Hepatocytes Contribute to Soluble CD14 Production, and CD14 Expression Is Differentially Regulated in Hepatocytes and Monocytes*

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CD14 presents as a glycosylphosphatidylinositol-linked membrane protein on the surface of monocytes/macrophages and as a soluble protein in the serum. Our previous studies have shown that an 80-kilobase pair (kb) genomic DNA fragment containing the human CD14 gene is sufficient to direct CD14 expression in a monocyte-specific manner in transgenic mice. In addition, we discovered that human CD14 is highly expressed in hepatocytes. Here, we report the generation of transgenic mice with either a 24- or 33-kb human CD14 genomic DNA fragment. Data from multiple transgenic lines show that neither the 24- nor the 33-kb transgenic mice express human CD14 in monocytes/macrophages. However, human CD14 is highly expressed in the liver of the 33-kb transgenic mice. These results demonstrate that human CD14 expression is regulated differently in monocytes and hepatocytes. Furthermore, we identified an upstream regulatory element beyond the 24-kb region, but within the 33-kb region of the human CD14 gene, which is critical for CD14 expression in hepatocytes, but not in monocytes/macrophages. Most importantly, the data demonstrate that the liver is one of the major organs for the production of soluble CD14. These transgenic mice provide an excellent system to further explore the functions of soluble CD14.
MATERIALS AND METHODS

Generation of Transgenic Mice—The P1 phagemid containing the human CD14 genomic sequence (P1-CD14) was described previously (30). A 24-kb BamHI fragment and a 33-kb KpnI fragment of P1-CD14 (Fig. 1) were prepared for microinjection by gel electrophoresis and subsequent extraction with a Geneclean kit (Bio 101, Vista, CA) following BamHI or KpnI digestion and prepared as described previously (30). Transgenic mice were produced in the transgenic facility of Beth Israel Deaconess Medical Center using zonaotes from FVB/N mice.

Southern Blot Analysis—Murine genomic DNA was prepared and analyzed as described previously (30). The relative copy number of the transgene was estimated by comparing the transgenic murine tail DNA samples using [32P]dNTP-labeled human or murine CD14 cDNA fragment. The human murine DNA fragments were analyzed as described previously (30). The level of human CD14 expression relative to GAPDH was demonstrated that the Sp1 sites and C/EBP site in the proximal promoter region of the CD14 gene are critical for CD14 expression in monocytes/macrophages and in the liver of transgenic mice (30). To further understand the molecular basis of CD14 expression, we isolated a 24-kb BamHI genomic DNA fragment and a 33-kb KpnI CD14 genomic DNA fragment and used them to generate transgenic mice (Fig. 1). Five founder lines of 24-kb transgenic mice and six founder lines of 33-kb transgenic mice were obtained. All five founder lines of 24-kb transgenic mice and four of six founder lines of 33-kb transgenic mice were germine transmitted. These founder lines, which exhibited germine transmission of the transgene, were used in further investigations.

Tissue-specific Expression of Human CD14 in Transgenic Mice—In the 80-kb transgenic mice, human CD14 was highly expressed in peritoneal macrophages and liver, which is consistent with its expression pattern in human macrophage and liver (30). To investigate the expression of human CD14 in the 24- and 33-kb transgenic mice, RNA was prepared from various tissues of these transgenic mice. Northern hybridization analyses with human CD14 as a probe showed that there was no highly detectable human CD14 expression in any of the tested tissues from the 24-kb transgenic mice (data not shown). However, human CD14 was highly expressed in the liver and mildly expressed in the heart, thymus, and lung of the 33-kb transgenic mice (Fig. 2A). There is no highly detectable human CD14 expression in the macrophages of the 33-kb transgenic mice. To further verify the relative level of CD14 expression in the liver, heart, thymus, and lung in the transgenic mice, RNA samples prepared from these tissues were analyzed again with a more evenly loaded gel (Fig. 2B). These results indicate that liver is the major organ for human CD14 expression in 33-kb transgenic mice; lung also expresses significant amounts of CD14. All four founder lines showed a similar pattern of human CD14 expression. Comparing this with the results from the 80-kb transgenic mice, the data indicate that regulatory elements beyond 33 kb, but within the 80-kb flanking regions of human CD14, are necessary for human CD14 expression in macrophages. The results also suggest that the regulatory elements involved in human CD14 expression in macrophages and liver are different, and that the region located outside of 24-kb but within 33-kb flanking regions of human CD14 gene contains important regulatory element(s) for human CD14 expression in the liver.

