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Original Article

Milk fat globule-EGF factor 8 mRNA expression in rat splanchnic tissues during postnatal development

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Abstract: Milk fat globule-EGF factor (MFG-E8) is a protein that binds to αvβ3/5 integrin and phosphatidylserine. It plays a role in apoptotic cell removal, tissue remodeling and intestinal epithelial wound-healing process. In the present study, we examined the expression of MFG-E8 mRNA in the small intestine, liver and lungs during postnatal development. Sprague-Dawley rats were sacrificed at different ages (E20, P0, P5, P10 and P21). Total RNA was extracted from various tissues including the small intestine, liver and lungs. The MFG-E8 mRNA expression was quantified by real-time PCR. We found that the MFG-E8 mRNA was expressed at a low level on E20 in the liver. Within 24 hours after birth, its expression was markedly increased. It was then stably expressed at high level during the postnatal period. In contrast, in the small intestine, MFG-E8 mRNA significantly decreased by more than 60% ($p<0.001$) within 24 hours after birth compared to E20, then it gradually regained E20 values by P10. It was persistently expressed until P21. In the lungs, MFG-E8 is constitutively expressed prenatally at E20. Its expression did not change during the postnatal period. In summary, our study indicated that MFG-E8 is extensively expressed in the small intestine, liver, and lungs. As opposed to other organs, the expression of intestinal MFG-E8 is decreased during the first week of life. It is possible that this could contribute to the predisposition of the neonatal intestine to inflammation.

Key Words: MFG-E8, postnatal gene expression, intestines, liver, lung

Introduction

Milk fat globule-EGF factor 8 (MFG-E8) is a glycoprotein derived from macrophages in splanchnic organs [1, 2]. It participates in multiple physiological processes associated with tissue remodeling. For example, MFG-E8 has been found to play an important role in mediating clearance of apoptotic cells *in vivo* [3]. It also promotes angiogenesis [4, 5]. Previously, Atabai et al. showed that MFG-E8 is critical for mammary gland remodeling during involution [6]. In addition, Ensslin and Shur showed that MFG-E8 regulates mammary gland development by participating in the intercellular signaling between luminal and myoepithelial cells which leads to branching morphogenesis of the mammary gland [7]. It is generally accepted that splanchnic organs such as the liver, lung, and intestines are constantly remodeled during postnatal development. Recently, we showed that MFG-E8 plays an important role in intestinal epithelial cell migration and mucosal repair [8]. Therefore, we examined the changes in MFG-E8 gene expression during the postnatal period in rats by measuring its mRNA with real-time RT-PCR.

Materials and methods

Tissue collection and RNA isolation

All animal experiments were conducted in accordance with the NIH guidelines under protocols approved by the Institutional Animal

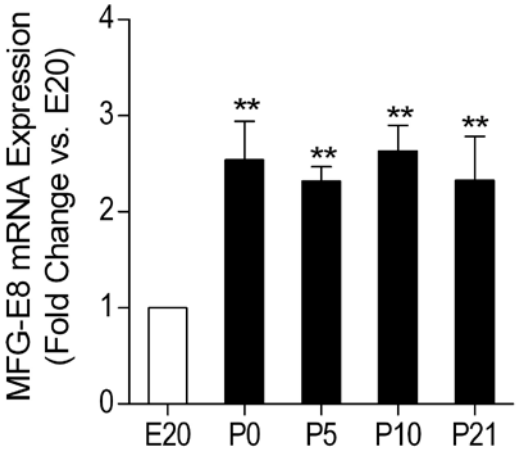

Figure 1. Expression of MFG-E8 mRNA in the liver during postnatal development. Liver samples from E20 and P0-5-10-15 were collected. Total RNA was extracted as described in the Methods. The MFG-E8 RNA transcript was quantified by real-time RT-PCR at each time point. The transcript abundance was normalized to the amount present in the liver at E20. Data are representatives of 3 independent experiments and displayed as their means ± SD. $**P<0.0001$ compared with E20.

