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Dexamethasone Alters the Hepatic Inflammatory Cellular Profile Without Changes in Matrix Degradation During Liver Repair Following Biliary Decompression

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Abstract

Background—Biliary atresia is characterized by extrahepatic bile duct obliteration along with persistent intrahepatic portal inflammation. Steroids are standard in the treatment of cholangitis following the Kasai portoenterostomy and were liberally adopted advocated for continued suppression of the ongoing immunological attack against intrahepatic ducts. Recent reports however have failed to demonstrate an improved patient outcome or difference in the need for liver transplant in postoperative patients treated with a variety of steroid regimes compared to historical controls. In the wake of progressive liver disease despite biliary decompression steroids are hypothesized to suppress inflammation and promote bile flow without any supporting data regarding their effect on the emerging cellular and molecular mechanisms of liver repair. We have previously shown in a reversible model of cholestatic injury that repair is mediated by macrophages, neutrophils and specific matrix metalloproteinase activity (MMP8); we questioned whether steroids would alter these intrinsic mechanisms.

Methods—Rats underwent biliary ductal suspension for 7 days followed by decompression. Rats were treated with IV dexamethasone or saline at the time of decompression. Liver tissue obtained at the time of decompression or after 2 days (D2) of repair was processed for morphometric analysis, immunohistochemistry, and quantitative RT-PCR.

Results—There was a dramatic effect of dexamethasone on the inflammatory component with the initiation of repair. Immunohistochemistry revealed a reduction of both ED1+ hepatic macrophages and ED2+ Kupffer cells in repair when compared to saline controls. Dexamethasone treatment also reduced infiltrating neutrophils by D2. TNF- α expression, increased during injury in both saline and dexamethasone groups, was markedly reduced by dexamethasone during repair (D2) whereas IL-6, IL-10 and CINC-1 remained unchanged when compared to saline controls. Dexamethasone reduced both MMP8 and TIMP1 expression by D2, whereas MMP9, 13, and 14 were unchanged when compared to sham controls. Despite substantial cellular and molecular changes during repair, collagen resorption was the same in both groups

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Conclusion—Dexamethasone has clear effects on both the hepatic macrophage populations and infiltrating neutrophils following biliary decompression. Altered MMP and TIMP gene expression might suggest that steroids have the potential to modify matrix metabolism during repair. Nevertheless, successful resorption of collagen fibrosis proceeded presumably through other MMP activating mechanisms. We conclude that steroids do not impede the rapid intrinsic repair mechanisms of matrix degradation required for successful repair.

INTRODUCTION

Biliary atresia is characterized by extrahepatic bile duct obliteration along with persistent intrahepatic portal inflammation. Several hypotheses have proposed various etiologies that can broadly be considered congenital or acquired. Reports have pointed at faulty ductal development during embryogenesis, abnormal bile acid metabolism, ischemia and viral infection as possible causes. (1,2) Despite the multiple theories, the exact etiology still remains unknown. Biliary atresia is generally considered an acquired condition with potential immunologic, inflammatory, infectious and abnormal developmental pathway contributing to the progressive destruction of the extra- and intrahepatic biliary tree leading to hepatic fibrosis. (3–9)

The Kasai portoenterostomy was developed theoretically to address the extrahepatic obstruction and restore bile flow. Despite providing a conduit for bile, the long-term success of the Kasai procedure is diminished by ongoing progression of the intrahepatic cholangiopathy and fibrosis leading to hepatic failure and the need for liver transplantation. Medical therapies including steroids have been advocated to ameliorate the continuing immunologic, inflammatory, and infectious components of the disease despite biliary decompression. The use of steroids has become an almost universal part of the management of post Kasai cholangitis. (10,11–14) Due to the success of liberal steroid use in many early and subsequent studies of cholangitis, their potential application for all patients with biliary atresia has been proposed and even tested in a limited trial. They are hypothesized to suppress the immunologic attack on intrahepatic biliary epithelia, depress the secondary inflammatory cascade, and promote bile flow. Steroid use following Kasai portoenterostomy is not without controversy and their use has come under scrutiny recently as two reports recently demonstrated no significant difference in progressive liver disease and the need for liver transplantation. (11, 14) Moreover, all this has been proposed without any supporting data regarding their effect on the emerging knowledge of the cellular and molecular mechanisms necessary for intrinsic liver repair that would be essential for restoration of hepatic function and architecture following biliary decompression via the Kasai portoenterostomy. Clinical experience as evidenced by successful Kasai procedures and experimental data suggest that there is a critical time period during which restoration of bile flow can halt or reverse fibrosis, indicating an intrinsic capacity for repair. Successful liver repair must be associated with removal of collagen matrix and restoration of normal hepatic architecture, hepatocyte gene expression and metabolic function. Due to limited models that recapitulate the inflammatory and fibrogenic consequences of biliary obstruction and decompression there have been few studies examining the cellular and molecular mechanisms that control hepatic matrix metabolism and net resorption. Moreover, few models, including ours, accurately reproduces biliary atresia secondary to the poorly defined multifactorial etiology however, using a reversible model of bile duct obstruction which induces cholestatic injury and simulates biliary decompression, we have previously demonstrated that repair is mediated by hepatic macrophages, neutrophils and the finding of specific matrix metalloproteinase activity (MMP-8). (15,16) In this study we sought to address the resolution of hepatic fibrosis following biliary decompression and questioned whether steroids would alter these intrinsic mechanisms.

