsbad, CA, USA). The sections were then incubated with a second primary antibody CD163 labeled with fluorescent dye-conjugated Alexa 488 (1:200, AbD Serotec) for 3 h. The sections were visualized in Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories Inc) for nuclear staining and analyzed using a virtual slide scanner (VS-120, Olympus, Tokyo, Japan).

Total RNA was extracted from the fetal, neonatal and adult renal tissues using RNeasy[™] RNA Stabilization Reagent (Qiagen, Duesseldorf, Germany) and an SV Total RNA Isolation System (Toyobo Co. Ltd., Osaka, Japan). The RNA was reverse-transcribed to cDNA using a Super Script First Strand Synthesis System[™] (Invitrogen, Thermo Fisher Scientific Inc, Waltham, MA, USA). All PCR experiments were performed with SYBR Green Real-time PCR Master Mix (Toyobo Co. Ltd.). The colony stimulating factor (CSF-1) (forward, ccaccgagagctacaggaa; reverse, gttgcaatcaggcttggtca) and insulin growth factor (IGF-1) (forward, taagaccctctatccaacac; reverse, ctctgctgtgatccacat) primers were used. CSF-1 and IGF-1 are important for the postnatal growth and organ maturation; local production of IGF-1 may depend on CSF-1 production by *in situ* macrophages, and in turn CSF-1-stimulated macrophages are the most abundant source of IGF-1^{5,6}. The relative expression values were normalized to the expression value of β -actin. The data were analyzed using the comparative Ct method (ΔCt method).

To analyze the kinetics and characteristics of macrophages in the developing kidney, the numbers of positive cells of each macrophage marker (CD68, CD163, or MHC class II) were counted for 5 different randomly selected points in each of cortex and medulla using a light microscope (400 magnification). Clearly fragmented nuclei (for possible apoptotic bodies) were also counted by the same method in HE-stained sections, as this may be useful for evaluate possible apoptosis. Obtained data were expressed as the mean \pm standard deviation (SD). Statistical analysis was performed using Dunnet's test. $P < 0.05$ was considered significantly different for comparisons with fetuses at day 18, neonates at day 1, and adult rat.

In HE-stained sections on fetal days 18 and 20, round-, comma-, and S-shaped bodies, which are characteristic components in the developing kidney, were observed in the cortical areas. In addition, loosely-arranged blastemal cell-derived mesenchymal cells were seen frequently, but developing renal tubules were less frequently observed (Fig. 1A). After birth, S-shaped bodies and interstitial spindle-shaped cells (possibly mesenchymal cells) were still frequently observed on days 1 and 4 (Fig. 1B). With advancement of age, the number of mesenchymal cells gradually decreased, and developing renal tubules and glomeruli became more dominant (Fig. 1C). On days 15 to 21, renal tissues were quite similar in histology to those of adult rats (Fig. 1D).

The kinetics of macrophages reacting to CD68, CD163, and MHC class II in the developing kidney are shown in Fig. 2A and B. CD68⁺ macrophages appeared in the developing kidney as the major macrophage immunophenotype (Fig. 2A–E). Particularly, CD68⁺ cells appeared in both the cortex and medulla on fetal days 18 and 20. The number of CD68⁺ macrophages quickly increased on neonatal day 4, and the cells retained their increased numbers until neonatal day 21, which showed statistical significance compared with the number on fetal day 18; on the other hand, CD68⁺ cells in the adult kidney were very small in number and showed