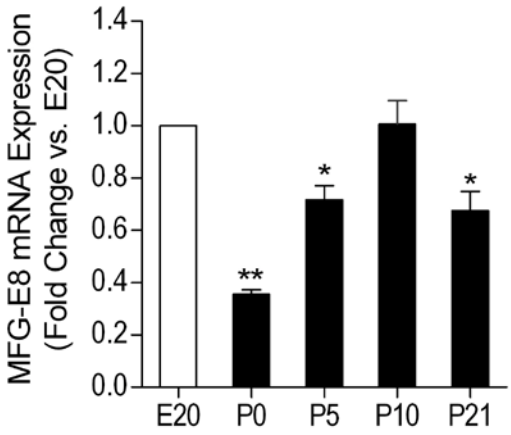

Figure 2. Expression of MFG-E8 mRNA in intestines during postnatal development. The small intestinal samples from E20 and P0-5-10-15 were collected. Total RNA was extracted as described in the Methods. The MFG-E8 RNA transcript was quantified by real-time RT-PCR at each time point. The transcript abundance was normalized to the amount present at E20. Data are representatives of 3 independent experiments and displayed as their means ± SD. $*P<0.01$ and $**P<0.0001$ compared with E20.

Care and Use Committee of the Children's Memorial Hospital. Sprague-Dawley rats were purchased from Harlan Sprague Dawley (Madison, WI). Tissues were collected after sacrificing animals at embryonic day 20 (E20), postnatal day 0 (P0), P5, P10, and P21. Total RNA from various tissues was extracted by using the RNeasy kit (QIAGEN, Valencia, CA) according to the protocol of the manufacturer. RNA concentration was determined by optical densitometry at 260 with Smart Spec™ plus spectrophotometer (Bio-Rad, Hercules, CA).

cDNA synthesis from total RNA by reverse transcription

cDNA was synthesized using iScript ™ cDNA sythesis kit (Bio-Rad) according to the protocol provided by the manufacturer. Briefly, 0.7μg of RNA from each tissue sample was added to 25 μl of reaction mixture containing dNTP mix, 1 X random hexamers primer, 1 X reaction buffer, 1 μl MMLV-derived iScript reverse transcriptase which was pre-blended with RNAse inhibitor. The reaction was run at 25°C for 5min, 42°C for 30 min and stopped by incubation at 85°C for 5 min. The resulted cDNA was used for the following quantitative real-time PCR.

Quantitative real time PCR (q-PCR)

QuantiTect™ SYBR Green PCR kit (QIAGEN, Valencia, CA) was used for the study. In brief, mastermix (73 μl) containing 0.4 nM primers and 1× SYBR Green PCR Universal Mastermix (QIAGEN) was added to 2 μl cDNA before aliquoting in triplicate to a 96-well microtiter plate (25 μl/well). The cDNA was amplified using a Fast 7500 real-time PCR system (AB Applied Biosystems, Foster City, CA) under the following conditions: 50 °C for 5 min, 95 °C for 10min, and then 40 cycles of amplification (95°C for 15s and 60° C for 1 min). All PCR reactions were performed in 96-well plate using a final volume of 25 μl. The cycle at which each sample crossed a fluorescence threshold, C_T (at 0.1 - 0.2 fluorescence unites), was determined. The triplicate values for each cDNA were averaged. Sequences for forward (F) and reverse (R) primers for real-time PCR were rat 18S rRNAF 5'-TTGATTAAGTCCCTGCCCTTT GT-3', rat 18S rRNAR 5'-CGATCCGAGGGCCTAACTA-3', rat MFG-E8F 5'-GGGCCTGAAGAATAACACGA-3',

and rat MFG-E8R 5'-GCATTGATCTTGCCCTGATT-3'. In a pilot study, we found that the C_T values for 18S ribosomal RNA from the different tissue at the different postnatal development stages fell in a close range with no specific pattern of spatial or temporal variation detected. Thus, 18S rRNA served as a control gene for calculating the ΔC_T, ($\Delta C_T = C_{T\text{mfge8}} - C_{T18S}$). Fold changes in expression levels of MFG-E8 mRNA in different tissues and different postnatal stages were calculated using the $2^{-\Delta\Delta CT}$ method [9] using 18S rRNA as the internal reference. The $\Delta\Delta C_T$ value is defined as the CT difference between the normalized amount of sample and the normalized amount of calibrator.

