Contribution of Hepatic Parenchymal and Nonparenchymal Cells to Hepatic Fibrogenesis in Biliary Atresia

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Extrahepatic biliary atresia is a severe neonatal liver disease resulting from a sclerosing cholangiopathy of unknown etiology. Although biliary obstruction may be surgically corrected by a “Kasai” hepatopancreaticoenterostomy, most patients still develop progressive hepatic fibrosis, although the source of increased collagen deposition is unclear. This study examined the role of hepatic stellate cells (HSCs) and assessed the source of transforming growth factor-β (TGF-β) production in hepatic fibrogenesis in patients with biliary atresia. Liver biopsies from 18 biliary atresia patients (including 5 pre- and post-Kasai) were subjected to immunohistochemistry for α-smooth muscle actin and in situ hybridization for either procollagen α1 (I) mRNA or TGF-β1 mRNA. Sections were also subjected to immunohistochemistry for active TGF-β1 protein. The role of Kupffer cells in TGF-β1 production was assessed by immunohistochemistry for CD68. Procollagen α1 (I) mRNA was colocalized to α-smooth muscle actin-positive HSCs within the region of increased collagen protein deposition in fibrotic septa and surrounding hyperplastic bile ducts. The number of activated HSCs was decreased in only one post-Kasai biopsy. TGF-β1 mRNA expression was demonstrated in bile duct epithelial cells and activated HSCs and in hepatocytes in close proximity to fibrotic septa. Active TGF-β1 protein was demonstrated in bile duct epithelial cells and activated HSCs. This study provides evidence that activated HSCs are responsible for increased collagen production in patients with biliary atresia and therefore play a definitive role in the fibrogenic process. We have also shown that bile duct epithelial cells, HSCs, and hepatocytes are all involved in the production of the profibrogenic cytokine, TGF-β1. (Am J Pathol 1998, 153:527–535)

Extrahepatic biliary atresia is a progressive, sclerosing, inflammatory process in neonates, causing atresia of all or part of the extrahepatic biliary system and rapidly extending to involve the major intrahepatic biliary ducts.1,2 This bile duct obliteration may be relieved by hepatopancreaticoenterostomy (HPE) or the “Kasai procedure,”3–5 in which >80% of infants will develop some biliary flow, particularly if HPE is performed within 60 days of birth (reviewed in Ref. 6). However, the majority of patients still develop progressive hepatic fibrosis, with approximately one-third developing liver failure and requiring liver transplantation within 12 to 14 months and a further one-third by the teenage years, and the remainder will live with some form of liver disease, including mild transaminase elevations, recurrent cholangitis, or an inactive cirrhosis with portal hypertension.7–9 Overall, biliary atresia accounts for up to 70% of all pediatric cases progressing to liver transplantation.10,11 Therefore, despite surgical relief of the obstruction deposition of collagen, progressive hepatic fibrosis and portal hypertension usually occur. Indeed, the development of hepatic fibrosis in this disease is more rapid and aggressive than any other disorder in adults.

The mechanisms responsible for increased collagen production and hepatic fibrosis in neonatal liver diseases such as biliary atresia are unknown. A population of nonparenchymal cells known as hepatic stellate cells (HSCs) have been shown to be “activated” and therefore responsible for the increased production of type I collagen leading to hepatic fibrosis in pathological conditions of the adult human liver,12–14 and in a number of experimental models of adult liver injury,15–20 including cholestasis.21–24 In liver injury, HSCs are transformed into myofibroblasts (activated HSCs), which produce increased levels of fibrillar collagen and express an intracellular microfilament protein, α-smooth muscle actin (SMA), which is traditionally used as a marker protein of the activated HSC phenotype (reviewed in Ref. 25). Ac-

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tivated HSCs also express a number of different cytokine receptors, including the transforming growth factor (TGF-\(\beta\)) receptor. TGF-\(\beta\) is an important profibrogenic cytokine and has been shown to increase collagen gene expression at the transcriptional level via binding of the transcription factors AP-1 and Sp-1.27,28

This study was designed to evaluate whether activated HSCs are the cellular source of increased collagen production in infants with biliary atresia and to determine the role of hepatic parenchymal and nonparenchymal cells in the expression of the profibrogenic cytokine, TGF-\(\beta\), in this age group. We were particularly interested in bile duct epithelia in view of the unique bile ductule hyperplasia seen in this disorder.