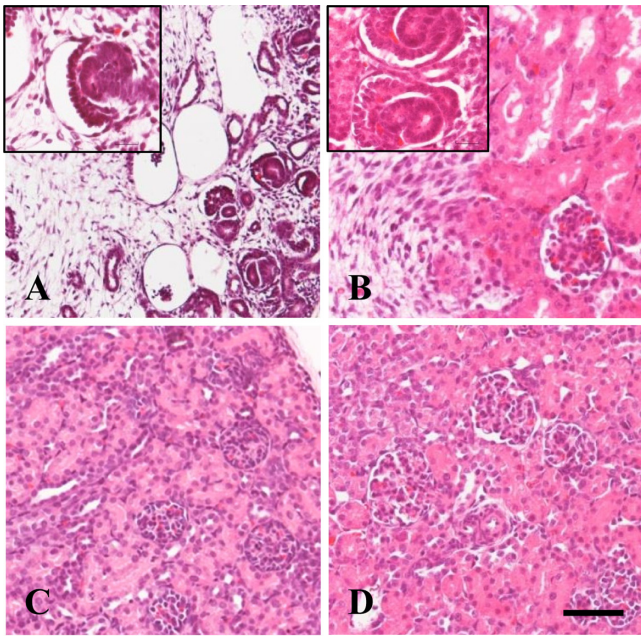


Fig. 1. Histology of the developing rat kidney. A, B: Loosely arranged undifferentiated spindle-shaped cells (possibly mesenchymal cells) were frequently observed among developing renal tubules and glomeruli in the cortical area on fetal day 18 (A) and neonatal day 1 (B). The inset shows an S-shaped body with a vesicle at higher magnification. C: The number of spindle-shaped cells was decreased, and developing renal tubules and glomeruli became prominent in the cortex on neonatal day 8. D: On neonatal day 15, the cortical area comprised developing glomeruli and renal tubules with a few interstitial spindle-shaped (mesenchymal) cells, which was almost the same as the adult kidney. HE stain. Bar = 40 μ m.

significant differences compared with the developing fetal or neonatal kidney (Fig. 2A and B). CD68⁺ macrophages had a round to oval configuration with short cytoplasmic processes and a nucleus and were scattered evenly throughout the developing cortex and medulla (Fig. 2C).

On the other hand, CD163⁺ and MHC class II⁺ cells were not seen on fetal days 18 and 20 or neonatal day 1 in the cortex and medulla. CD163⁺ first appeared on neonatal day 8 in the medulla and on neonatal day 12 in the cortex (Fig. 2A and B). After that, the number of CD163⁺ cells gradually increased in the medulla, although it did not change in the cortex. CD163⁺ cells in the adult kidney were also very small in number and showed significant differences compared with the developing neonatal kidney (Fig. 2A and B). The CD163⁺ cells appeared scatteredly in the developing kidney, and the cells were round or fusiform in shape with cytoplasmic processes (Fig. 2D). MHC class II⁺ cells were first seen in the cortex and medulla on neonatal day 4. The number of MHC class II⁺ cells was subsequently found to be significantly increased on neonatal days 8 and 12 in the cortex and medulla, respectively. Thereafter, the number of MHC class II⁺ cells did not change until neonatal day 21, which was almost the same as in the adult kidney. The dis-

tribution of MHC class II⁺ cells was sporadic in the cortex and medulla (Fig. 2E).

The number of CD163⁺ and MHC class II⁺ cells was much smaller than that of CD68⁺ cells in the late stage of the developing kidney on neonatal days 12–21 (Fig. 2A and B). In addition, double immunofluorescence for CD68 and CD163 showed that there were CD68⁺ macrophages co-expressing CD163 in the developing kidney (Fig. 2F–H).

Apoptotic bodies in HE-stained sections were more frequently seen in the cortex and medulla on fetal day 18 to neonatal day 4 (Fig. 3A–C). The number of apoptotic bodies then gradually decreased until neonatal day 21, with a significant difference observed compared with fetal day 18 (Fig. 3A and B). On neonatal day 21, the number of apoptotic bodies was almost the same as that in the adult rat. Apoptotic bodies in adult kidney were very small in number and showed significant differences compared with those in the developing fetal or neonatal kidney (Fig. 3A and B). Interestingly, there were apoptotic bodies phagocytized by CD68⁺ macrophages, which were seen in immunohistochemical sections (Fig. 3D).

CSF-1 mRNA expression was significantly increased in the developing kidney on neonatal days 12 to 21 as compared with that of the fetus, and a significantly increased level of CSF-1 was also seen on neonatal day 21 in contrast to adulthood (Fig. 4A). IGF-1 mRNA expression was significantly increased in the developing kidney on neonatal days 12 and 15 as compared with that on neonatal day 1, although there was no difference compared with the fetal levels (Fig. 4B).