Statistical analysis

Data were expressed as means ± SD analysis of variance and one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test were used to assess the significant of differences; $P< 0.05$ was considered significant.

Results and discussion

MFG-E8 mRNA expression patterns in the liver during postnatal rat development

By using real-time PCR, we initially characterized the expression pattern for MFG-E8 mRNA in the rat liver during postnatal development. MFG-E8 mRNA was detected in the fetal liver at E20. After birth, the expression rapidly increased 2.57 ± 0.59 (means ± SD, $P<0.001$) in the liver of newborn pups and was stable at a high level during the postnatal period (**Figure 1**). The liver contains large amounts of residential macrophages (i.e. Kupffer cells). MFG-E8 has been shown to be expressed in macrophages [2]. Thus, it is not surprising that MFG-E8 is constitutively expressed in the liver. However, it is interesting that the MFG-E8 transcripts are rapidly increased at birth in the newborn liver. Previously, MFG-E8 has been shown to play an important role in mediating phagocytosis of apoptotic cells. Our results suggest that tissue remodeling and associated apoptosis occurs in the liver after birth. Newly produced MFG-E8 may play a role in mediating the clearance of apoptotic cells by Kupffer cells in the neonatal liver during the remodeling process.

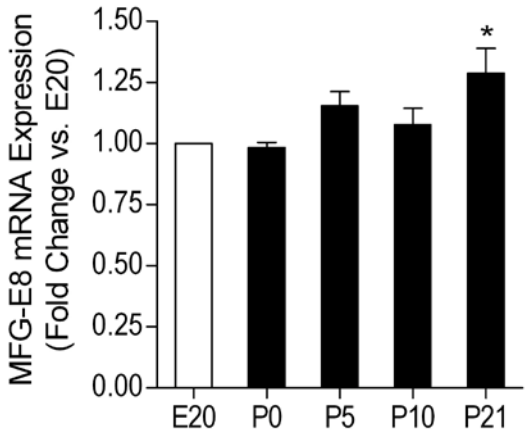

Figure 3. Expression of MFG-E8 mRNA in the lung during postnatal development. The lung samples from E20 and P0-5-10-15 were collected. Total RNA was extracted as described in the Methods. The MFG-E8 RNA transcript was quantified by real-time RT-PCR at each time point. The transcript abundance was normalized to the amount present in the lung E20. Data are representatives of 3 independent experiments and displayed as their means ± SD. *$P<0.005$.

MFG-E8 mRNA expression in the rat small intestine during postnatal development

Recently, we showed that MFG-E8 plays an important role in the maintenance and repair of the murine intestinal lining [8]. Here, we further determined the expression pattern of intestinal MFG-E8 mRNA at E20 and during development. MFG-E8 mRNA was present in the small intestine at E20. The expression of MFG-E8 mRNA was markedly down-regulated immediately after birth (**Figure 2**). It gradually regained levels similar to E20 by day 10 of life and remains persistently expressed at 3 weeks of age in rats. The regulation of MFG-E8 gene in development and gut immunity remains to be elucidated. It is puzzling that the rat fetus intestine expresses higher level of MFG-E8 comparing to the postnatal intestine. Miksa et al. recently showed that LPS induced a decrease in MFG-E8 gene expression in macrophages [10]. During the postnatal period, the intestinal development is associated with the establishment of the bacterial flora in the gut. Thus, we could speculate that bacteria down-regulate MFG-E8 during the colonization process or that a low level of MFG-E8 has to be maintained in the

intestinal mucosa to allow for proper bacterial colonization to take place.

MFG-E8 mRNA expression during postnatal development of the lung

The neonatal lung undergoes massive remodeling during the neonatal period as the newborn adjusts to air breathing. It is an organ rich in macrophages. Thus, we examined the MFG-E8 mRNA expression in the lung before birth and during the first 3 weeks of age. We found that MFG-E8 is constitutively expressed at E20 in the lung. The data goes along with a previous report that MFG-E8 is constitutively expressed in the adult lung [1]. In contrast to the gene expression in the liver and small intestine, the lung MFG-E8 mRNA expression did not change during 10 days after birth and then slightly increased at P21 (**Figure 3**). However, the function of MFG-E8 in the lung remains to be determined.