**Materials and Methods**

**Biopsy Collection and Patient Data**

Eighteen patients (6 male and 12 female) with extrahepatic biliary atresia and failed HPE were studied. Diagnosis of extrahepatic biliary atresia was confirmed in all cases at the time of HPE by histopathological evaluation, which revealed characteristic observations of portal or perilobular fibrosis, ductular proliferation, and canalicul and cellular biliary stasis. All patients were referred for liver transplantation assessment because of progressive liver disease, and orthotopic liver transplantation was performed at a mean age of 2.6 ± 0.63 years (range, 7 months to 11.75 years).

Twenty-three percutaneous liver biopsies, fixed in formalin and embedded in paraffin, were studied in these 18 patients. In 5 patients, both pre- and post-HPE biopsies were collected at a mean age of 1.8 ± 0.4 (mean ± standard error) and 8.2 ± 0.4 months, respectively. In the remaining 13 patients, liver biopsies were obtained at a mean age of 2.5 ± 0.8 years.

**In Situ Hybridization**

For detection of procollagen \(\alpha_1\) (I) mRNA, a 1500-bp fragment of human procollagen \(\alpha_1\) (I) cDNA was subcloned into pGEM 11Z vector. For detection of TGF-\(\beta_1\) mRNA, a 1000-bp fragment of human TGF-\(\beta_1\) cDNA was subcloned into pGEM-3zf(+) vector. Both fragments were then subjected to alkaline hydrolysis to produce a 300-bp fragment for use in situ hybridization.

Digoxigenin-labeled riboprobes, for sense (control) and antisense, were produced for both procollagen \(\alpha_1\) (I) and TGF-\(\beta_1\) by in vitro transcription with SP6 and T7 polymerases. *In situ* hybridization was performed on 5-μm human liver sections, deparaffinized by xylol, and rehydrated by gradient alcohol before exposure to hydrochloric acid (0.2 mol/L), as previously described.30 Sections were permeabilized with 5 μg/ml proteinase K at 37°C for 15 minutes, followed by fixation in 4% paraformaldehyde for 20 minutes at room temperature. Prehybridization (50% formamide, 1% sodium dodecyl sulfate, 5× standard saline citrate, 500 μg/ml tRNA, and 50 μg/ml heparin) was performed at 70°C for 3 hours followed by hybridization for 16 hours at 70°C in a solution containing 1 μg/ml of digoxigenin-labeled riboprobe. Sections were then washed to remove unbound probe and incubated with alkaline phosphatase-conjugated anti-digoxigenin polyclonal sera (1:200) at room temperature for 2 hours. Unbound antibody was removed by washes, followed by visualization with nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate in the dark at room temperature for 16 hours. Unbound complex was removed by washing, and sections were subjected to immunohistochemistry for SMA as previously described31 to colocalize procollagen \(\alpha_1\) (I) mRNA to activated HSCs (see Immunohistochemistry, below).

**Immunohistochemistry**

**SMA**

All liver sections were incubated with a mouse monoclonal anti-SMA primary antibody (1:400, clone 1A4; Sigma Chemical Co., St. Louis, MO), followed by a biotinylated rabbit anti-mouse immunoglobulin G as the secondary antibody, as previously described.12 The detection system used was a DAKO (Glostrup, Denmark) streptavidin-biotin complex/horseradish peroxidase kit, with 3,3-diaminobenzidine tetrahydrochloride as the chromogenic substrate. Sections were counterstained with eosin.

Biopsies were graded histologically for SMA expression as previously described24 using the following classification: 0, normal staining pattern for SMA with expression in smooth muscle cells within portal blood vessels only; 1+, mild perisinusoidal staining for SMA within activated HSCs; 2+, perportal staining for SMA, proliferation of SMA-expressing HSCs, and moderate SMA expression in perisinusoidal HSCs; 3+, septal and bridging SMA expression between portal tracts; and 4+, SMA expression within cirrhotic bands linking portal tracts.