METHODS

Animals

Adult male rats (225–250g, Harlan Sprague-Dawley, Indianapolis, IN) were housed in an artificial 12-hour light-dark cycle with access to rat chow and water *ad libitum* according to the NIH publication *Guide for the Care and Use of Laboratory Animals*. Experiments were carried out in compliance with guidelines prescribed by the Institutional Animal Care and Use Committee of Rhode Island Hospital and The Alpert Medical School of Brown University.

Biliary obstruction (liver injury)

Animals (n=45) were divided into saline controls (operated; n=12 and sham; n=7) or Dexamethasone treated (operated; n=16 or sham; n=10) groups. The saline and Dexamethasone-treated operated animals underwent bile duct loop suspension surgery for biliary obstruction as previously described.(15,16,18,19) Briefly, animals were anesthetized with vaporized isoflurane, weighed and prepared for surgery. A midline laparotomy was performed and the common bile duct was dissected sufficiently to allow passage of a 5cm length of silicone vessel loop (Surg-I-Loop, Scanlan International, Saint Paul, MN). A mid-segment of this loop was pre-marked to 1cm and the ends were brought through each side of the abdominal wall, 1cm lateral to the midline at the costochondral margin. The vessel loop was stretched and sutured to the perichondrium bilaterally at the pre-marked points displacing the common bile duct ventrally. Bile duct obstruction was verified and the abdomen was closed. Sham-operated animals underwent an identical laparotomy where the common bile duct was identified, but not obstructed.

Biliary decompression (liver repair)

Following 7 days of bile duct obstruction, rats were anesthetized, weighed and prepared for decompression/repair. The skin of the midline incision was opened exposing the vessel loop. The anchoring sutures were cut and the vessel loop removed allowing the bile duct to decompress, re-establishing bile flow. Dexamethasone (n=16) or saline (n=12) was injected intravenously as follows. Treated animals received intravenous dexamethasone or saline at the time of decompression and at day two of repair. Dexamethasone (American Reagent Laboratories, Inc, Shirley, NY) (4mg/ml) was diluted 1:100 in 0.9% normal saline to a concentration of 40 µg/ml. A dose of 7.2 µg/100g was administered through the penile vein. A corresponding volume of 0.9% injectable normal saline was similarly administered to the saline group. The kinetics of Dexamethasone dosing was previously reported by Melgert et al. (17) This was the determined dose that effectively reduced TNF- α release from endotoxin-activated Kupffer cells in vitro and in vivo when targeted against rat hepatic endothelial and Kupffer cells. Moreover, Dexamethasone was the glucocorticoid of choice based on its accepted use in the treatment of premature babies with chronic lung disease. Its short-term tolerance and safety profile are well known. It is 5 to 6 times as potent as prednisolone with respect to its anti-inflammatory potential with a longer half life and is rapidly and well absorbed. A comparative dose of prednisolone requires a prohibitive dose of 4mg/kg/d.(13)

The abdominal skin was closed and the animals were allowed to recover. This procedure is referred to as decompression and the time point as day 0 of repair (Fig. 1).

Necropsy

Two time points were chosen for necropsy analysis. Our previous studies revealed that 7 days of biliary obstruction achieved ~66% of maximal fibrosis observed at 21 days which resolved to near sham levels by 2 days after decompression. (15,16) Animals were necropsied following 7 days of bile duct obstruction leading to cholestatic injury (day 0 of repair) or 2 days following

bile duct decompression to initiate intrinsic repair (day 2 of repair); all sham-operated groups were necropsied on day 0 of repair i.e. (after 7 days of bile duct obstruction) (Fig. 1). Necropsy was performed 6 hours after the Dexamethasone or saline injection on both days. Rats were anesthetized and weighed, the abdomen was opened with a U-shaped incision and the abdominal wall was reflected superiorly. Blood and serum (5–10ml) was collected in K2 EDTA and clotting enhancement collection tubes, respectively, by venipuncture of the inferior vena cava. Samples were sent to Marshfield Laboratories (Marshfield, WI) for the independent determination of Complete Blood Count with differential, liver enzymes and bilirubin. Hepatectomy was performed and livers were divided and processed as follows: frozen with dry ice in O.C.T. Compound (Sakura, Torrance, Ca); flash frozen in liquid nitrogen; fixed in 10% neutral phosphate buffered formalin (Fisher Scientific, Fair Lawn, NJ); or fixed in formalin free zinc fixative (BD Pharmingen, San Diego, Ca). Weight, bilirubin and liver enzymes were utilized as markers of the progression of cholestatic injury and subsequent repair. Animals that did not demonstrate obstruction or decompression were excluded from the study.

