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Nuetrophil Elastase Mediated Damage in Infants with Extrahepatic Biliary Atresia: A Prospective Cohort Study

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Abstract

Background: Neutrophils are part of our innate immune system. They are capable of accumulation in tissues, chemotaxis, phagocytosis and digests bacteria and fungi by their lysosymes. Yet, paradoxically neutrophils are mediators in acute liver injury. Extrahepatic biliary atresia (EHBA) is a progressive chronic cholangiopathy of infancy, even in those who undergo timely portoenterostomy.

Objective: Assessment of neutrophil involvement in EHBA.

Subjects and Methods: 32 Infants known to have EHBA attendants of Hepatology Clinic, New Children Hospital, Cairo University, were enrolled in this study. It commenced by October, 1999 and ended by October, 2002. Percutaneous biopsied liver tissue (using Menghini needles) required for confirmation of diagnosis of extra-hepatic biliary atresia was assessed for fibrosis, neutrophil infiltration, neutrophil elastase and CD14+.

Results: All biopsies (100%) demonstrated fibrosis of them 2 (6.3%) demonstrated cirrhosis. Neutrophil counts ranged from 2 to 12 cell per high power field (mean ± SD= 5.8±2.5). Anti-neutrophil elastase stained strong positive in all (100%) biopsies. All biopsies stained positive for CD 14+ where 24 (75%) demonstrated moderate and 8 (25%) strong staining. Strength of CD14+ staining correlated positively with neutrophil counts per high power field (p=0.0000), but not to fibrosis score (p=0.252). CD14+ staining negatively correlated with cirrhosis (p=0.0000). Fibrosis score had a trend for positive correlation with neutrophil counts that did not mount to statistical significance (p=0.065). Both did not predict favorable outcome (CD14+ p=0.356, fibrosis score p=0. 812) or deterioration (CD 14+ p=0. 13 1, fibrosis score p=0.584). Neutrophil elastase stain was strongly positive in all cases. Only predictors of good outcome in a multiregression model with 95% confidence interval was younger age at Kasai portoenterostomy (p=0.012) and less fibrosis (p=0.000).

Conclusion: Neutrophil elastase is integral in liver histopathology in EHBA yet neutrophil exact role in tissue injury await exploration.

Key Words: *Extrahepatic biliary atresia – Immuno - histochemical staining – CD14+ monocytes – Neutrophil elastase – Fibrosis.*

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Introduction

EXTRAHEPATIC biliary atresia (EHBA) is a progressive inflammatory cholangiopathy that affects neonates. It presents by total or partial sclerosis of extrahepatic biliary system and spreads to intrahepatic bile ducts [1]. Aetiology is obscure, outcome is punctuated by repeated attacks of cholangitis, and liver transplantation is often necessary [2]. Proposed aetiologies varied from ductal plate malformation, embryonic/fetal development arrest of biliary system, viral infection (hepatitis B virus/ reovirus type 3), vascular accidents, or genetic susceptibility. In any case immune-mediated destruction was the proposed underlying pathogenesis, with evidence of heavy infiltration of portoenterostomy core by CD4+ helper T lymphocytes, CD8+ suppressor T lymphocytes and CD68+ macrophages [1,3,4]. Anticytoplasmic antineutrophil antibodies were reported to be present in some cases with EHBA [5]. There is evidence for endotoxin circulation and up regulation of lipopolysaccharide endotoxin receptor CD14+ monocytes in EHBA [6].

Neutrophils degranulate elastase, metalloproteinases, collagenases, defensins, reactive oxygen species, cathepsin, gelatinase, proteases and others locally, thus mediate tissue damage involved in some diseases with chronic march e.g.: Anti-neutrophil cytoplasmic antibody (ANCA)-related glomerulonephritis [7] and diabetic retinopathy [8].

This work aimed to study involvement of neutrophil elastase and CD14+ in EHBA.

Subjects and Methods

Subjects:

Infants known to have EHBA, who were attendants of Hepatology Clinic, New Children Hospital,

Cairo University, were enrolled in this study. It commenced by October, 1999 and ended by October, 2002.

