Contribution of Apoptosis and Apoptosis-Related Proteins to the Malformation of the Primitive Intrahepatic Biliary System in Meckel Syndrome

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In the developing liver, the complete or partial persistence of the primitive double-layered cylinder of biliary-type cells that surrounds the branches of portal vein and its mesenchyme gives origin to portal tracts with an increased number of bile duct structures. The term “ductal plate malformation of the liver” was coined to label the insufficient remodeling of the primitive intrahepatic biliary system. Meckel syndrome is an autosomal recessive inherited disease characterized by occipital encephalocele, postaxial polydactyly, diffuse cystic renal dysplasia, and malformation of the ductal plate of the liver. We studied 52 fetuses with Meckel syndrome from five German centers (Berlin, Freiburg, Heidelberg, Mainz, and Marburg). Analysis of apoptosis and cell proliferation (Ki-67) was performed by terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) and immunohistochemistry in the liver of 24 normal fetuses of different gestational ages (14–38 weeks of gestation) and in 14 fetuses with Meckel syndrome (17–38 weeks of gestation). The expression of two apoptosis-related proteins, Fas (a transmembrane cell surface protein involved in the apoptosis) and Bcl-2 (an anti-apoptotic protein), was studied by immunohistochemistry in the liver of 11 normal fetuses of different gestational ages (14–40 weeks of gestation) and in 40 fetuses with Meckel syndrome (16–38 weeks of gestation). In control fetuses, apoptosis rate and cell proliferation were high in the remodeling ductal plate and moderate in the ductal plate and in remodeled bile ducts. During gestation, expression of Fas and Bcl-2 decreased and increased, respectively. The malformed ductal plates in the fetal livers with Meckel syndrome showed a marked decrease in the apoptotic rate and Fas expression and an increase in proliferative activity and Bcl-2 expression in comparison with control fetuses. Furthermore, by linear regression analysis, we found that both proliferation activity and apoptosis rate in the ductal plate malformation of fetuses with Meckel syndrome were practically constant along the gestation. These results, which represent the first systematic study of apopto-

sis in ductal plate malformation of the liver, indicate that 1) animals harboring the gene defect of Meckel syndrome could be a good model for the study of the abnormal development of the primitive intrahepatic biliary system, 2) a decreased cell turnover occurs in the ductal plate malformation of fetuses with Meckel syndrome, and 3) the increase of Bcl-2 expression contributes to the pathogenesis of the lack of remodeling of ductal plate of the liver in Meckel syndrome. (*Am J Pathol* 2000, 156:1589–1598)

Programmed cell death, or apoptosis, is a key mechanism in developing organisms, playing an important role in their differentiation and maturation. In evolutionary biology, its role is quite well determined. In fact, the different expression of a bone morphogenetic protein in limb buds creates an evolutionarily important morphological alteration during the extremely similar embryogenesis of duck and chick. At the same developmental stage, its expression in the interdigital webbing induces cells to undergo apoptosis in the chick limb, and the lack of its expression allows webbed feet to be retained in the duck.¹ In ontogenesis, apoptosis plays a basilar role in the organogenesis of several systems.^{2,3} In the liver, the ductal plate is the protostructure of the intrahepatic biliary system and consists of a double-layered cylinder of biliary-type cells with a slit-like lumen forming around the portal vein and its surrounding mesenchyme (stage of ductal plate). The remodeling of the ductal plate is characterized by the incorporation of a few ductal plate cells into the mesenchyme surrounding the portal vein to form bile ducts as well as by the disappearance of nonmigrating ductal plate cells (stage of remodeling ductal plate and stage of remodeled bile ducts). The development of intrahepatic bile ducts proceeds from the hilar to peripheral portions. Two or more of these developmental stages may be present in the same liver specimen.^{3–5} During the three stages of development of the intrahepatic bile

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ducts, apoptosis and cell proliferation are quantitatively different, being high in the stage of remodeling ductal plate and moderate in the stages of ductal plate and remodeled bile ducts.³ During gestation, Fas (CD95/Apo-1 antigen),⁶ a transmembrane cell surface protein that plays a major role in the programmed sequence of events leading to apoptosis, and Bcl-2,⁷ an antiapoptotic protein, decrease and increase, respectively.

The complete or partial persistence of the primitive double-layered cylinder of biliary-type cells in the developing liver gives rise to portal tracts with an increased number of bile duct structures. The term "ductal plate malformation of the liver" was coined to label this complex biliary plexus with an excess of primitive bile duct structures.⁸ The factors controlling the balance between proliferation and cell death in the malformation of ductal plate of the liver remain to be studied.