Macrophages are a heterogeneous cell population with high plasticity, playing crucial roles in both kidney development and diseases. They show various immunophenotypes and functions. The present immunohistochemical analyses revealed that CD68⁺ macrophages appeared at the early and late stages of renal development in much greater numbers than other macrophages reacting to CD163 and MHC class II. Among these renal macrophages, CD68⁺ macrophages may play a greater role in rat nephrogenesis. CD68 is an antigen of the lysosomal membrane of macrophages, and its increased expression implies activated phagocytosis of macrophages¹¹. In organogenesis, apoptosis is essential for tissue modeling¹⁴. In the present study, a large number of apoptotic bodies in HE-stained sections were seen mainly in the early stage of renal development on fetal days 18 and 20, as well as on neonatal days 1 and 4 in the cortex and medulla; simultaneously, CD68⁺ cells appeared in the fetal kidney and the number increased gradually in the neonatal developing kidney, indicating that CD68⁺ macrophages might be related to phagocytosis of apoptotic bodies to remove cell debris. In fact, there were CD68⁺ cells phagocytizing apoptotic bodies, which were seen in the immunohistochemistry. Although the number of apoptotic bodies decreased with age, the number of CD68⁺ macrophages retained still increased at the late stage of renal development. It has been reported that CD163-expressing macrophages are the major sources of IGF-1 in developing tissue¹⁵. IGF-1 is an impor-