**TGF-\(\beta\)**

All liver sections were subjected to antigen retrieval by heating in a microwave oven on high power for 8 minutes in 0.01 mol/L citrate buffer (pH 6.0) and then incubated with a mouse monoclonal anti-TGF-\(\beta_1\), \(\beta_2\), and \(\beta_3\) primary antibody to active TGF-\(\beta\) (150 μg/ml; Genzyme Diagnostics, Cambridge, MA) for the cellular localization of TGF-\(\beta\) protein. The sections were then subjected to the identical detection methodology as for SMA.12

**CD68**

All liver sections were subjected to antigen retrieval by autoclaving in 0.01 mol/L citrate buffer (pH 6.0) at 121°C for 10 minutes. Immunohistochemistry for CD68, a specific marker for Kupffer cells, was performed by incubating sections with a mouse monoclonal anti-CD68 primary antibody (1:50, clone PG-M1; DAKO), followed by identical detection methodology as described for SMA.12 This technique allowed assessment of Kupffer cells as a po-
tential source of TGF-β, mRNA in the livers of patients with biliary atresia.

The negative controls used for each immunohistochemical assessment used nonimmune normal mouse immunoglobulin G antisera (Santa Cruz, San Diego, CA) in place of the primary antibody for either SMA, TGF-β, or CD68 (results not shown).

Hematoxylin/Van Gieson Histology

All sections were subjected to hematoxylin/Van Gieson stain for the detection of collagen protein deposition.

Results

Hepatic Histopathological Assessment of Biliary Atresia

There was evidence of canaliculacinar and cellular biliary stasis, variable inflammatory changes, bile duct hyperplasia with expanded portal tracts, periportal and bridging fibrosis, and mild to severe cirrhosis in all biopsies examined, including pre- and post-Kasai HPE livers.

Identification of Activated HSCs: Cellular Source of Procollagen α1 (I) mRNA Expression

Liver biopsies were subjected to immunohistochemistry for the intracellular micromlant protein, SMA, which has been shown to be an excellent marker for the activated HSC phenotype. Activated HSCs were demonstrated morphologically both by their stellate shape and by the expression of SMA (Figure 1A) in the extracellular matrix surrounding hyperplastic bile ducts and within fibrous septa bridging between portal tracts (Figure 1B). Furthermore, procollagen α1 (I) mRNA expression was shown to colocalize to SMA-positive HSCs (Figure 1, A and B), demonstrating that activated HSCs are the cellular source of increased collagen leading to hepatic fibrosis in biliary atresia. Procollagen α1 (I) mRNA expression was not seen in hepatocytes, bile duct epithelial cells, or smooth muscle cells of the portal tract vasculature. Procollagen α1 (I) mRNA signal specificity for the antisense probe was demonstrated by the absence of signal over SMA-expressing stellate cells using the sense probe (results not shown).

Pre- and Post-Kasai HPE

Five patients were examined both pre- and post-Kasai HPE for evidence of HSC activation. Only one of five patients showed a decrease in the expression of SMA (grade 4+ to 2+) and hence in the number of activated HSCs surrounding hyperplastic bile ducts and within fibrous bridging septa after HPE (Figure 1, C and D). All five of these patients subsequently progressed to liver transplantation.

Colocalization of Activated HSCs and Increased Collagen Protein Deposition

Liver biopsies were examined histologically for collagen protein deposition using hematoxylin/Van Gieson stain. Figure 2A demonstrates grossly enlarged bile ducts surrounded by excessive collagen protein deposition. Figure 2B demonstrates increased numbers of activated HSCs showing colocalization of SMA and procollagen α1 (I) mRNA in the identical region of increased collagen protein deposition. Elevated numbers of procollagen α1 (I) mRNA-expressing, activated HSCs (Figure 2D) were also demonstrated in the identical region of collagen protein deposition within fibrous tissue between portal tracts (Figure 2C).

Cellular Source of the Profibrogenic Cytokine, TGF-β

Immunohistochemistry for TGF-β

Immunohistochemistry for TGF-β protein revealed that TGF-β was predominantly expressed by bile duct epithelial cells within hyperplastic bile ducts and also by activated HSCs in the extracellular matrix of scar tissue (Figure 3A). TGF-β was also expressed to a lesser extent in hepatocytes in close proximity to areas of fibrosis at the interface of the regenerative nodule (Figure 3B). TGF-β expression was not evident in hepatocytes at a distance from scar tissue (results not shown).