Expression of Human CD14 in Liver Is Copy Number-dependent and Position-independent—To study the regulation of CD14 expression in 33-kb human CD14 transgenic mice, we first analyzed the copy number of the human CD14 fragment in four germine transmitted founder lines using Southern blot hybridization (Fig. 3A). We then studied the level of human CD14 expression in four founder lines using Northern blot
hybridization with RNA prepared from these transgenic mice (Fig. 3B). Densitometry analysis of the results revealed that human CD14 expression levels relative to GAPDH expression in different founder lines and their copy numbers have a linear relationship (Fig. 3C). This indicates that human CD14 expression in the 33-kb transgenic mice is copy number-dependent. Since the integration of the 33-kb fragment is random in the murine genome, the data also suggest that the expression of human CD14 in the liver of transgenic mice is position-independent.

A Distal Positive Regulatory Element Was Identified for Human CD14 Expression in Hepatocytes—To further identify the regulatory elements within the 33-kb region of human CD14, a DNase I-hypersensitive site study was performed. We developed a 1.2-kb probe located at the 5’ end of a SacI-digested fragment (Fig. 4A) and applied this probe to SacI-digested DNA from the livers of transgenic mice following DNase I treatment of the liver nuclei for varying lengths of time. The results revealed two DNase I-hypersensitive sites within the 33-kb region. One site is 6.3 kb, and another is 7 kb upstream of the transcription initiation site of the human CD14 gene (Fig. 4A and B).

To investigate the function of the region containing DNase I-hypersensitive sites for CD14 expression, a 4.2-kb BglII-BamHI fragment as shown in Fig. 4A was subcloned into a human CD14 proximal promoter-luciferase reporter construct p-227CD14-luc (27). We made luciferase constructs, which included the 4.2-kb BglII-BamHI fragment in either sense orientation (p-4.2K(s)227CD14-luc) or antisense orientation (p-4.2K(a)227CD14-luc) (Fig. 5). With the CD14 proximal promoter-luciferase construct (p-227CD14-luc) as a control, these constructs were transiently transfected into a hepatocytic cell line, HepG2; a monocytic cell line, Mono Mac 6; and a cervical carcinoma cell line, HeLa. The results showed that the 4.2-kb fragment enhanced the activity of the CD14 proximal promoter in HepG2 cells by 25-fold when it is in the sense orientation, but only weakly enhanced the activity in the antisense orientation. Furthermore, it did not significantly enhance the promoter activity in either orientation in Mono Mac 6 cells or HeLa cells (Fig. 5). These results indicate that this 4.2-kb fragment contains distal regulatory element(s), whose activity is orientation-dependent and tissue-specific.

Deletion constructs from the 4.2-kb fragment were generated to further study the regulation of CD14 expression in hepatocyte. As shown in Fig. 6, the sense oriented 4.2-kb BglII-BamHI fragment, 3-kb SacI-BamHI fragment, and the 0.7-kb SacII-BamHI fragment had similar positive effect on the CD14 proximal promoter in HepG2 cells. The data indicate that the positive distal regulatory element is located in the 0.7-kb fragment. Furthermore, the 4.2- and 3-kb fragments showed strong orientation dependence, and the 0.7-kb fragment only showed partial orientation dependence.
Liver Is One of the Major Sources of Soluble CD14—Soluble CD14 is able to mediate LPS signaling and initiate the cytokine cascade in normal human monocytes (37), as well as in cells that lack membrane-bound CD14, such as epithelial and endothelial cells (38, 39). Furthermore, soluble CD14 has a potential involvement in other lipid transfer processes (17). As shown in the above sections, the human CD14 gene is clearly expressed in the livers of 33-kb transgenic mice although it is not expressed in the monocytes/macrophages of these transgenic mice. To further study the biological significance of our transgenic mice, we investigated human CD14 expression at the protein level in the liver and serum of four transgenic founder lines. Due to the high background of liver proteins with human CD14 antibodies, we were unable to clearly detect the CD14 protein in liver protein extracts by Western blot analysis (data not shown). However, when an ELISA was used to analyze the level of soluble human CD14 in the serum of transgenic mice, we detected a clear expression of human CD14 (Fig. 7). Furthermore, the amount of soluble human CD14 expression had a direct correlation with the copy number of the human CD14 transgene in the transgenic mice. These data indicate that liver is one of the major tissues in which soluble human CD14 is produced.

DISCUSSION

Since CD14 is specifically expressed in mononuclear cells during hematopoiesis and has been used as a differentiation marker for monocytes/macrophages, our original goal was to study the expression of CD14 in the monocytic lineage in order to gain information about myeloid cell differentiation. However, in the analysis of our 80-kb transgenic mice and subsequent investigation with human tissues and cell lines, we have demonstrated a strong human CD14 expression in both monocytic and hepatocytic cells (30). Using 24- and 33-kb CD14 genomic DNA fragments in current studies in transgenic mice, we have further revealed the differential regulation of CD14 expression in mononuclear and hepatocytic cells. The data indi-