A few genetic syndromes may demonstrate a malformation of the ductal plate of the liver.⁹ Meckel syndrome is an autosomal recessive inherited disease characterized by occipital encephalocele, postaxial polydactyly, diffuse cystic renal dysplasia, and malformation of the ductal plate of the liver.¹⁰ We chose to investigate the ductal plate malformation of the liver in Meckel syndrome because of standardized histological criteria.

In this study, we examined the livers of fetuses with Meckel syndrome for evidence of apoptosis, cell proliferation, and expression of Fas and Bcl-2 proteins to determine their roles in the abnormal development of ductal plate cells.

Materials and Methods

Subjects

Formalin-fixed and paraffin-embedded human liver tissues from 52 fetuses with Meckel syndrome (gestational age 21.4 ± 5 weeks; mean $\pm$ SD) were retrieved from the files of five university institutions over a 12-year period, 1988–1999 (Berlin, Freiburg, Heidelberg, Mainz, and Marburg; see Appendix) and were used for these studies. Meckel syndrome was diagnosed according to the criteria of Salonen and Paavola.¹⁰ Because degeneration and autolysis of liver cells can generate a sufficiently high number of stainable DNA ends, only fetuses or infants without autolytic livers were enrolled in the study.

For the control specimens, we collected liver sections from the right lobe from 24 human fetuses and infants without external or internal malformations of gestational ages approximately uniformly distributed from 14 to 40 weeks of gestation (weeks 14–16, 19–25, 27, 29, 38, 40). Gestational age was confirmed by measuring somatometric findings.¹¹ The fetuses were either the products of spontaneous abortions (placental or amniotic cause of death: premature rupture of the membranes, placenta previa, premature rupture of the membranes, retroplacental hematoma, amnionitis, or acute placenta dysfunction) or elective terminations of pregnancy (eg, hysterectomy in a mother suffering from mammary carcinoma). In each control case, the extrahepatic and intrahepatic bil-

iary systems were normal. Autopsies were performed within 36 hours after death and always with the consent of the parents. Chromosomal studies, when performed by amniocentesis or at the time of autopsy, revealed a numerically and structurally normal karyotype (46, XY or 46, XX). In the fetuses without karyotype analysis aneuploidy phenotypes were carefully excluded according to current guidelines.¹⁰ Pregnant women with a history of cigarette smoking, carbohydrate intolerance, or drug addiction were not included in the study, to minimize possible influences that could cause deviation from the normal pattern of development during organogenesis. Tissue collection was always done in accordance with the appropriate German federal laws. This study is related to an investigation of ductal plate malformation, performed on fetal material and approved by the Ethics Committee of the University of Heidelberg (Resolution 186/98, September 22, 1998).

Classification of Intrahepatic Bile Duct Development

The development of the intrahepatic bile ducts was classified into three stages according to the methods of previous reports.^{3,4} The intrahepatic bile ducts develop out of sheets of primitive hepatic epithelial cells. Development is mostly determined by the progressive growth and branching of the portal vein with its surrounding mesenchyme. The primitive hepatic epithelial cells in direct contact with the portal vein mesenchyme transform into bile duct type cells by first forming one layer and later two layers. First, a cleft forms between the two layers of bile duct-type epithelial cells (ductal plate stage, approximately 9–12th gestational week). Subsequently, there is a migration of the biliary cells from the ductal plate into the mesenchyme, with biliary structures still partially in contact with the progressively disappearing ductal plate (remodeling ductal plate stage, approximately 13–17th gestational week). Finally, remodeled bile ducts are formed that are centrally located in the portal tracts without contact with ductal plate remnants (remodeled bile ducts stage, approximately 18–40th gestational week). The intrahepatic bile duct development progresses from the hepatic hilum peripherally. Frequently, two or more of these stages may be observed in the same liver, and at 40 weeks of gestation the smallest portal veins are still surrounded by a discontinuous ductal plate.

TUNEL Reaction

Cells with a morphology characteristic of apoptosis (ie, nuclear condensation, fragmentation, apoptotic bodies) may be supported by the existence of DNA fragmentation in these cells. Double-stranded DNA breaks were detected on paraffin sections of the liver by the terminal deoxynucleotide transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) method, basically according to the method described by Gavrieli et al,¹² with some modifications. We used the *in situ* cell death Detection Kit for TUNEL (Boehringer Mannheim, Mannheim, Germany) ac-

Figure 1. Malformation of the ductal plate of the liver of a fetus affected with Meckel syndrome with dilatation of the primitive ductal plate structure and portal tract fibrosis. In small portal tracts the dilatation in the portal tract suggested a "polypoid"-like image. AE 1 + 3. Original magnifications: **a**, $\times 62.5$; **b**, $\times 200$.

cording to the manufacturer's recommendations. To inhibit false positivity caused by the release of endogenous endonucleases as a result of proteinase treatment, the slides were also incubated in an ethanolic solution of diethyl pyrocarbonate (DEPC) (4% v/v; Sigma-Aldrich, Deisenhofen, Germany) for 30 minutes at 4°C according to the method of Stähelin et al.¹³ As a negative control, we omitted the TdT enzyme in the TUNEL reaction. As a positive control, we used normal tonsil tissue.