In Situ Hybridization for TGF-β

In situ hybridization for TGF-β, mRNA demonstrated that TGF-β1 mRNA was expressed in bile duct epithelial cells within hyperplastic bile ducts (Figure 3, C and E) and was also observed colocalized to SMA-positive HSCs (Figure 3C). Increased expression of TGF-β1 mRNA was also demonstrated in hepatocytes along the interface of the regenerative nodules and fibrotic scar tissue (Figure 3D). TGF-β1 mRNA was not detected in hepatocytes within the acinus distal from scar tissue (results not shown).

Role of Kupffer Cells in TGF-β Production

Figure 3E demonstrates the localization of increased numbers of Kupffer cells as assessed by CD68 immunohistochemistry, in sinusoidal and perisinusoidal regions of the regenerative hepatocyte nodule, and within scar tissue. TGF-β1 mRNA expression was not detected in CD68-positive Kupffer cells in close proximity to the interface of the fibrotic scar tissue, indicating that Kupffer cells may not contribute to the TGF-β1 mRNA expression seen in Figure 3D. In addition, CD68-positive cells within the scar tissue did not demonstrate colocalization of TGF-β1 mRNA, and therefore, these cells do not appear to play a role in collagen production by HSCs surrounding bile ducts. TGF-β1 mRNA signal specificity for the antisense probe was demonstrated by the absence of...
signal over bile duct epithelial cells, hepatocytes, and HSCs using the sense probe (Figure 3F).

**Discussion**

This study has demonstrated that activated HSCs, identified by the colocalization of procollagen \( \alpha_1 \) (I) mRNA expression to cells expressing the HSC activation marker, SMA, are responsible for the production of increased levels of type I collagen leading to hepatic fibrosis in young patients with biliary atresia. In addition, this study has shown that the hyperplastic bile duct epithelium is the predominant source of the profibrogenic cytokine TGF-\( \beta_1 \), within the portal tract and that hepatocytes produce increased levels of TGF-\( \beta_1 \) along fibrous septa bridging portal tracts, which forms the fibrotic scar leading to cirrhosis. TGF-\( \beta_1 \) was also produced by activated HSCs within the fibrous matrix but to a lesser degree than other cells. Finally, this study has demonstrated that the number of activated HSCs was decreased in only one of five patients after Kasai HPE.

Many different theories have been proposed to explain the pathogenesis of biliary atresia, including infectious, genetic, and immune-mediated etiologies, although convincing evidence to support these hypotheses is lacking (reviewed in Refs. 6 and 32). Furthermore, there is a

**Figure 1.** Identification of activated HSCs as cellular source of increased collagen production in biliary atresia. **A:** Colocalization of SMA (brown) and procollagen \( \alpha_1 \) (I) mRNA (blue) within stellate-shaped activated HSCs in a liver biopsy from an infant with biliary atresia, using immunohistochemistry and in situ hybridization, respectively. Original magnification, \( \times 400 \). **B:** Bile duct hyperplasia within fibrotic bands in a liver biopsy from an infant with biliary atresia. Intense staining for SMA (brown) and procollagen \( \alpha_1 \) (I) mRNA (blue), colocalized in activated HSCs. Original magnification, \( \times 400 \). **C:** Immunohistochemistry for SMA (brown) within activated HSCs surrounding hyperplastic bile ducts in a pre-Kasai HPE. **D:** A post-Kasai HPE liver biopsy from the same infant shown in **C** with biliary atresia. Original magnification, \( \times 100 \).
paucity of knowledge concerning the mechanisms involved in the fibrogenesis associated with this condition. In a recent study, Malizia and colleagues examined five patients with advanced biliary atresia and cirrhosis and showed increased expression of procollagen mRNA associated with "spindle-shaped fibroblast-like cells" in the fibrous tissue surrounding regenerative hepatocyte nodules and some proliferating bile ductules. The identification of the responsible cell type was not established in this study, although the cells were described as "vimentin-positive mesenchymal cells," which could describe either Kupffer cells, endothelial cells, or ductal plate or biliary epithelial cells. These authors also described the collagen-producing cells as desmin negative, suggesting that HSCs may not be the major cell type involved in fibrogenesis, based on a previous report that identified human HSCs as desmin-positive cells. However, the literature on desmin reactivity of human HSCs is conflicting. Other studies have shown that the detection of desmin in human HSCs, either in vitro or in vivo, is quite variable and often unsuccessful. Our study, however, clearly documents the identification of activated HSCs, as evidenced by both SMA expression and cell morphology, as the cellular source of in-