Diagnosis relied upon clinical picture, specific percutaneous liver biopsy findings [1], and operative findings. Infants received protocol metrinidazole and third-generation cephalopsporin during the immediate post-operative month, and co–trimoxazole (trimethoprim- sulfamethoxazole) during the following year. Failure of bile flow after initial successful portoenterostomy warranted steroids for one month, which was weaned over 10 days. Cholangitis was managed by antibiotics according to culture and sensitivity. Outcome grading was according to Grosfeld et al., (1989) [9] where successful was coined to no jaundice, hepatic aminotransferases were within or less than double fold of high normal. Improved outcome was coined to decrease in bilirubin level and hepatic transferases within four folds of normal in a stable disease, with normal colored stools. Failure was labeled to outcome of infants with progressive disease, minimal or no bile flow.

Methods:

Percutaneous biopsied liver tissue (using Menghini needles) required for confirmation of diagnosis of extra-hepatic biliary atresia was assessed for fibrosis, neutrophil infiltration, neutrophil elastase and CD 14+.

Histopathology studies:

Hematoxylin and eosin stained sections were assessed for density of portal tract and lobular infiltrate. Neutrophils were counted per high power field (HPF). At least 7 HPF were studied, and mean number of positive cells was calculated for each slide. Mason trichrome staining of sections allowed scoring of fibrosis [10]. Fibrosis was graded from 1 to 6. Where grade 1: Fibrous expansion of some portal areas, grade 2: Fibrous expansion of most portal areas, grade 3: Fibrous expansion of most portal areas plus occasional portal to portal (P-P) bridging, grade 4: Fibrous expansion of most portal areas plus marked P-P and portal to central (P-C) bridging, grade 5: Marked P-P and P-C bridging with incomplete nodules, and 6: Frank cirrhosis with parenchymal nodules and disruption of liver architecture [11].

Immunohistochemical staining:

Liver tissue sections were stained by monoclonal mouse antihuman neutrophil elastase (Dako cytomation, Denmark) [12] and monoclonal mouse antihuman CD 14+, clone TUK4 (Dako cytomation, Denmark) [13]. Sections from paraffin block were treated with monoclonal antibodies, utilizing Ultravision Plus Detection System, Anti-polyvalent HRP with DAB chromogen, from Lab Vision Corporation, UK. Positive results were indicated by cytoplasmic staining of the chromogen. The intensity of the staining as well as the rate of expression were taken into consideration. Expression was graded into: Negative, mild, moderate and strong.

Statistical analysis:

Statistical analysis in this study were conducted using the Statistical Package for Social Sciences version 15 [SPSS, Chicago, IL, USA]. We employed simple frequency, cross-tabulation, descriptive analysis, tests of significance (*t*-test for parametric data, and χ^2 tests for non parametric data). Regression analysis was conducted to define predictors of outcome in biopsies of the 32 children known to suffer from biliary atresia.

Results

Demography and outcome:

Mean age and standard deviation (SD) of onset of cholestasis among the 32 enrolled infants was 12±13 days (range day 1-day 38), while the mean age±SD at presentation was 65.6±27 days (range day 1-day 127) and at portoenterostomy was 81±22.5 days (range 50-137 days). Only 24 (75%) underwent portoenterostmy, and 10 (31.3%) were lost to follow-up. Mean±SD age at Kasai portoenterostomy was 81±22.5 days (range=50-137 days). Duration of follow-up ranged from 14 days till 2yrs 10 months (mean=161 days). 15 (46.9%) were males, and only 8 (25%) were known to be product of consanguineous marriages (10 (31.3%) were not and 14 (43.7%) consanguinity could not be determined). Duration of follow-up ranged between 1-896 days (mean=161 days). 21 (65.6%) received ursodeoxycholic acid and 20 (62.5%) received fat soluble vitamins and co-trimoxazole (trimethoprim-sulfamethoxazole). Three had initial resolution that was maintained in only one, while the other 2 received UDCA and suffered from deterioration. 11 suffered from attacks of cholangitis, 3 from chest infections, 2 from otitis media, 5 from diarrhea, 6 from liver cell failiure, and 6 from intractable ascites and 1 from spontaneous bacterial peritonitis among those who received UDCA. Among those who did not receive UDCA 1 suffered from portal vein thrombosis and ascites. Outcome of studied infants is shown in Table (1).