Inflammatory Cell Identification and Quantification

Formalin- and zinc-fixed tissues were embedded in paraffin and sectioned at 7µm. Inflammatory cells were identified for quantification using both histochemistry, naphthol AS-D chloroacetate esterase (esterase, Sigma-Aldrich Co.) specific for granulocytes,(20) and immunohistochemistry, mouse anti-rat CD68 monoclonal antibody (ED1+, Serotec Inc., Raleigh, NC) specific for the majority of macrophages(21) and mouse anti-rat CD163 monoclonal antibody (ED2+, Serotec Inc.) specific for Kupffer cells.(21,22) Liver sections, stained for ED1, ED2 or esterase, were digitally imaged at 400X with a minimum of ten fields per section and every stained cell was counted within the field. Results are expressed as cells per mm².

Matrix proteins, primarily collagen type I, were identified and quantified using Sirius red stained images. Sections were scanned (SprintScan 35 Plus, Polaroid, Cambridge, Ma) into a PowerMac G4 (Apple computer, Inc. Cupertino, Ca) using a PathScan Enabler optical card (Meyer Instruments, Inc. Houston, TX). NIH ImageJ imaging software (NIH, Bethesda, MD) was used to determine total tissue section area and collagen area. Results are expressed as a percentage of total section area.

Real-time RT-PCR

Total RNA was extracted with the RNeasy Mini Kit (Qiagen, Inc., Valencia, Ca) from liver samples flash frozen in liquid nitrogen. RNA quality and quantity were determined using the RNA 6000 Nano LabChip kit on the 2600 BioAnalyzer (Agilent Technologies, Santa Clara, Ca). Five micrograms of total RNA was converted into cDNA using First Strand cDNA Synthesis kit (GE Healthcare Life Sciences, Piscataway, NJ) with random primers. The Quantitect SYBR Green real-time PCR kit (Qiagen, Inc., Valencia, Ca) was used for all quantitative PCR and run on the Stratagene MX4000 thermocycler. Primer sequences (Table 1) were chosen using appropriate GENBANK sequences, NCBI (www.ncbi.nlm.nih.gov) and Primer 3 software (Whitehead Institute for Biomedical Research: Source code available at <http://fokker.wi.mit.edu/primer3/>).(23) Sequences were purchased from Integrated DNA Technologies, Inc. Melting curves validated the utility and specificity of each primer set. Data were evaluated using the Comparative Ct Method ($2^{-\Delta\Delta C_t}$) of relative quantification standardized to 18S rRNA and are reported as fold increases over sham controls. Because of the relative abundance of 18S rRNA, standardizing samples were diluted 1:1000. The results were equivalent to those obtained using GAPDH as an alternate reference gene (data not shown).

Statistics

Data are expressed as means $\pm$ standard error of the mean and were analyzed by ANOVA and Fisher's PSLD (Stat-View 4.1+, SAS Institute Inc.). $P < 0.05$ was considered significant.

RESULTS

Measuring injury and repair

Our unique rat model of reversible biliary fibrotic cholestatic injury was achieved by bile duct obstruction for seven days via bile duct suspension followed by relief of obstruction by release of the occluding band. As previously reported, (15,16) compared to the sham operated controls, the obstructed animals developed the typical cholestatic injury pattern, which mimicked the human condition. Transaminase and bilirubin levels increased over the period of obstructive injury and rapidly decreased to sham levels after relief of obstruction and return of bile flow. Furthermore, the cholestatic injured animals demonstrated signs of systemic illness as evidenced by weight loss during bile duct obstruction that returned toward baseline following return of bile flow after relief of obstruction. Most importantly, compared to sham operated controls, the bile duct obstructed rats rapidly developed collagen fibrosis during injury, which improved after reversal of bile duct obstruction and restoration of bile flow. (Data not shown). (15,16,18)