Immunohistochemistry

Five-micrometer sections were obtained from each formalin-fixed paraffin-embedded block. Two different immunolabeling methods were used: the avidin-biotin-peroxidase complex (ABC) method, according to the method of Hsu et al.¹⁴ and the highly sensitive catalyzed signal amplification (CSA) method, based on the peroxidase-catalyzed reaction of biotinyl tyramide with electron-rich aromatic amino acids in tissue specimens, according to the method of Bobrow et al.¹⁵ The ABC immunolabeling method was used to demonstrate cyto-keratin epitopes (CK) (intermediate filaments of the cy-

Figure 2. Proliferation activity (MIB-1 Rate) expressed as the percentage of MIB-1-positive cells on all counted cells in a determinate structure during the stages of human fetal liver development (ductal plate (DP), remodeling ductal plate (RDP), and remodeled bile ducts (RBD)) and in the malformation of the primitive embryonic ductal plate or ductal plate malformation (DPM). For statistical significance see text.

toskeleton) to better localize ductal plate and bile duct cells (AE 1 + 3, 1:2, mouse IgG1, CK nos. 1–8, 10, 14, 15, 16, 19 (acid CK: 40, 48, 50, 46.5 kd; basic CK: 58, 65–67 kd), Linaris, Camon, Wiesbaden, Germany) and the proliferation activity (MIB-1, 1:10, mouse IgG1, cell proliferation-associated Ki-67 epitope). Sections were deparaffinized and always either treated with a proteolytic agent (pronase 0.1%; Merck, Darmstadt, Germany) or heated using a domestic microwave (700 W for 10 minutes) in citrate-phosphate buffer (pH 7.6) or, alternatively, in a Tris-citrate target retrieval buffer (TRS-Citrate; Dako, Hamburg, Germany) to demask the antigenic epitopes. Endogenous avidin-binding activity was blocked with a Avidin/Biotin Blocking Kit (Lenco Technologies, Ballwin, MO). The secondary antibody was a biotinylated anti-mouse IgG (1:200; Vector Labs, Burlingame, CA), and the first antibodies were localized with a Vector Elite ABC Kit (Vector Labs). The expression of the apoptosis regulatory protein Fas was studied using three commercial antibodies: CD 95, 1:100, mouse, clone DX2 (Pharmingen, San Diego, CA); CD 95, 1:100, mouse, clone DX2 (Dako); and Apo-1, 1:50, mouse, clone A11 (Alexis, San Diego, CA). Preliminary studies indicated comparable results for the first two antibodies, no signal was seen with the third one (data not shown). The expression of the apoptosis regulatory protein Bcl-2 was studied by an antibody to Bcl-2 (anti-human Bcl-2 oncoprotein, 1:100, mouse, clone 124; Dako). The best results were obtained with the following pretreatment methods: 10 minutes of proteolytic digestion at 37°C for Fas and microwave treatment for Bcl-2. Both the expression of Fas and the expression of Bcl-2 were investigated on paraffin sections of livers according to the CSA method (Dako Catalyzed Signal Amplification System; Dako). Preliminary studies with the avidin biotin peroxidase method for immunostaining of Fas and Bcl-2 showed no or faint staining (data not shown). In fact, although these antibodies are suitable for labeling formalin-fixed, paraffin-embedded tissue reactions, the duration of formalin fixation (recommended not to exceed 24–48 hours) was frequently exceeded in many interinstitutional cases of Meckel syndrome. This rendered the ABC method insufficient to visualize the antigens related to apoptosis. The

Figure 3. Proliferation activity (Ki-67) during fetal development of intrahepatic bile ducts and in ductal plate malformation of the liver of fetuses with Meckel syndrome. Cells of remodeling ductal plate in a normal fetus (**a**, arrow) and in several biliary cells of malformed ductal plates in Meckel syndrome (**b**, **c-d**, arrows). Original magnifications: **a**, $\times 200$; **b**, $\times 160$; **c**, $\times 220$; **d**, $\times 320$.