Table (1): Outcome of studied infants with extrahepatic biliary atresia.

	Number (32 infants)	%
Resolved	1	3.1
Improved	0	0
Stationary	3	9.4
Failed	5	15.6
Death	13	40.6
Dropped out	10	31.3

Histopathology findings:

Fibrosis:

All biopsies (100%) demonstrated fibrosis of them 2 (6.3%) demonstrated cirrhosis. Grade 1 fibrosis was encountered in 4 biopsies (12.5), grade 2 in 12 (37.5%), grade 3 in 10 (31.3%) and grade 4 in 4 (12.5%). Neutrophil counts ranged from 2 to 12 cell per high power field (mean ±SD=5.8±2.5).

Immunohistochemical staining:

Anti-neutrophil elastase stained strong positive in all (100%) biopsies. Staining was accentuated in portal tracts within fibrosis of varied densities. All biopsies stained positive for CD14+ where 24 (75%) demonstrated moderate and 8 (25%) strong staining.

Correlations:

Strength of CD 14+ staining correlated positively with neutrophil counts per high power field (p=0.0000), but not to fibrosis score (p=0.252). CD14+ staining negatively correlated with cirrhosis (p=0.0000). Fibrosis score had a trend for positive correlation with neutrophil counts that did not mount to statistical significance (p=0.065). Both did not predict favorable outcome (CD 14+ p=0.3 5 6, fibrosis score p=0.812) or deterioration (CD14+ p=0.131, fibrosis score p=0.584). Neutrophil elastase stain was strongly positive in all cases. Only predictors of good outcome in a multiregression model with 95% confidence interval was younger age at Kasai portoenterostomy (p=0.012) and less fibrosis (p=0.000).

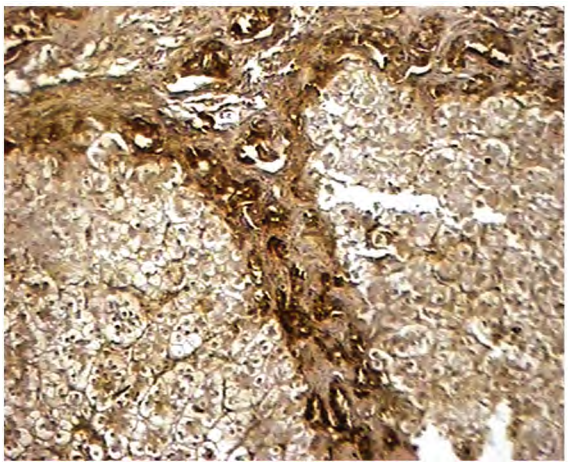

Fig. (1-A): Section of biopsied liver tissue in biliary atresia with proliferated bile ductules stained with monoclonal mouse antihuman neutrophil elastase (using DAB chromgen and magnification x100). Note strong staining and dense intralobular fibrosis.

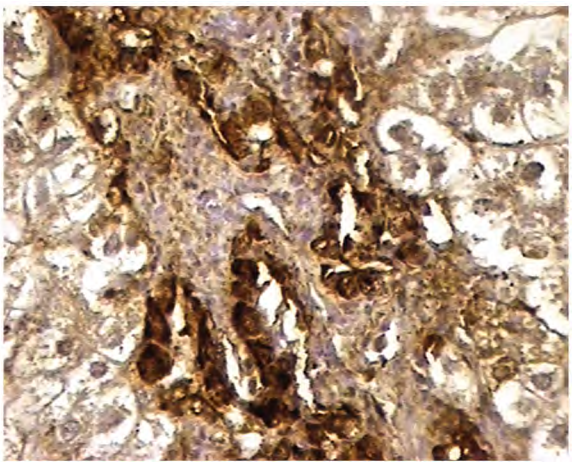

Fig. (1-B): Magnified previous section of biopsied liver tissue in biliary atresia with proliferated bile ductules stained with monoclonal mouse antihuman neutrophil elastase (using DAB chromgen and magnification x200). Note strong staining.