Inflammatory cell infiltrate

Bile duct obstruction induced a pattern of liver injury characterized by an inflammatory cell infiltrate that included monocytes and neutrophils. In response to dexamethasone, there was a dramatic effect on the inflammatory cellular component at the initiation of repair. Immunohistochemistry revealed a reduction of both ED1+ hepatic macrophages (Fig. 2A) and ED2+ Kupffer cells (Fig. 2B) in repair when compared to saline controls. Dexamethasone markedly reduced ED2+ Kupffer cell counts after two days of biliary decompression-initiated repair. Similarly, ED1+ infiltrating macrophages were significantly reduced following two days of repair but dexamethasone treatment also markedly reduced the ED1+ hepatic macrophages at the time of biliary decompression suggesting that dexamethasone had an impact on infiltration and or clearance of the macrophages in response to injury. A unique feature of the neutrophil response to bile duct cholestatic injury that differs from the ED1+ and ED2+ response is the persistence of the neutrophils at the site of injury during the repair phase following reversal of bile duct obstruction and restoration of bile flow. Dexamethasone markedly reduced the neutrophil cell count after two days of repair. (Fig. 2C).

Cytokine response to dexamethasone

In response to bile duct obstruction, it is hypothesized that the resident hepatic macrophages, the Kupffer cells, play a central role and mediate the cytokine and chemokine response to regulate physiologic hepatocellular function. Kupffer cell activation leads to upregulation of multiple proinflammatory cytokines to commence recruitment of inflammatory cells and initiate downstream activation of hepatic stellate cells leading to deposition of extracellular matrix collagen fibrosis. Compared to sham operated rats, gene expression of TNF- α was markedly upregulated in response to cholestatic injury secondary to bile duct obstruction and remained elevated during repair despite biliary decompression and bile flow. (Fig. 3A) Conversely, dexamethasone treated rats demonstrated a profound reduction in hepatic TNF- α expression at two days of repair following reversal of biliary obstruction. In opposition to the response of TNF- α to dexamethasone, IL-6 (Fig. 3B) and IL-10 (Fig. 3C) gene expression did not change from sham-operated controls. There was also no difference in the hepatic gene expression of IL-6 or IL-10 during cholestatic injury or following reversal of bile duct obstruction in repair. The neutrophil chemokine, CINC-1 gene expression was markedly

upregulated in response to biliary obstruction and similar to the neutrophil findings, remained elevated following biliary decompression compared to controls. (Fig. 3D) Importantly, the hepatic CINC-1 gene expression was unchanged by dexamethasone treatment which paralleled the untreated animal expression.

The effect of dexamethasone on the extracellular matrix

Matrix metalloproteinases (MMP's) are zinc-dependent endoproteinases known to digest components of the extracellular matrix in substrate-specific fashion and are primarily regulated by tissue inhibitors of matrix metalloproteinases (TIMP's). In response to bile duct obstructive cholestatic injury, only MMP-8 (Fig. 4A) and TIMP-1 (Fig. 5A) were affected by dexamethasone treatment. Gene expression of the two most prominent gelatinases, MMP-2 and MMP-9 (Fig. 4B, C) was unchanged during cholestatic injury and following biliary decompression. Membrane-associated MMP-14 (not shown) and the collagenase MMP-13 (Fig. 4D) were similarly unchanged. Moreover, dexamethasone treatment had no appreciable change in respective gene expression. In contrast, following bile duct obstruction there was an 8 to 10 fold increase in MMP-8 (neutrophil collagenase) gene expression that remained elevated following decompressive repair. MMP-8 hepatic gene expression was decreased in dexamethasone treated rats during repair. Following seven days of bile duct obstruction, both TIMP-1 and TIMP-2 gene expression were elevated. (Fig. 5A, B) However, while TIMP-2 expression rose 4 to 5 fold in response to injury and remained elevated after decompression compared to sham-controls, TIMP-1 gene expression was significantly elevated in response to injury by 30 fold compared to sham-controls. Following biliary decompression, there was a marked reduction in TIMP-1 expression toward sham levels after two days of repair. Dexamethasone treated animals demonstrated even further reductions of TIMP-1 expression during repair. Despite substantial cellular and molecular changes during repair in response to dexamethasone treatment, collagen resorption was equal in both groups. (Fig. 6).