relationship between the duration of formalin fixation and the intensity of the immunostaining was not studied. Briefly, after the endogenous peroxidase was blocked and of the nonserum block was applied, the sections were incubated with primary antibodies for 15 minutes. The sections were washed in 0.05 mol/L Tris-HCl buffer (pH 7.6) and 0.1% Tween 20 and incubated with the anti-mouse biotin-marked secondary antibody. The sections were then rinsed and incubated with the streptavidin-biotin-peroxidase complex for 30 minutes, rinsed, and incubated with the amplification reagent biotin-marked tyramine for 15 minutes and with horseradish-conjugated streptavidin for 15 minutes. Finally, the sections were stained with diaminobenzidine for 5 minutes, counterstained, and mounted. All incubations were performed at room temperature. A negative control slide was always included in each immunostaining, with the first antibody replaced by normal serum.

Immunostaining results were evaluated, graded, and scored by extent (0 = none, 1 = 1–25%, 2 = 26–50%, 3 = 51–75%, 4 = 76–100% of the ductal cells) and intensity (0 = negative, 1 = weak, 2 = moderate, and 3 = intense). An immunohistochemical score was calculated for each case in which the percentage of positive rating

was multiplied by the intensity rating. The immunostaining was judged to be antibody-specific by following two criteria: staining use of normal serum produced no consistent immunostaining of any cells and signal intensity diminished as the dilution of antibody was increased.

Counting and Statistical Analysis

In each specimen, at least 200 cells of developing bile ducts for control cases and at least three malformed ductal plates of fetuses with Meckel syndrome were randomly selected and counted. The fields were selected independently by two authors (C.S., P.K.) without knowledge of the gestational age of the cases. Due to extreme sensibility of the TUNEL method and to avoid artifacts, only 14 well formalin-fixed livers of fetuses with Meckel syndrome were studied for TUNEL and MIB labeling (17–38 weeks of gestation: 21.9 ± 6 weeks of gestation, mean $\pm$ SD). Apoptotic or MIB-positive cells in the three stages of bile duct development (ductal plate stage, remodeling ductal plate stage, remodeled bile ducts stage) and in ductal plate malformation were separately counted. Mitotic cells and blood cells were carefully ex-

Figure 4. Results of linear regression analysis in all three stages of normal bile duct development (ductal plate (DP), remodeling ductal plate (RDP), remodeled bile ducts (RBD)) of 24 normal fetuses and in the ductal plate malformation (DPM) of 14 fetuses with Meckel syndrome. The best-fit lines are shown as a solid line for DP, a dashed line for RDP, a dotted line for RBD, and a mixed broken line for DPM. The proliferative activity (MIB - Rate) was expressed as the percentage of MIB-positive cells on all counted cells in a determinate structure during the stages of human fetal liver development. Two or more of these developmental stages may be present in the same liver specimen of normal fetuses. Replicate values were averaged and treated as single data points. For statistical significance and r^2 see text.

cluded. The rate of apoptotic cells (apoptosis rate) and the rate of MIB labeled cells (MIB rate) were expressed as a percentage (number of positive cells to all counted cells in a determinate structure). The total number of counted cells in a determinate structure (ductal plate, remodeling ductal plate, remodeled bile ducts, and ductal plate malformation) and the results of the percentages of positive cells were presented as mean $\pm$ SE, if not otherwise specified. We compared simultaneously the apoptosis rate and the MIB rate in the different four groups using the Kruskal-Wallis rank-sum test and the Dunnett's posttest (GraphPad Software data analysis, version 2.01, San Diego, CA, USA: <http://www.graphpad.com>). We analyzed also the linear regression models of MIB rate and apoptosis rate along the gestation in all three stages of normal bile duct development (ductal plate, remodeling ductal plate, remodeled bile ducts) of normal fetuses and in the ductal plate malformation of fetuses with Meckel syndrome (GraphPad Software data analysis). Moreover, we compared the linear regression analysis of Fas - score and Bcl-2 score during human fetal bile duct development in normal fetuses and in fetuses with Meckel syndrome using the Mann Whitney test (GraphPad Software data analysis). All p values were two-sided. Values of less than 0.05 were considered to indicate statistical significance. Intra- and interobserver differences were less than 5% and non-concordant cases were re-evaluated simultaneously by both observers (C.S., P.K.).