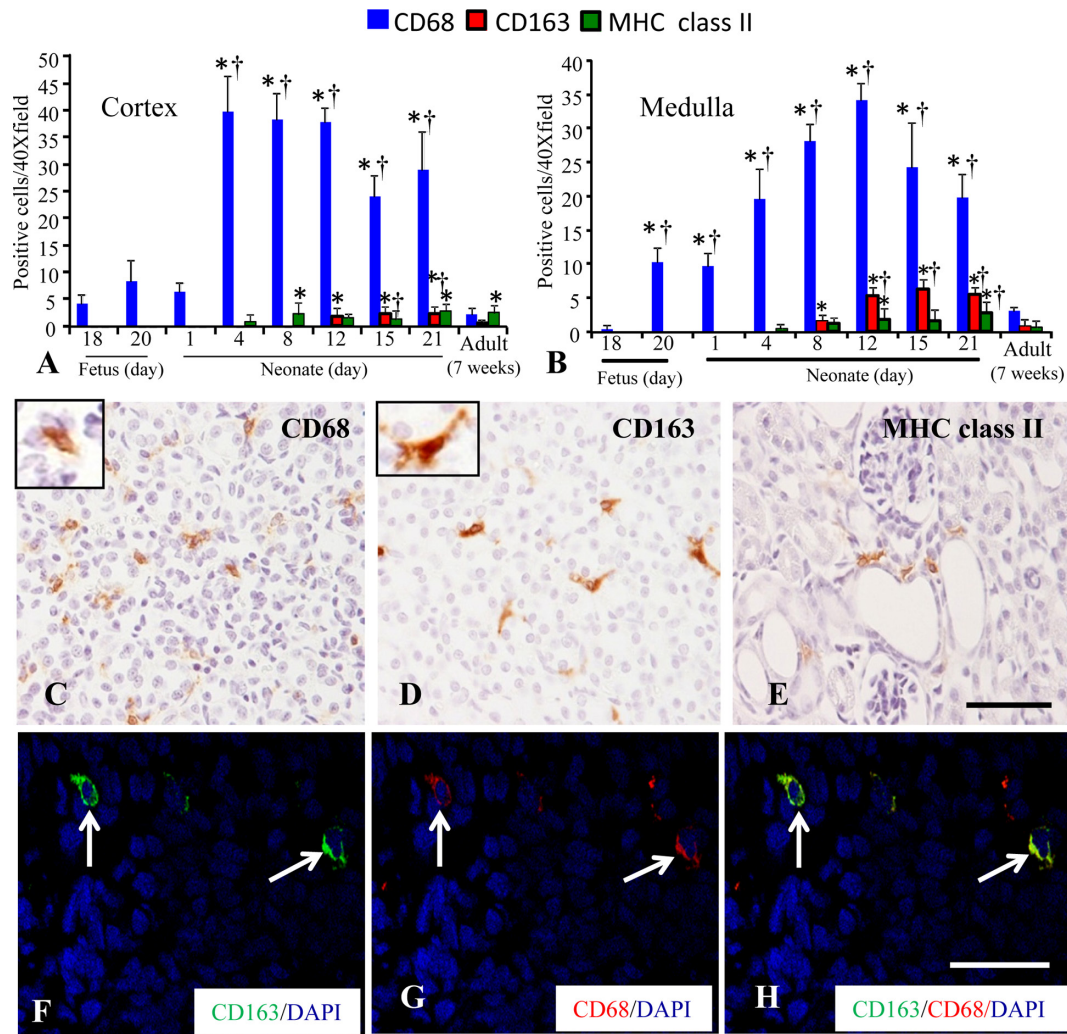


Fig. 2. Macrophages in the developing and adult rat renal cortex and medulla. A, B: The kinetics of macrophages reacting to CD68, CD163, and MHC class II in the cortex and medulla of the fetal, neonatal, and adult rat kidney. C-E: Immunohistochemistry showed a scattered or sporadic distribution of macrophages reacting to CD68 (C), CD163 (D) and MHC class II (E) in the cortical area on neonatal day 12. The inset shows each reactive macrophage at higher magnification. F-H: Double immunofluorescence for CD163 (green) and CD68 (red) in the cortex on day 12. Areas of yellow indicate a double positive reaction (arrows). Nuclear staining with 4'-6-diamidino-2-phenylindole (DAPI). Bar = 40 μ m for C and D, 50 μ m for F-H. Dunnett's test. *Significantly different from the fetal day 18 ($P < 0.05$). †Significantly different from the adult rats (7 weeks old; $P < 0.05$).

tant factor for renal tissue modeling⁶ as mentioned below. Using double immunofluorescence, it was confirmed that some CD68⁺ macrophages co-expressed CD163 in the present study. In addition to phagocytosis of apoptotic bodies, CD68⁺ macrophages might have been possible cells capable of producing IGF-1, and they would have been related to renal development in rats; because, it has been considered that macrophages may contribute to postnatal growth and organ maturation through release of growth factors such as CSF-1 and IGF-1; CSF-1-stimulated macrophages may be capable of producing of IGF-1^{5, 6}. In order to confirm the definite production of these factors by macrophages or by other kidney-constituting cells, further analyses such as *in situ* hybridization are needed.

CD163⁺ macrophages and MHC class II⁺ cells appeared

exclusively in the late stage of renal development on neonatal days 4 to 21, especially in the medulla. CD163⁺ and MHC class II⁺ cells have been reported to produce growth factors during tissue development⁶, suggesting that these macrophages might be responsible for maturation of renal tissue.

On the basis of M1/M2 macrophage polarization, CD68 is considered an M1 type macrophage, and CD163 is considered an M2 type macrophage¹⁶. The present findings indicated that CD68⁺ M1 macrophages appear in the early and late stage of renal development and that CD163⁺ M2 macrophages appear mainly in the late stage of renal development; MHC class II⁺ cells participate in renal tissue modeling during maturation in renal development like M2 macrophages.