DISCUSSION

Despite improvements in the surgical management of biliary atresia, the long-term incidence of progressive liver failure remains high. Although the etiology of neonatal biliary atresia is not known, the disease is characterized by a progressive sclerosing and inflammatory process. Liver biopsy in biliary atresia classically shows bile duct proliferation, canalicular stasis, swelling and vacuolization of bile duct epithelial cells, portal tract edema and fibrosis and monocytic and lymphocytic cell infiltration of the portal tracts. (10) A number of cellular inflammatory markers have been studied including CD14-positive macrophages in the monocytic infiltrate, which, when activated by endotoxin, secrete a number of inflammatory cytokines into the periductular tissue. (6) Expression of intracellular adhesion molecule-1 (ICAM-1) by the bile duct epithelium may play a role in the recruitment of lymphocytes. (24) Since chronic inflammation including CD4+ and CD8+ T-lymphocytic infiltration of both bile ducts and liver parenchyma contributes to the pathology, many have hypothesized that the liver damage may be altered using immunosuppressive therapy. Clinically, Muraji and Higashimoto (7) reported the use of steroids as an adjuvant therapy after Kasai portoenterostomy and Dillon et al (25) were the first to advocate long-term high-dose steroids and achieved impressive jaundice-free 4 year results in 19 of 25 patients (76%). Currently, most surgeons use postoperative steroids in biliary atresia patients. The clinical use of steroids in post-Kasai patients has exploded following the publication of a series of reports attesting to the better outcomes with routine postoperative steroid therapy compared to a variety of historical controls. (7,10,25) However the route, dose, duration and general use of steroids following the Kasai procedure remains highly controversial and all authors agree on the need for a well-conducted prospective, randomized controlled trial. The accepted hypothesis that steroid use would be beneficial to ameliorate the progressive injury rests on its anti-inflammatory

properties targeting infiltrating neutrophilic and cytotoxic T-cell-mediated biliary epithelial injury. Importantly, there is little to no supporting data regarding the effect of steroids on the cellular and molecular mechanism during intrinsic repair. The intention of the scope of our work presented here was not to support or refute the clinical use of steroids following the Kasai portoenterostomy, but rather to potentially provide further scientific justification of their effect on resolution of extracellular matrix collagen fibrosis after biliary decompression.

Steroids have been used to augment antibiotic treatment of refractory cases of postoperative cholangitis in patients with biliary atresia for more than 20 years. The two most cited reasons for steroids relate to its potential choleric and antiinflammatory properties. The choleric effect of steroids involves induction of the Na⁺-K⁺ ATPase, which increases canalicular electrolyte transport and stimulates bile flow independent of the bile salt concentration. In high doses, steroids have pronounced antiinflammatory and immunosuppressive properties decreasing edema and collagen deposition, inhibiting scarring, and arresting migration of infiltrating monocytes and lymphocytes. They have a broad range of specific immune response mediated by T cell and B cells as well as suppressive effect on the effector function of monocytes, macrophages, and neutrophils. Once bound to its ligand, the glucocorticoid-receptor complex is translocated to the nucleus where it binds to promoter regions of genes susceptible to steroid regulation. Glucocorticoids inhibit synthesis of many proinflammatory cytokines by inhibiting the transcription factor for nuclear factor kappa B (NF- κ B) and activator protein-1 (AP-1). Our results are in agreement with the conventional understanding of steroid action on TNF- α synthesis. Of the cytokines examined by these experiments, only TNF- α was affected by dexamethasone treatment. This might be partially explained by the effect of dexamethasone on ED1+ macrophage and ED2+ Kupffer cell populations as both were markedly reduced following steroid treatment. Dexamethasone had an immediate effect on the ED1+ macrophages noted shortly after administration. This action might be explained by changes in ED1+ macrophage-monocyte migration and infiltration. Our results further indicate a reduction in ED2+ Kupffer cells in response to dexamethasone treatment. Dexamethasone has previously been shown to be preferentially taken up by Kupffer cells and liver endothelial cells. (17) Taken together, the reduction in the hepatic macrophages and Kupffer cells may explain the markedly reduced hepatic gene expression of TNF- α following dexamethasone treatment.

Glucocorticoids are also known to affect neutrophil function. Our previous results demonstrate that there is an increase in neutrophil numbers into the portal regions in response to bile duct obstructive cholestatic injury. (15,16) This neutrophil response is persistent following biliary decompression and restoration of bile flow and we hypothesize is the basis for the neutrophil-dependant repair observed in our model.(16) It is interesting to note that despite the dexamethasone-induced reduction in macrophage and Kupffer cells, the hepatic gene expression of the neutrophil chemoattractant CINC-1 was not significantly changed in the steroid treated rats. This is in contradistinction to previous work demonstrating decreased CINC-1 expression with Kupffer cell depletion. This might be partial explained by the efficacy of Kupffer cell depletion compared to the immunosuppressive effect of glucocorticoid on CINC-1 message. Nonetheless, despite CINC-1 message persistence in the dexamethasone treated rats, neutrophils were markedly decreased compared to the non-treated injured rats. These results suggest an alternate explanation for the reduced neutrophil counts in the dexamethasone treated injured livers.