Results

Cases

The fetuses affected with Meckel syndrome usually showed a prominent abdomen, a Potter-like facies, and

occipital encephalocele at external examination. All fetuses with Meckel syndrome showed a normal extrahepatic biliary system (gall bladder, cystic duct, and common bile duct), but had malformed ductal plates with variable degrees of bile duct cystic dilatation and portal fibrosis. Cystic dilatation of the primitive biliary structures with little portal tract fibrosis was observed in 39 cases (gestational age: 20.9 ± 5.1 weeks of gestation, mean $\pm$ SD) to the epithelial-mesenchymal interface at the periphery of the portal tracts (Figure 1, a and b). This ductal plate malformation was seen both near the hepatic hilum and in the peripheral liver. In 13 cases (gestational age: 23 ± 4.1 weeks of gestation, mean $\pm$ SD), a dominance of no or little dilatation of the primitive biliary structures and an increased amount of connective tissue were observed in the portal tracts.

Figure 5. Apoptosis rate expressed as the percentage of TUNEL-positive cells on all counted cells in a determinate structure during the stages of human fetal liver development (ductal plate (DP), remodeling ductal plate (RDP), and remodeled bile ducts (RBD)) and in the malformation of the primitive embryonic ductal plate or ductal plate malformation (DPM). For statistical significance see text.

Figure 6. Apoptotic cells during fetal development of intrahepatic bile ducts and in ductal plate malformation of the liver of fetuses with Meckel syndrome are recognizable as nuclei stained by the TUNEL method. Shown are cells of remodeling ductal plate in a normal fetus (**a**, arrows) and in very few biliary cells of a malformed ductal plate in Meckel syndrome (**b**, arrow). Original magnifications: **a**, $\times 320$; **b**, $\times 200$.

Proliferation Activity

Immunoreactivity was detected only in the nucleus. MIB positive cells were counted during the development of intrahepatic bile ducts of 24 normal fetuses (counted cells: 220.3 ± 4.70) and in the ductal plate malformation of 14 fetuses with Meckel syndrome (counted cells: 314.9 ± 23.09). In control fetuses, MIB rate was high in the remodeling ductal plate ($14.10 \pm 1.62\%$), but moderate in the ductal plate ($7.97 \pm 0.96\%$) and remodeled bile ducts ($4.09 \pm 1.05\%$) (Figure 2). The MIB rate of remodeling ductal plate stage was significantly higher than that of remodeled bile duct stage ($P < 0.001$). There were no significant differences of MIB rate between ductal plate stage *versus* remodeling ductal plate stage and ductal plate stage *versus* remodeled bile duct stage (Figures 2 and 3).

In Meckel syndrome, MIB rate in biliary cells of ductal plate malformation ($14.97 \pm 1.38\%$) was significantly higher than that of ductal plate stage [$P < 0.05$] and remodeled bile duct stage [$P < 0.001$] (Figure 2, Figure 3b-d). The MIB rate of ductal plate malformation was no significantly different from that of remodeling ductal plate stage.

We performed also the linear regression analysis of MIB rate along the gestation in all three stages of normal bile duct development (ductal plate, remodeling ductal plate, remodeled bile ducts) of normal fetuses and in the ductal plate malformation of fetuses with Meckel syndrome (Figure 4). We found that all four best-fit regression lines showed insignificant P values and showed practically no linear relationship between MIB rate and gestational age (r^2 : 0.1 for ductal plate stage, r^2 : 0.01 for remodeling ductal plate stage, r^2 : 0.1 for remodeled bile ducts stage, and r^2 : 0.1 for ductal plate malformation). In all three stages of normal bile duct development and in

Figure 7. Results of linear regression analysis in all three stages of normal bile duct development (ductal plate (DP), remodeling ductal plate (RDP), remodeled bile ducts (RBD)) of 24 normal fetuses and in the ductal plate malformation (DPM) of 14 fetuses with Meckel syndrome. The best-fit lines are shown as a solid line for DP, a dashed line for RDP, a dotted line for RBD, and a mixed broken line for DPM. The apoptosis rate was expressed as the percentage of TUNEL-positive cells on all counted cells in a determinate structure during the stages of human fetal liver development. Two or more of these developmental stages may be present in the same liver specimen of normal fetuses. Replicate values were averaged and treated as single data points. For statistical significance and r^2 see text.

