Evidence of Disruption of p53 and Glutathione S Transferase Pi in Extrahepatic Biliary Atresia in Association with Neutrophil Elastase Mediated Damage

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Abstract

Background: Extrahepatic biliary atresia (EHBA) is a chronic obstructive cholangiopathy of infancy with obscure aetiology. I recently reported unanimous strong immunohistochemical staining against neutrophil elastase in EHBA liver biopsies, with variable degrees of fibrosis, and variable CD14+ monocytes intensity staining. Neutrophil inflicted cellular damage is a crucial step for regeneration by removal of cells that are unwanted, damaged or that do not demonstrate DNA fidelity. Cellular p53 and glutathione S transferase (GST) govern the ontogeny respected regeneration. EHBA is not a self-limiting condition despite fulfilling the neutrophil inflicted cellular damage step, and healing by regeneration results in cirrhosis. This necessitated further investigation of these biopsies for involvement of p53 that is responsible for DNA fidelity at cellular replication and regeneration and GST that is responsible for detoxification of a wide array of substances that affect cellular replication and DNA fidelity.

Aim of Work: Is to study p53 and GSTPi in EHBA.

Material and Methods: The liver biopsies of 32 neonates and infants with EHBA were studied by immunohistochemical staining for p53 and class Pi of GST family. The biopsies were collected percutaneously using Menghini needles. The findings were correlated to previously studied parameters: Fibrosis, neutrophil infiltration, staining against neutrophil elastase and staining for anti-CD14+ monocytes. Study commenced by October, 1999 till October, 2002, in New Children Hospital, Cairo University.

Results: All 32 biopsies (100%) demonstrated defective staining of p53 and GSTPi. Mean ± SD percentage of p53 staining was 30.37 ± 9.6% (range 12-46%). 25 (78.1%) did not stain for GSTPi, and 7 (21.9%) stained mildly. GST Pi staining did not correlate with other biopsy findings or prognosis. Staining of p53 correlated with neutrophil infiltration (p=0.001), fibrosis (p=0.026) and anti CD14+ staining (p=0.000), but not to outcome. Negative correlation between staining for p53 and resolution of symptoms did not amount to statistical significance (p=0.068).

Conclusion: Involvement of p53, GSTPi, CD14+ monocytes and neutrophil elastase in EHBA is unanimous. Their contribution to aetiology or to notorious chronic progressive course of EHBA remains to be defined.


Introduction

AETIOLOGY of extrahepatic biliary atresia (EHBA) is obscure, and its march is progressive and relentless. EHBA commences by partial or total sclerosis of extrahepatic biliary system and spreads to intrahepatic bile ducts [1]. Irrespective of hypothesized aetiologies, all pathogenesis described in EHBA involves immune mediated damage of bile ducts, with evidence of heavy infiltration by CD4+ helper T lymphocytes, CD8+ suppressor T lymphocytes, CD68+ macrophages and CD14+ [1-4]. Anticytoplasmic antineutrophil antibodies were reported to be present in some cases with EHBA [5]. The evidence for endotoxin circulation and up regulation of lipopolysaccharide endotoxin receptor CD14+ monocytes in EHBA is compelling [6,7]. The intriguing unanimous neutrophil infiltration and strong immunohistochemical staining against neutrophil elastase described in my previously published work [7] supported an integral role of neutrophil in the bile duct damage. Yet neutrophil elastase induced damage is an essential step for regeneration to remove cells that are apoptotic or damaged or demonstrating infidelity of DNA [8]. In view of strong involvement of neutrophil elastase in EHBA it would be expected that cellular debris is removed and EHBA would be a self limiting disease, instead regeneration in EHBA results in variable degree of fibrosis and cirrhosis [1]. Irrespective of aetiology, neutrophil elastase inflicted...
damage on cholangiocytes does not trigger the expected resolution in EHBA [7].

Resolution and ontogeny respected regeneration is a function of other cellular systems, of them p53 is a system responsible for DNA fidelity at cellular replication and regeneration [9] and glutathione S transferases (GST) that are responsible for detoxification [10]. GST is a super family involving 7 classes named from alfa to omega [11,12]. GST including GSTPi is responsible for phase II detoxification of mainly xenobiotics, by catalyzing the reduced glutathione (GSH) nucleophilic attack [13]. They are responsible for detoxification of a wide array of substances that affect cellular replication and DNA fidelity [14]. p53 is disrupted under stress, and its effects can mount to carcinogenesis and developmental malformations [9,15].

This work aimed to study p53 and GSTPi in previously studied liver biopsies of EHBA demonstrating strong staining against human neutrophil elastase [7].

Material and Methods

Material:
Liver biopsies of 32 neonates and infants known to have biliary atresia were studied. The biopsies were required for confirmation of diagnosis of EHBA. The same biopsies were previously studied in Kotb, 2014 [7]. The neonates and infants were attendants of Hepatology Clinic, New Children Hospital, Cairo University, during October 1999-October 2002.

Methods:
The liver biopsies were collected percutaneously using Menghini needles. The biopsies were known to demonstrate fibrosis, neutrophil infiltration, strong staining against neutrophil elastase and strong staining for anti-CD14+ monocytes as reported previously [7]. The biopsies were assessed for p53 and GSTPi by immunohistochemical staining.

Diagnosis depended upon clinical picture, lab investigations, abdominal sonar, typical percutaneous liver biopsy findings, and operative findings [1]. Outcome grading was according to Grosfeld et al., (1989) [16] successful was defined as no jaundice, hepatic aminotransferases were within or less than double fold of high normal. Improved outcome was defined as 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 [7].