The role of neutrophils during intrinsic repair following biliary decompression is yet to be fully determined. We hypothesize a neutrophil-derived or dependant activation of hepatic matrix metalloproteinases that alter the extracellular matrix balance of MMP to TIMP ratio in favor of MMP activity and collagen breakdown. We further hypothesized that dexamethasone, via its anti-inflammatory and immunosuppressive properties would alter the intrinsic repair

mechanisms and affect collagen resorption. Although the cellular and cytokine mediators were altered, collagen resorption was not affected by dexamethasone. There are two main gelatinases, MMP-2 and MMP-9 and two main collagenases, MMP-8 and MMP-13 implicated in hepatic fibrinolysis. These data are consistent with our previous results that revealed no change in the expression of MMP-2, MMP-9 and MMP-13 in response to bile duct obstructive injury and following decompressive repair. Likewise, only the neutrophil collagenase, MMP-8 was upregulated in response to bile duct obstruction and remained elevated after biliary decompression and bile flow. Dexamethasone reduced the hepatic gene expression of MMP-8, which might be related to the effect of dexamethasone on neutrophils or on intrinsic hepatic MMP-8 expression by the biliary epithelia. Dexamethasone had a significant effect on TIMP-1 expression during repair. As anticipated from previous work, TIMP-1 expression increase in response to injury and reduced following biliary decompression. The dexamethasone treated rats demonstrated a further reduction of TIMP-1 hepatic expression to near sham levels. This change in gene expression between MMP-8 and TIMP-1 ratio is suggested to result in intrinsic repair in our reversible cholestatic model. It is surprising that despite changes in the cellular inflammatory component in response to dexamethasone and the cytokine differences displayed including MMP-8, collagen resorption and intrinsic repair of fibrosis proceeded without apparent change.

If our model is clinically accurate and reflects the successful repair of hepatic fibrosis through metabolism of extracellular matrix then our results indicate that steroids do not interfere with these intrinsic mechanisms. Extrapolating the data beyond that would be speculative since there is seemingly good scientific justification for pursuing therapies that would target critical inflammatory pathways involved in hepatic injury. It is plausible that the ideal use of steroids would involve a regimen which would strike a balance between anti-inflammatory benefits targeting cytotoxic lymphocyte action on biliary epithelia without inhibition of the native hepatic mechanisms crucial for extracellular matrix degradation. It is important to note that our reversible model of cholestatic injury is a valuable tool to investigate the robust development of fibrosis and extracellular matrix metabolism. We conclude that successful resorption of collagen fibrosis may carry on through MMP activating mechanisms and that steroids do not impede the rapid intrinsic repair mechanisms of matrix degradation required for successful repair.

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References

1. Nio M, Ohi R. Biliary atresia. *Semin Pediatr Surg* 2000;9:177–186. [PubMed: 11112835]
2. Ahmed AF, Ohtani H, Nio M, Funaki N, Shimaoka S, Nagura H, Ohi R. CD8+ T cells infiltrating into bile ducts in biliary atresia do not appear to function as cytotoxic T cells: a clinicopathological analysis. *J Pathol* 2001;193:383–389. [PubMed: 11241420]
3. Ohya T, Fujimoto T, Shimomura H, Miyano T. Degeneration of intrahepatic bile duct with lymphocyte infiltration into biliary epithelial cells in biliary atresia. *J Pediatr Surg* 1995;30:515–518. [PubMed: 7595823]
4. Haas JE. Bile duct and liver pathology in biliary atresia. *World J Surg* 1978;2:561–569. [PubMed: 741760]
5. Altman RP, Lilly JR, Greenfield J, Weinberg A, van Leeuwen K, Flanigan L. A multivariable risk factor analysis of the portoenterostomy (Kasai) procedure for biliary atresia: twenty-five years of experience from two centers. *Ann Surg* 1997;226:348–353. [PubMed: 9339941]discussion 353–345