Figure 8. Expression of Fas and Bcl-2 antigens during fetal development of intrahepatic bile ducts and in ductal plate malformation of the liver of fetuses with Meckel syndrome. Moderate Fas expression in cells of remodeling ductal plate stage (**a**, $\times 320$), and negative to faintly positive Fas expression in the malformed ductal plates of Meckel syndrome (**b**, $\times 200$). Bcl-2 is faintly positive in the remodeling ductal plate stage (**c**, $\times 200$), whereas it is strongly positive in the malformed ductal plates of Meckel syndrome (**d**, $\times 200$).

the ductal plate malformation of fetuses with Meckel syndrome the scatter of the values was evident. However, both the y intercept (ie, the place where a line crosses the y axis) of the best-fit regression lines of the ductal plate stage (14.89 ± 4.9), the y intercept of the remodeling ductal plate stage (12.08 ± 8.7), and the y intercept of the ductal plate malformation (19.46 ± 6.9) showed higher values in comparison with the y intercept value of the best-fit regression line of the remodeled bile ducts (9.775 ± 4.5).

Apoptosis

Staining was confined to the nucleus. TUNEL-positive cells were counted during the development of intrahepatic bile ducts of 24 normal fetuses (counted cells: 270.8 ± 11.47) and in the ductal plate malformation of 14 fetuses with Meckel syndrome (counted cells: 370.9 ± 34.08). In control fetuses, the apoptotic rate was high in the remodeling ductal plate stage ($19.93 \pm 2.41\%$) and intermediate in the ductal plate stage ($6.47 \pm 1.87\%$) and in the remodeled bile duct stage ($8.95 \pm 2.30\%$) (Figure 5). The apoptotic rate of remodeling ductal plate stage

was significantly higher than that of the remodeled bile duct stage ($P < 0.01$) and than that of the ductal plate stage ($P < 0.001$) (Figure 6a). There were no significant differences in apoptotic rate in biliary cells between ductal plate and remodeled bile ducts.

In Meckel syndrome, the apoptotic rate in biliary cells of ductal plate malformation ($3.43 \pm 0.64\%$) was significantly lower than that of the remodeling ductal plate stage ($P < 0.001$) (Figures 5 and 6b). Although not significant, the apoptotic rates of malformed ductal plates were lower than those of the ductal plate stages and remodeled bile duct stage. Apoptosis occurred in particular in malformed ductal plates with pronounced portal tract fibrosis and little dilatation of the primitive biliary structures.

We also performed a linear regression analysis of the apoptosis rate during the gestation in all three stages of normal bile duct development (ductal plate, remodeling ductal plate, remodeled bile ducts) of normal fetuses and in the ductal plate malformation of fetuses with Meckel syndrome (Figure 7). We found a modest coefficient of determination (r^2 : 0.3) for the remodeling ductal plate stage of normal fetuses ($P < 0.05$). All three of the other

Figure 9. Fas score during human fetal bile duct development (x axis: weeks of gestation) of 14 normal fetuses (○) and 40 fetuses with malformation of the primitive embryonic ductal plate (●). In normal fetuses the Fas score comprises the analysis in all three stages of bile duct development (DP, RDP, RBD). Replicate values for the same week of gestation are averaged and treated as single data points. For statistical significance and linear regression analysis see text.

regression analyses showed very low coefficients of determinations (r^2 : 0.2 for the ductal plate stage, r^2 : 0.03 for the remodeled bile ducts stage, and r^2 : 0.01 for the ductal plate malformation), with insignificant P values. The best-fit regression line for the ductal plate malformation was shown to be horizontal by showing no linear relationship between apoptosis rate and gestational age. The best-fit regression line for the ductal plate malformation showed a higher 1/slope (ie, the reciprocal of the slope of a straight line, which represents the change in y for every unit change in x) of 28.9 in comparison with the 1/slopes of the best-fit regression lines during normal fetal bile duct development (-1.5 for the ductal plate stage, 1.1 for the remodeling ductal plate stage, 2.6 for the remodeled bile ducts stage).

Apoptosis-Related Proteins (Fas and Bcl-2)

The expression of apoptosis-related proteins was studied in 11 normal fetuses (25 ± 8 weeks of gestation, mean $\pm$ SD) and in 40 fetuses with Meckel syndrome (21.1 ± 5.1 weeks of gestation, mean $\pm$ SD). Fas immunoreactivity was observed in the cytoplasm and at the cell membrane. Bcl-2 immunoreactivity resided exclusively in the cytosol, often in a punctate pattern suggestive of association with intracellular organelles.

In control fetuses, Fas antigen was expressed in decreasing order in the ductal plate stage, remodeling ductal plate stage, and remodeled bile duct stage. In the ductal plate stage, the extension of the Fas expression was $>50\%$ of the ductal cells, and the intensity was moderate to strong, whereas the expression in remodeled bile ducts was very weak in $>50\%$ of the biliary cells (Figure 8a). In Meckel syndrome, the expression of Fas antigen was negative or faintly positive in the malformed ductal plates ($>50\%$ of the biliary cells) (Figure 8b).