Figure 2. Colocalization of activated HSCs to collagen protein deposition in biliary atresia liver. A: Collagen protein deposition surrounding two bile ducts in liver biopsy from an infant with biliary atresia (pink). B: Activated HSCs surrounding bile ducts, showing colocalization of SMA (brown) and procollagen α1 (I) mRNA (blue). Original magnification, ×1000. C and D: Fibrotic region in liver biopsy from an infant with biliary atresia showing deposition of collagen protein fibrils (C, pink) and activated HSCs (D), as evidenced by colocalization of SMA (brown) and procollagen α1 (I) mRNA (blue). Original magnification, ×200.
increased procollagen α₁ (I) mRNA in extrahepatic biliary atresia.

The hepatic histopathological presentation of biliary atresia is classically characterized by ductular proliferation, canalicular and cellular biliary stasis, swelling and vacuolization of biliary epithelial cells, portal tract edema and fibrosis, and monocytic inflammatory cell infiltration of portal tracts. Although the mechanisms responsible for many of these phenomena are not known, portal fibrosis and cirrhosis are arguably the most damaging and have the greatest prognostic significance. It is now clear that activated HSCs are responsible for the increased production of type I collagen leading to hepatic fibrosis in biliary atresia similar to that of pathological conditions of...
the adult liver\textsuperscript{12–14} and in experimental models of cholestatic liver injury.\textsuperscript{21–24} The factors that are responsible for initiating the activation of HSCs are unclear, although it has been established that the profibrogenic cytokine, TGF-\(\beta\), and the proliferative cytokine, platelet-derived growth factor, are involved in perpetuating the activated HSC phenotype (reviewed in Ref. 14).

In the present study we have demonstrated that the bile duct epithelium is a major source of TGF-\(\beta\) in biliary atresia as evidenced by immunohistochemistry for active TGF-\(\beta\) protein and increased expression of TGF-\(\beta\) mRNA. We have also shown that activated HSCs surrounding hyperplastic bile ducts produce both TGF-\(\beta\) protein and mRNA. However, our results suggest that bile duct epithelial cells may be the predominant source of the TGF-\(\beta\) responsible for increased transcription of collagen type I genes in HSCs surrounding bile ductules leading to periductular fibrosis. Few previous studies have examined the cellular source of cytokine production in neonatal biliary obstruction. Milani and colleagues\textsuperscript{40} demonstrated increased TGF-\(\beta\) mRNA in biliary epithelial cells and low levels of TGF-\(\beta\) transcripts in hepatocytes, mesenchymal cells, and some inflammatory cells in bile duct-ligated adult rats. Others have observed a significant increase in TGF-\(\beta\) immunohistochemistry in bile duct epithelium after ligation of the common bile duct.\textsuperscript{41}

In biliary atresia, Tan and colleagues\textsuperscript{42} demonstrated increased TGF-\(\beta\) peptide immunoreactivity within bile duct structures at the porta hepatitis and within intrahepatic portal tracts, whereas Malizia and colleagues\textsuperscript{33} demonstrated TGF-\(\beta\) protein associated with the extracellular matrix in fibrous septa and in areas of periductular fibrosis. In addition, this group examined TGF-\(\beta\) mRNA expression and demonstrated increased message in scar tissue and associated with proliferating bile ductules at the interface of the regenerative nodules and the scar, although the precise cellular source of this TGF-\(\beta\) mRNA was not clearly defined.\textsuperscript{33} They also found that bile ductules expressed increased levels of platelet-derived growth factor-A and -B mRNA.