6. Tracy TF Jr, Dillon P, Fox ES, Minnick K, Vogler C. The inflammatory response in pediatric biliary disease: macrophage phenotype and distribution. *J Pediatr Surg* 1996;31:121–125. [PubMed: 8632264]discussion 125–126
7. Muraji T, Higashimoto Y. The improved outlook for biliary atresia with corticosteroid therapy. *J Pediatr Surg* 1997;32:1103–1106. [PubMed: 9247243]discussion 1106–1107
8. Nietgen GW, Vacanti JP, Perez-Atayde AR. Intrahepatic bile duct loss in biliary atresia despite portoenterostomy: a consequence of ongoing obstruction? *Gastroenterology* 1992;102:2126–2133. [PubMed: 1375180]
9. Lunzmann K, Schweizer P. The influence of cholangitis on the prognosis of extrahepatic biliary atresia. *Eur J Pediatr Surg* 1999;9:19–23. [PubMed: 10207698]
10. Meyers RL, Book LS, O’Gorman MA, Jackson WD, Black RE, Johnson DG, Matlak ME. High-dose steroids, ursodeoxycholic acid, and chronic intravenous antibiotics improve bile flow after Kasai procedure in infants with biliary atresia. *J Pediatr Surg* 2003;38:406–411. [PubMed: 12632357]
11. Davenport M, Stringer MD, Tizzard SA, McClean P, Mieli-Vergani G, Hadzic N. Randomized, double-blind, placebo-controlled trial of corticosteroids after Kasai portoenterostomy for biliary atresia. *Hepatology* 2007;46:1821–1827. [PubMed: 17935230]
12. Muraji T, Nio M, Ohhama Y, Hashimoto T, Iwanaka T, Takamatsu H, Ohnuma N, et al. Postoperative corticosteroid therapy for bile drainage in biliary atresia--a nationwide survey. *J Pediatr Surg* 2004;39:1803–1805. [PubMed: 15616935]
13. Stringer MD, Davison SM, Rajwal SR, McClean P. Kasai portoenterostomy: 12-year experience with a novel adjuvant therapy regimen. *J Pediatr Surg* 2007;42:1324–1328. [PubMed: 17706489]
14. Escobar MA, Jay CL, Brooks RM, West KW, Rescorla FJ, Molleston JP, Grosfeld JL. Effect of corticosteroid therapy on outcomes in biliary atresia after Kasai portoenterostomy. *J Pediatr Surg* 2006;41:99–103. [PubMed: 16410116]discussion 199–103
15. Harty MW, Papa EF, Huddleston HM, Young E, Nazareth S, Riley CA, Ramm GA, et al. Hepatic macrophages promote the neutrophil-dependent resolution of fibrosis in repairing cholestatic rat livers. *Surgery* 2008;143:667–678. [PubMed: 18436015]
16. Harty MW, Huddleston HM, Papa EF, Puthawala T, Tracy AP, Ramm GA, Gehring S, et al. Repair after cholestatic liver injury correlates with neutrophil infiltration and matrix metalloproteinase 8 activity. *Surgery* 2005;138:313–320. [PubMed: 16153442]
17. Melgert BN, Weert B, Schellekens H, Meijer DK, Poelstra K. The pharmacokinetic and biological activity profile of dexamethasone targeted to sinusoidal endothelial and Kupffer cells. *J Drug Target* 2003;11:1–10. [PubMed: 12852435]
18. Roggin KK, Papa EF, Kurkchubasche AG, Tracy TF Jr. Kupffer cell inactivation delays repair in a rat model of reversible biliary obstruction. *J Surg Res* 2000;90:166–173. [PubMed: 10792959]
19. Posner MC, Burt ME, Stone MD, Han BL, Warren RS, Vydelingum NA, Brennan MF. A model of reversible obstructive jaundice in the rat. *J Surg Res* 1990;48:204–210. [PubMed: 2314093]
20. Moloney WC, McPherson K, Fliegelman L. Esterase activity in leukocytes demonstrated by the use of naphthol AS-D chloroacetate substrate. *J Histochem Cytochem* 1960;8:200–207. [PubMed: 14423467]
21. Damoiseaux JG, Dopp EA, Calame W, Chao D, MacPherson GG, Dijkstra CD. Rat macrophage lysosomal membrane antigen recognized by monoclonal antibody ED1. *Immunology* 1994;83:140–147. [PubMed: 7821959]
22. Dijkstra CD, Dopp EA, van den Berg TK, Damoiseaux JG. Monoclonal antibodies against rat macrophages. *J Immunol Methods* 1994;174:21–23. [PubMed: 8083524]
23. Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* 2000;132:365–386. [PubMed: 10547847]
24. Dillon P, Belchis D, Tracy T, Cilley R, Hafer L, Krummel T. Increased expression of intercellular adhesion molecules in biliary atresia. *Am J Pathol* 1994;145:263–267. [PubMed: 7519822]
25. Dillon PW, Owings E, Cilley R, Field D, Curnow A, Georgeson K. Immunosuppression as adjuvant therapy for biliary atresia. *J Pediatr Surg* 2001;36:80–85. [PubMed: 11150442]

Experimental Design

Figure 1. Experimental design

Biliary suspension surgery was performed and cholestatic injury occurred over the following 7 days. Saline (n=12) or Dexamethasone (n=16) (7.2 μ g/100g per rat) was administered IV on day 7 of injury at bile duct decompression and on day 2 of repair prior to necropsy. The day of decompression is defined here as day 0. Rats were sacrificed 6 hours following injections at day 0 and at 2 days of repair for each group

Figure 2. Inflammatory cells of the liver

ED1+ (A), ED2+ (B) and PMN (C) stained liver sections derived from sham, injured (day 0) and repairing (day 2) animals treated with saline or Dexamethasone were digitally imaged and counted. The number of ED1+, ED2+ cells and PMN following cholestatic injury are significantly elevated when compared to saline sham controls. 2A and 2B * indicates significant ($p \leq 0.05$) increase in cell number over sham controls and Dexamethasone treatment at day 0 compared to saline controls. 2C * indicates significant ($p \leq 0.05$) increase in cell number over sham controls. 2A, 2B and 2C ** indicates significant decrease in cell number in Dexamethasone-treated animals compared to saline controls ($p \leq 0.05$). Declines in ED1+ and ED2+ cells following decompression are in contrast with PMN cell counts that remain elevated in saline controls suggesting a role for the persistence of neutrophils during repair.