The expression of Bcl-2 protein was negative or faintly positive in the ductal plate stage and in the remodeling ductal plate stage, but was moderately to strongly posi-

Figure 10. Bcl-2 score during human fetal bile duct development (x axis: weeks of gestation) of 14 normal fetuses (○) and 40 fetuses with malformation of the primitive embryonic ductal plate (●). In normal fetuses the Bcl-2 score comprises the analysis in all three stages of bile duct development (DP, RDP, RBD). Replicate values for the same stage of gestation are averaged and treated as single data points. For statistical significance and linear regression analysis see text.

tive in the remodeled bile duct stage ($>50\%$ of the biliary cells) (Figure 8e). In Meckel syndrome, the expression of Bcl-2 protein was moderately to strongly positive ($>50\%$ of the biliary cells) (Figure 8d).

Moreover, we compared the linear regression models of Fas score (Figure 9) and Bcl-2 score (Figure 10) during human fetal bile duct development in normal fetuses and in fetuses with Meckel syndrome. With reference to the Fas score, two different best-fit regression lines were found for both control fetuses during normal fetal bile duct development and fetuses with Meckel syndrome. y Intercepts of the regression models (Fas score) for control fetuses and fetuses with Meckel syndrome were 11.6 ± 1.4 and 5.5 ± 1.5 , respectively. The two best-fit regression lines showed different scatters of data (control fetuses: r^2 : 0.8 and $P < 0.01$; fetuses with Meckel syndrome: r^2 : 0.2 and insignificant P value). The comparison of the Fas score in both groups was not significant, however.

With reference to the Bcl-2 score, two different best-fit regression lines were found for both control fetuses during normal fetal bile duct development and fetuses with Meckel syndrome. y Intercepts of the regression models (Bcl-2 score) were -2.5 ± 1.6 for control fetuses and 3.3 ± 1.3 for fetuses with Meckel syndrome. Both best-fit regression lines showed low scatters of data (control fetuses: r^2 : 0.7 and $P < 0.01$; fetuses with Meckel syndrome: r^2 : 0.7 and $P < 0.001$). The comparison of the Bcl-2 scored in both groups was significant ($P < 0.001$).

Repeated staining and scoring gave essentially identical results for both Fas and Bcl-2 antigens, thus confirming the reproducibility of the results (data not shown).

Discussion

Apoptosis plays a pivotal role in normal organ development, deletion of vestigial structures, control of cell numbers, and elimination of nonfunctional, abnormal, or misplaced cells. During the normal development of the intrahepatic biliary system in the human, apoptosis is important for the balance between cell proliferation and

cell elimination. The apoptotic rate and cell proliferation are high in the remodeling ductal plate stage and moderate in the ductal plate stage and in remodeled bile duct stage. This demonstrates an increased cell turnover at the remodeling ductal plate stage. In this study, we investigated the kinetic state of the malformed ductal plate in Meckel syndrome. Investigation proceeded by the TUNEL method and immunohistochemistry with antibodies to Ki-67, Fas, and Bcl-2. We used Ki-67 immunostaining according to the method of Hall et al,¹⁶ because the cellular over expression of proliferating cell nuclear antigen, an auxiliary protein for DNA polymerase δ , is not necessarily associated with actual cell proliferation. Although the fields were selected randomly by the observers, the possibility that this may have influenced the results cannot be completely ruled out. The present study revealed that the abnormal development of the intrahepatic biliary system in fetuses with Meckel syndrome shows a decrease in the apoptotic rate and Fas expression and an increase in Ki-67 and Bcl-2 expression in comparison with control fetuses.

The earliest form of bile ducts in the embryo assumes the shape of a cylinder, called the "ductal plate" by Hammar.¹⁷ Several congenital bile duct diseases are usually described as being characterized by ductal proliferation with an excess of ductal structures. First, in his published doctoral thesis, Jørgensen suggested that a lack of sufficient breakdown of the primitive embryonic ductal plate structure was an additional mechanism causing excessive bile duct cell proliferation.⁸ Such insufficient remodeling of a persistent ductal plate was termed the "ductal plate malformation." Incomplete or deficient remodeling of the ductal plates is probably due to a perturbation of the delicate, precisely timed, and complex epithelial-mesenchymal interactions.¹⁸ We found a down-regulated cell turnover with an increase in cell proliferation and a decrease in cell apoptosis in the malformed ductal plate of Meckel syndrome. Bcl-2 overexpression seems to account for a protection against various apoptotic stimuli (with the function of deleting vestigial structures).