We have also demonstrated increased expression of both TGF-\(\beta\) mRNA and active TGF-\(\beta\) protein by hepatocytes at the interface of the regenerative nodules and fibrous septa forming fibrotic and cirrhotic bands. We propose that the production of TGF-\(\beta\) at this interface may be intimately involved in the induction of collagen gene transcription by activated HSCs due to the close histological association between these two cell populations. Only one other study has previously observed TGF-\(\beta\) protein expression by hepatocytes in biliary atresia, although it is unclear whether this represented active TGF-\(\beta\).\textsuperscript{42}

Our study did not demonstrate a role for Kupffer cells in TGF-\(\beta\) production in biliary atresia. Although we demonstrated evidence of increased numbers of perisinusoidal CD68-positive macrophages within both regenerative hepatocyte nodules and scar tissue, Kupffer cells did not demonstrate TGF-\(\beta\) mRNA expression. Others have previously demonstrated hepatic Kupffer cell proliferation and monocyte migration to the liver in biliary atresia\textsuperscript{43,44} and bile duct-ligated rats.\textsuperscript{45} Tracy and colleagues have also shown increased numbers of resident CD68-positive Kupffer cells that also express CD14, which confers susceptibility to activation by low doses of lipopolysaccharide.\textsuperscript{43} We did not show increased TGF-\(\beta\) expression by Kupffer cells, although they may take part in the local inflammatory response by releasing other cytokines such as tumor necrosis factor-\(\alpha\), interleukin-1, and interleukin-6.\textsuperscript{43,46}

Although the present study clearly implicates activated HSCs in the fibrogenic process and bile duct epithelial cells and hepatocytes in the production of the profibrogenic cytokine, TGF-\(\beta\), the mechanisms involved in HSC and bile duct proliferation and the induction of TGF-\(\beta\) remain elusive. Bile duct hyperplasia appears to be an early event in cholestatic liver injury, and some groups have suggested that in the bile duct-ligated rat, this may result from an increase in intraductal pressure.\textsuperscript{47} Others suggest that circulating cholangiotrophic factors released from the liver in cholestasis may induce the bile duct proliferative response.\textsuperscript{48} Supporting evidence is derived from studies that have demonstrated that the proliferation of liver epithelial cells appears to accompany the increased hepatic expression of the growth-related proto-oncogenes, such as c-ras and c-erb-B2\textsuperscript{49} and H-ras and c-myc\textsuperscript{49} in bile duct-ligated rats. More recent mechanistic hypotheses center on the injurious effect of hydrophobic bile acids on specific cell populations (reviewed in Ref. 6). Some groups have demonstrated increased levels of chenodeoxycholic acid in human cholestatic liver disease,\textsuperscript{50} and others have reported the hepatotoxic effects of hydrophobic bile acids.\textsuperscript{51,52} Varying the dose of chenodeoxycholic acid in vitro has been shown to induce either hepatocyte necrosis or apoptosis,\textsuperscript{53,54} which may in turn alter mitochondrial function through the generation of oxygen free radicals.\textsuperscript{51,55} Cholestatic hepatotoxicity may also be induced via the depletion of hepatic or mitochondrial antioxidants, such as vitamin E and glutathione.\textsuperscript{56,57} Thus, it has been proposed that oxidant stress may play a major role in the induction of hepatocellular injury by bile acids such as chenodeoxycholic acid in cholestatic liver disease.\textsuperscript{6,51,55} Hydrophobic bile acids and oxidant stress may also directly alter Kupffer cell or HSC viability and function. The result of any of these scenarios may be the induction of cytokine expression by injured hepatocytes or activated Kupffer cells, which may ultimately lead to the activation of HSCs and fibrogenesis. Additional investigations will be required to fully elucidate the association between increased biliary levels of hydrophobic bile acids, hepatocellular injury, and HSC activation in patients with biliary atresia.

The results of the present study suggest that important interactions exist between different hepatic cell populations, and these interactions are essential in the fibrogenic response in biliary atresia. In summary, this study has provided evidence that activated HSCs are responsible for increased collagen production in biliary atresia and are therefore involved in the development of hepatic fibrosis. This study has also shown that the profibrogenic cytokine, TGF-\(\beta\), is predominantly produced by bile duct...
epithelial cells and to a lesser extent by hepatocytes and activated HSCs. Although the results presented here have demonstrated the potential for bile duct epithelial cell-derived TGF-β to induce collagen production by periductular activated HSCs, the initiating stimulus to bile duct injury and the role of hydrophobic bile acid hepatotoxicity remains the subject of future investigation.

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