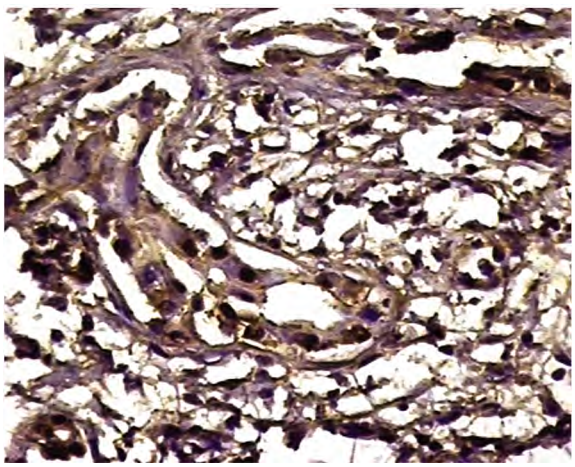

Fig. (2): Section of biopsied liver tissue in biliary atresia, demonstrating distorted bile ductile and cellular infilterate stained with monoclonal mouse antihuman CD14+ monocytes (using DAB chromgen and magnification x400). Note dispersion of CD14+ monocytes throughout section.

Discussion

Neutrophil elastase is an Integral Part of Pathogenesis of EHBA:

Neutrophil degranulation is known to follow priming by cytokines and mobility into tissues [14,15]. It is interesting however, that neutrophil elastase is a marker of acute liver injury in response to ischemia-reperfusion injury, endotoxemia, acetaminophen and alcohol intoxication [16]. All our studied EHBA biopsies demonstrated strong staining of neutrophil elastase, which could be attributed to lipopolysaccharide receptor stimulation as demonstrated by the CD14+ monocytes infiltration and by the subacute ischemia associated with EHBA [17]. Neutrophil degranulation is associated with acute liver injury as it causes exacerbation of another underlying aetiology. It seems that neutrophil degraulation is a common pathway of liver damage in EHBA. The strong staining against neutrophil elastase in portal areas explain the poor outcome of our studied infants.

Neutrophil elastase induced liver injury and lipopolysaccaride endtoxin:

Lipopolysaccharide endotoxin of gram-negative bacteria binds to CD 14+ monocytes and results in a cascade of pro-inflammatory cytokines [18]. Others have reported endotoxemia in infants with EHBA [19]. It seems that EHBA is a disease of multiple "hits" involving more than a factor working in concert, followed by a common pathway of neutrophil degranulation and tissue damage.

Vascular hepatic hypo perfusion is revisited:

Ischemia reperfusion injury is known to induce bile duct epithelial proliferation [20]. Proliferated bile ducts are also encountered in EHBA [21], yet attributed to the obstruction, and not to hypoperfusion. Liver ischemia or vascular accidents should not be undermined in EHBA pathogenesis. Hepatic perfusion by pressure and volume need to be assessed in EHBA. Hepatic artery is a medium sized artery that should need a filling diastolic pressure, and volume [22].

It is empirical to study central venous pressure effect, as an index of hepatic perfusion to be studied as a denominator of outcome along with urine output measurement.

Immunemodulatory medications role in EHBA:

The lack of unanimous response to steroids in controlling EHBA March [23,24] provides compelling evidence that the immune pathogenesis is not the sole determinant of outcome. The CD14+ up-regulation in response to endotoxemia provides enough rationale for co-trimoxazole prophylaxsis, yet it is not a sole effector in outcome of disease.

Conclusion:

Immune-mediated injury is integral in EHBA, involving lymphocytes, monocytes and neutrophils. Cascade of events is well orchestrated yet not fully elucidated by our current knowledge. Hepatic perfusion, and ischemia-reperfusion injury role in pathogenesis of EHBA remain to be defined.

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