By linear regression analysis of MIB rate in all three stages of normal bile duct development (ductal plate, remodeling ductal plate, remodeled bile ducts) of normal fetuses along the gestation, we found no correlation with the gestational age. This is in relation to the fact that two or more of the normal bile duct developmental stages may be present in the same liver specimen of normal fetuses, and overlap of the stages is a common issue. However, we have not applied to our data multiple regression models, and we cannot exclude the possibility that such an analysis could display interesting peaks and flexion points. A correlation between the apoptosis rate and the gestational age was only found for the remodeling ductal plate stage of normal bile duct development. No correlation was found in the other two stages of normal bile duct development (ductal plate, remodeled bile ducts) of normal fetuses between apoptosis rate and weeks of gestation. Interestingly, the fetuses with Meckel syndrome showed two practically horizontal best-fit regression lines for both proliferation activity and apoptosis

rate. Both proliferation activity and apoptosis rate were practically constant along the gestation. The comparison of Fas and Bcl-2 scores during human fetal bile duct development in normal fetuses and in fetuses with Meckel syndrome showed two different best-fit regression lines. However, only the comparison of the Bcl-2 score was significant (Figure 10). Other factors may be involved in altering the immunoexpression of Fas in the biliary system. The Fas (CD95/Apo-1)-Fas ligand has been recognized as a major pathway for the induction of apoptosis in cells and tissues. Because binding of Fas ligand to its receptor (Fas) initiates signal transduction pathways that result in the induction of apoptosis, studies on the Fas-ligand expression in frozen liver tissue might be needed to strengthen these data.

Fas-mediated apoptosis can be blocked by several mechanisms: 1) production of soluble Fas, 2) lack of cell-surface Fas expression, 3) overexpression of inhibitory proteins in signal transduction pathways such as Fas-associated phosphatase-1, and 4) mutation of the primary structure of Fas cDNA located at chromosome 10q24.1.¹⁹⁻²⁴ It is intriguing to postulate a molecular basis for the altered cell turnover of ductal plate malformation in Meckel syndrome. Further studies are warranted in this area. Although on different chromosomes, a relationship of the two candidate genes for Meckel syndrome localized at chromosome 17q22-q23 and 11q13 through a third gene or a positional effect may be suggested.^{25,26} It is possible that animal models may be provided in the future.

A counterpart of the ductal plate malformation is extrahepatic biliary duct atresia, with abnormal development of intrahepatic bile ducts. In a recent study, Funaki et al²⁷ demonstrated an increased cell turnover of bile ducts in 34 patients with biliary duct atresia. The frequency of both apoptotic and proliferative cells in biliary duct atresia was higher when compared to bile ducts in normal livers. Increased cell turnover in biliary structures of patients with biliary duct atresia was also considered an ongoing abnormal developmental process in the ductal plate.⁴ Recent studies support the thesis that both severe and protracted inflammatory reactions as well as a developmental aberration play a significant role in the onset and prosecution of the abnormal process.^{18,28-30} In their study, Funaki et al²⁷ studied also five surgical pathology specimens with "congenital biliary duct dilatation," in which dilatation of the extrahepatic biliary duct was also present. They found a decrease in apoptotic rate, but no increase in cell proliferation.

Our results represent the first systematic study of apoptosis in ductal plate malformation of the liver and indicate that 1) animals harboring the gene defect of Meckel syndrome could be a good model for the study of the abnormal development of the primitive intrahepatic biliary system, 2) a decreased cell turnover occurs in the ductal plate malformation of fetuses with Meckel syndrome, and 3) the increase in Bcl-2 expression contributes to the pathogenesis of the lack of remodeling of ductal plate of the liver in Meckel syndrome.

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Appendix

The following centers participated at this study and sent paraffin-embedded formalin-fixed liver blocks and pathological records of fetuses affected with Meckel syndrome: Abteilung für Paidopathologie und Placentologie, Campus Virchow-Klinikum, Humboldt-Universität, Berlin (Dir.: Prof. Dr. M. Vogel); Sektion Kinderpathologie, Pathologisches Institut, Albert-Ludwigs-Universität Freiburg (Dir.: Prof. Dr. N. Böhm); Institut für Allgemeine Pathologie und Pathologische Anatomie, Ruprecht-Karls-Universität Heidelberg (Dir.: Prof. Dr. H. F. Otto); Abteilung für Kinderpathologie, Johannes Gutenberg-Universität Mainz (Dir.: Prof. Dr. H. Müntefering); and Abteilung für Klinische Genetik, Medizinisches Zentrum für Humangenetik, Philipps-Universität Marburg (Dir.: Prof. Dr. H. Rehder).

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