In the developing kidney, the CSF-1 and IGF-1 expres-

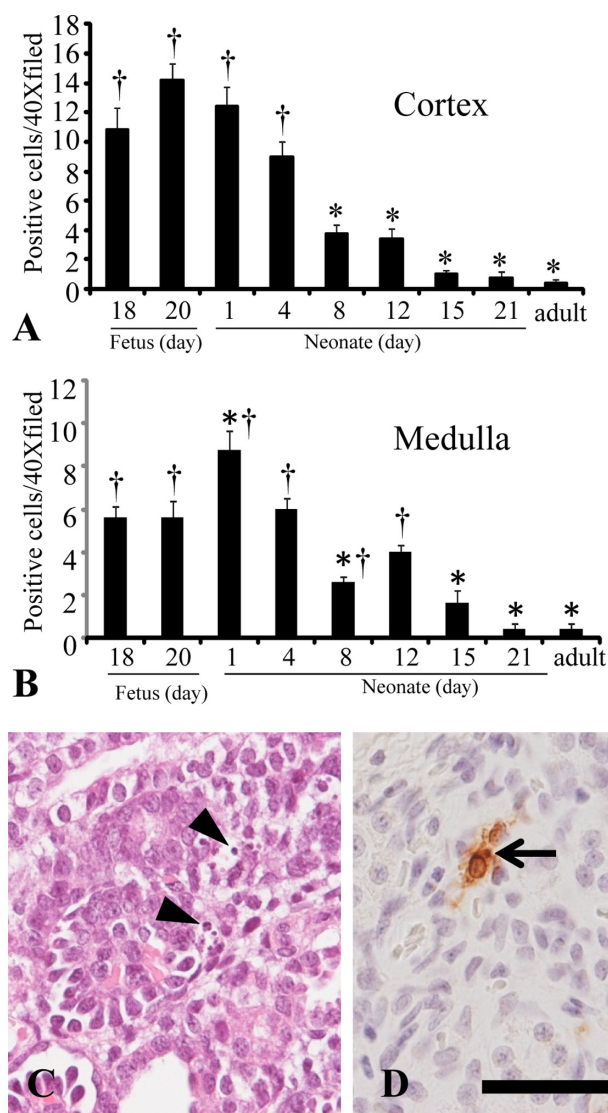


Fig. 3. A, B: The number of apoptotic bodies in the cortex (A) and medulla (B) of the fetal and neonatal kidney. C, D: HE-stained sections showed apoptotic bodies in the developing cortex on neonatal day 1 (C, arrowheads), and immunohistochemistry for CD68 showed the apoptotic bodies phagocytized by a CD68⁺ cell on neonatal day 12 (D, arrow). Bar = 40 μ m. Dunnet's test. *Significantly different from the fetal day 18 ($P < 0.05$). †Significantly different from the adult rats ($P < 0.05$).

sion levels simultaneously increased in the late stage. On neonatal days 12 to 21 after birth, tissue formation was almost complete, implying completion of tissue maturing. CSF-1 stimulates macrophages to infiltrate into the appropriate region and to produce IGF-1, thereby actively participating in renal tissue development^{5, 6}. Therefore, local production of IGF-1 may depend on CSF-1 production by *in situ* macrophages^{6, 15}. In fact, CSF-1 and IGF-1 has been reported to be necessary for efficient tissue modeling^{17, 18}. Local source of IGF-1 may be M2 macrophages (CD163)⁵. Based on these findings, CSF-1 and IGF-1, which increased exclusively in the late stage of renal development, might contribute to renal modeling and maturation; these factors

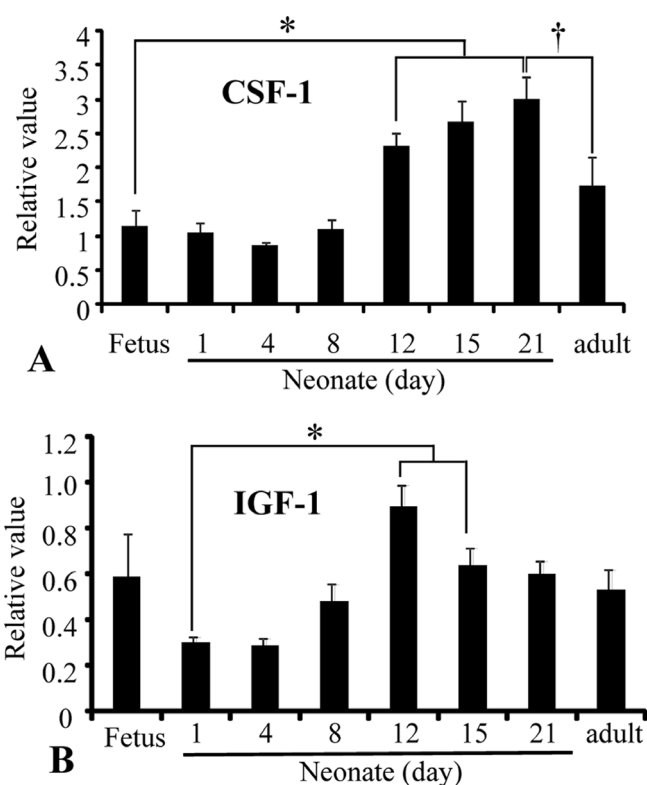


Fig. 4. Real-time PCR for CSF-1 (A) and IGF-1 (B) in the developing rat kidney. Expression levels were normalized to the β -actin mRNA level. Dunnet's test. *Significantly different from the fetal or neonatal day 1 ($P < 0.05$). †Significantly different from the adult rats ($P < 0.05$).

appear to be produced mainly by M2 macrophages reacting to CD163 and MHC class II.

In conclusion, many CD68⁺ M1 macrophages were observed in the early and late stages of renal development, indicating their roles in phagocytosis of apoptotic bodies as well as renal tissue maturation. On the other hand, CD163⁺ M2 macrophages and MHC class II-reacting cells appeared in the late stage of renal development, corresponding to increase levels of IGF-1 and CSF-1 mRNAs, which may be important for renal tissue modeling. Expression and functions of renal macrophage may be dependent on micro-environmental condition or age^{7, 8}. Therefore, the findings obtained in this study provide basic information regarding the participation of renal macrophages in normal kidney development and may be useful for evaluation of macrophage involvement in the pathogenesis of renal toxicity.

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