FIG. 4. A, schematic diagram of the upstream region of the human CD14 gene. The DNA probe is indicated by the filled box. The C/EBP and Sp1 binding sites in the proximal promoter region are shown, as well as the transcription initiation site of human CD14 (horizontal arrow). The DNase I-hypersensitive sites were located 6.5 and 7 kb upstream from the human CD14 transcription initiation site (vertical arrows). Restriction enzyme digestion sites used to produce regulatory fragment-luciferase constructs were indicated. B, DNase I-hypersensitive site analysis of the region upstream from the human CD14 gene in liver. Genomic DNA was isolated from the nuclei of liver cells from a transgenic mouse of founder line 3 following DNase I treatment for various times as indicated. After SacI digestion, 10 μg of genomic DNA from each time point were electrophoresed in a 0.8% agarose gel, transferred to a positively charged nylon membrane, and hybridized with the probe indicated in panel A. Two DNase I-hypersensitive sites were detected. The sizes of the fragments are indicated on the right side of the panel.

FIG. 5. Identification of distal regulatory elements in the upstream region containing DNase I-hypersensitive sites. The human CD14 promoter-luciferase construct (p-227CD14-luc) and the constructs containing a 4.2-kb BgIII/BamHI region with the DNase I-hypersensitive sites in sense orientation (p-4.2K(s)227CD14-luc) or antisense orientation (p-4.2K(a)227CD14-luc) were transiently transfected into HepG2, Mono Mac 6, and HeLa cells. Luciferase activities were normalized by cotransfecting with a Renilla luciferase construct (pRL-CMV) as an internal control. The relative luciferase activities of the constructs were averaged together from three separate sets of experiments. The error bars represent the standard deviations.

FIG. 6. Deletion analysis of distal regulatory elements within 4.2 kb of BglII/BamHI region. The human CD14 promoter-luciferase construct (p-227CD14-luc) and the constructs containing 4.2-kb BgIII/BamHI region, 3-kb SacI/BamHI region, and 0.7-kb SacI/BamHI region in sense or antisense orientation were transiently transfected into HepG2 cells. Luciferase activities were normalized by cotransfecting with a Renilla luciferase construct (pRL-CMV) as an internal control. The relative luciferase activities of the constructs were averaged together from three separate sets of experiments. The error bars represent the standard deviations.
cate that the 24-kb DNA fragment lacks important regulatory elements to support CD14 expression. The sequence within the 33-kb fragment, but beyond the 24-kb fragment, is required for directing CD14 expression in a copy number-dependent and position-independent manner in hepatocytes; and monocytic CD14 expression requires further genomic information beyond the 33-kb fragment. Using DNase I hypersensitivity and transfection studies, we have identified a 0.7-kb DNA fragment within the 33-kb genomic sequence, which functions as a hepatocyte-specific regulatory element. Therefore, we have generated an animal model, which exhibits human CD14 expression in the liver but not the monocytes.

It is interesting to note that the critical transcription factors regulating CD14 promoter activity are Sp1 and C/EBP (27, 29). Sp1 is a ubiquitously expressed transcription factor (40). It directly interacts with basal transcription machinery, such as binding to TAF110, and cooperates with tissue-specific factors to promoter gene expression (41). C/EBPs are a family of transcription factors (42). Some of the family members are expressed in a tissue-specific fashion, and others are expressed during cell stress and acute phase response. Among these members, C/EBPα and C/EBPβ are highly expressed in both hepatocytes and myeloid cells (43). Mice with C/EBPα or C/EBPβ deficiency have shown significant impairment in the differentiation or function of these cells (44–46). Therefore, hepatocytes and monocytes share the same set of transcription factors and use the same CD14 upstream region as their proximal promoter. The different regulation of their expression in two cell types depends on further upstream regulatory elements. Two DNase I-hypersensitive sites were identified by a DNase I hypersensitivity and transfection studies. We found that human CD14 is highly expressed in the liver, but not in macrophages. Human soluble CD14 are detected in these transgenic mice. Although human CD14 expression is also detected in the lung, heart, and thymus, its expression in the liver is much higher and liver mass is much bigger than the other tissues. This indicates that the expression of human CD14 in the liver generates soluble CD14. More importantly, this shows for the first time in vivo that the liver is one of the major sources of soluble CD14 in circulation. The soluble CD14 level in the serum of normal adult human is about 5 μg/ml, which represents approximately a 1000-fold molar excess of the LPS level seen in fatal septic shock patients (17). This indicates that soluble CD14 may have other biological functions besides its function in LPS signaling. Both membrane-bound and soluble forms of CD14 have been recently reported as potential lipid transport proteins (17, 18). Level of soluble CD14 in normal mouse serum is much lower than in human serum (51). We have generated transgenic mice, which express different levels of soluble CD14. Some of these transgenic lines have soluble human CD14 expression at the similar level to CD14 in human sera. They should be good models for analyzing CD14 biological functions.

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