Comparison of MFG-E8 mRNA expression among splanchnic tissues during the postnatal development in rats

First, we found that the level of 18S ribosomal RNA (our internal control) was similar in the different tissue at the different postnatal development stages (**Figure 4A**). We then compared the level of MFG-E8 mRNA expression in rat splanchnic tissues during postnatal development by quantitative real-time RT-PCR and found that the lung constitutively expressed the highest levels of MFG-E8 mRNA. This was the case both at E20 and throughout the first 3 weeks of life (**Figure 4B**). In addition, before birth (E20), the small intestine had a higher level of MFG-E8 transcript compared to the liver. The gene expression was found to be markedly increased in the liver immediately after birth, whereas it decreased in the small intestine. During the first 3 weeks of life, MFG-E8 mRNA was present in the small intestine, but at much lower than levels in other splanchnic tissues. However, while we have characterized the tissue specific pattern of MFG-E8 mRNA expression during the first three weeks of life in rodents, its physiological relevance remains to be determined.

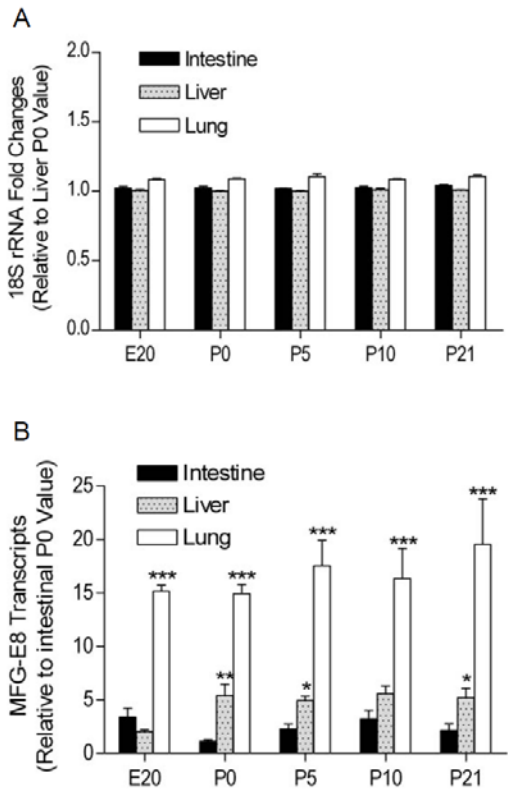

Figure 4. Comparison of MFG-E8 mRNA expression in different tissues after birth. Total RNA was extracted from various tissues at the indicated time points as described in the Methods. The 18S rRNA (Panel A) and MFG-E8 mRNA (Panel B) were quantified by real-time RT-PCR at each time point. The MFG-E8 transcript abundance was normalized to the amount present in the small intestine at P0. Data are representatives of 3 independent experiments and displayed as their means ± SD. **P*<0.05, ***P*<0.005, and ****P*<0.0001 compared with intestine at indicated time point.

Conclusions

In conclusion, we have documented that the MFG-E8 mRNA expression patterns in the liver, small intestine, and lung differ during postnatal development. Our work provides a direction for investigators interested in exploring the role of MFG-E8 in splanchnic organs during development.

Previously, we have demonstrated that MFG-E8 plays an important role in maintaining intestinal epithelial homeostasis [8]. In addition, MFG-E8 has been shown to be involved in mediating the clearance of apoptotic cells by macrophages [3]. In the present study, we found that MFG-E8 mRNA expression is strikingly higher in the lung and liver than in intestines during the first 3 weeks

of life. The lung and liver contain a large amount of macrophages, which play an important role in regulating host immunity. In addition, the lung and liver undergo thorough tissue remodeling during the postnatal period that are necessary to adjust to postnatal life. Thus, it is possible that the high level of MFG-E8 in the lung and liver is critical for insuring adequate postnatal remodeling and/or maintaining immune status in splanchnic tissues in neonates.

Our current study is focused on characterizing the pattern of mRNA expression in MFG-E8 gene. However, MFG-E8 protein expression and the mechanisms through which MFG-E8 gene expression is regulated in the postnatal period remain to be addressed.

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