Figure 3. Quantitative real time RT-PCR

TNF- α (A), IL-6 (B), IL-10 (C), CINC-1 (D) mRNA expression was quantified in liver homogenates derived from sham, injured and repairing animals following saline or Dexamethasone treatment. Data are relative to 18S expression and are given as fold increase over saline sham. 3A * indicates a significant ($p \leq 0.05$) decrease in TNF- α expression in Dexamethasone-treated animals at day 2 compared to saline controls 3D * indicates a significant ($p \leq 0.05$) increase in CINC-1 expression in injured animals at day 0 compared to sham controls.

Figure 4. Quantitative real time RT-PCR

MMP-8 (A), MMP-2 (B), MMP-9 (C), MMP-13 (D) mRNA expression was quantified in liver homogenates derived from sham, injured and repairing animals following saline or Dexamethasone treatment. Data are relative to 18S expression and are given as fold increase over saline sham. 3A * indicates a significant ($p \leq 0.05$) increase in MMP-8 expression in injured animals at day 0 compared to sham controls whereas ** indicates significant ($p \leq 0.05$) decrease in expression in Dexamethasone-treated animals at day 2 of repair compared to saline controls.

Figure 5. Quantitative real time RT-PCR

TIMP-1 (A), TIMP-2 (B) mRNA expression was quantified in liver homogenates derived from sham, injured and repairing animals following saline or Dexamethasone treatment. Data are relative to 18S expression and are given as fold increase over saline sham. 5A and 5B * indicates significant increase in TIMP gene expression ($p \leq 0.05$) in the injured animals at day 0 compared to sham controls. 5A ** indicates a significant ($p \leq 0.05$) decrease in TIMP-1 expression in the Dexamethasone-treated animals compared to saline controls.

Figure 6. Fibrotic Repair

Collagen is expressed as a percentage of total area of Sirius red stained liver sections derived from injured and repairing animals treated with either saline or Dexamethasone. Biliary obstruction * significantly ($p \leq 0.05$) increased the deposition of collagen following 7 days of injury compared to sham operated controls. Collagen content, as measured by Sirius red, was significantly ($p \leq 0.05$) decreased in the saline-treated animals ** following decompression (day 2). Dexamethasone-treatment did not block the decompression related decline in collagen despite changes in the inflammatory cellular response. Measures of collagen content were not different from injured animals and significantly ($p \leq 0.05$) elevated when compared to saline treated matched controls.

TABLE 1

Primer List for quantitative RT-PCR.

Primer Name	Sequence
18S-Up	CGA AAG CAT TTG CCA AGA AT
18S-Down	AGT CGG CAT CGT TTA TGG TC
MMP2-Up	TCC GCG TAA AGT ATG GGA AC
MMP2-Down	CAT CAC TGC GAC CAG TGT CT
MMP8-Up	CCC ACC TGA GAT TTG ATG CT
MMP8-Down	GGA TGC CGT CTC CAG AAG TA
MMP9-Up	AGG GTC GGT TCT GAC CTT TT
MMP9-Down	ATA AAA GGG CCG GTA AGG TG
MMP13-Up	TGA CCT GGG ATT TCC AAA AG
MMP13-Down	ACA CGT GGT TCC CTG AGA AG
MMP14-Up	TGG GAA CTT TGA CAC CGT GG
MMP14-Down	TTG GGT ATC CGT CCA TCA CTT G
TIMP1-Up	TTC CCT GGC ATA ATC TGA GC
TIMP1-Down	ATG GCT GAA CAG GGA AAC AC
TIMP2-Up	AAG ATC ACA CGC TGC CCT AT
TIMP2-Down	GTG CCC ATT GAT GCT CTT CT
IL-6-Up	TCC TAC CCC AAC TTC CAA TGC TC
IL-6-Down	TTG GAT GGT CTT GGT CCT TAG CC
IL-10-Up	GGG AAG CAA CTG AAA CTT CG
IL-10-Down	GGC AAG TGA AAG GAC ACC AT
CINC-1-Up	ACC CAA ACC GAA GTC ATA GC
CINC-1-Down	ACT TGG GGA CAC CCT TTA GC
TNF- α -Up	AAA TGG GCT CCC TCT CAT CAG TTC
TNF- α -Down	TCT GCT TGG TGG TTT GCT ACG AC