Histopathology studies:
In this study we investigated the hematoxylin and eosin previously stained sections that were already 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 [7]. Fibrosis grading was according to Ishak et al., (1995) [17].

Immunohistochemical staining:
Liver tissue sections were treated by monoclonal mouse antihuman p53 protein, clone DO-7 (Dako cytation, Denmark) [18] and monoclonal mouse antihuman glutathione S-transferase enzyme \( \pi_\text{Pi} \) clone 353-10, (Dako cytation, Denmark) [19]. Sections from paraffin block were treated with monoclonal antibodies, utilizing Ultravision Plus Detection System, and anti-polivalent HRP with DAB chromogen, from Lab Vision Corporation, UK. Positive results were indicated by cytoplasmic staining of the chromogen. For p53, positive results were indicated by nuclear staining by chromogen and the percentage of positive cells were counted in 500 cells and the results were tabulated. For GSTPi 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. The same biopsies were previously studied [7] and all were known to exhibit strong staining of neutrophil elastase by monoclonal mouse antihuman neutrophil elastase (Dako cytation, Denmark) [20] and staining of CD 14+ monocytes staining by monoclonal mouse antihuman CD 14+, clone TUK4 (Dako cytation, Denmark) was strong in 8(25%) and moderate in 24 (75%) [21].

Statistical analysis:
Statistical analysis in this study was conducted using the Statistical Package for Social Sciences version 15 (SPSS, Chicago, IL, USA). Simple frequency, descriptive analysis, cross-tabulation, tests of significance \((t\text{-test for parametric data, and } \chi^2 \text{ tests for non parametric data})\) were employed. 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:
The enrolled infants had a mean age \( \pm \) standard deviation (SD) of onset of cholestasis of 12 \( \pm \) 13 days (range day 1- day 38), at presentation of 65.6 \( \pm \) 27 days (range day1- day 127) and at portoenteros-
to 81 ±22.5 days (range 50-137 days). 15 (46.9%) were males, follow-up duration was 14-896 days (mean=161 days). Only one (3.1%) had a successful outcome, none improved, 8 (25%) failed (of them 3 had a stationary course), 13 (40.6%) died and 10 (31.1%) dropped out [7].

Histopathology findings:

**Immunohistochemical staining:**

All the biopsies were known to exhibit strong staining of neutrophil elastase while 24 exhibited moderate staining of CD 14+ monocytes and 8 strong staining by monoclonal mouse antihuman CD14+. Staining was accentuated in portal tracts within fibrosis of varied densities. All biopsies had decreased expression of p53, where maximum expression was 46%, with a mean ± standard deviation (SD) of 30.37±9.6% (range=12-46). 12 (37.5%) expressed 25% or less of p53 in hepatocytes and bile duct epithelia. Anti GSTPi staining was mild in hepatocytes and bile duct epithelia in 7 (21.9%) biopsies and did not stain at all in 25 (78.1%) biopsies.

Biopsies hematoxylin and eosin stained sections identified fibrosis in all biopsies (100%), 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%). All demonstrated neutrophil infiltration, counts ranged from 2 to 12 cell per high power field (mean ± SD=5.8±2.5).

**Correlations:**

Staining of p53 correlated positively with neutrophil infiltration (p=0.001), fibrosis (p=0.026) and anti CD 14+ staining (p=0.000), but not to outcome. Negative correlation between staining for p53 and resolution of symptoms did not amount to statistical significance (p=0.068). Correlations of GSTPi staining with other biopsy findings or prognosis could not be computed as GSTPi defective staining was constant in all biopsies. Similarly correlations of neutrophil elastase staining with other biopsy findings or prognosis could not be computed as neutrophil elastase strong staining was constant in all biopsies.

**Discussion**

This study demonstrates unanimous defective expression of p53 and GSTPi, in the presence of strong staining for neutrophil elastase and CD 14+ monocytes in liver biopsies of neonates and infants suffering from EHBA. p53 is essential for “normal” cell cycle and ontogeny by responding to stresses that can disrupt DNA replication and cell division [22]. Disruption of p53 is genetically determined or acquired by mutagens (chemicals, radiation or viruses) and results in compromised tumor suppression [23]. Genetic inactivation of p53 is implicated in the developmental syndrome of ocular coloboma, heart defect, choanal atresia, retarded growth, genitourinary hypoplasia and ear abnormalities (CHARGE syndrome) [18].

GST is a super family of enzymes of multiple forms, i.e. of isozymes, present in all cells, and epithelium of normal bile ducts. They are divided into cytosolic, mitochondrial and membrane associated protein in eicosanoid and glutathione metabolism (MAPEG), and cytosolic is further classified into alfa, zeta, theta, mu, pi, sigma, and omega [11,12]. GST and GSTPi are responsible for phase II detoxification of mainly xenobiotics, by catalyzing the reduced glutathione (GSH) nucleophilic attack [13]. They are responsible for detoxification of a wide array of substances that include drugs, pesticides, herbicides, epoxides and carcinogens [24]. GST can be inhibited by raised bilirubin levels [14]. The absent GSTPi in our study in 25 biopsies and mild staining in the other 7, in the presence of cholestasis point to impaired detoxification of phase II in EHBA that require further investigation.

Compared to adults, in normal neonates, evidence supports higher expression of erythrocyte GST [25], satellite stem cell GST [26] but not neonatal hepatocyte GSTPi [27]. Contrary to our study Mathew and his co-workers [27] demonstrated intense GSTPi cytoplasmic and nuclear expression in the 15 biopsies of EHBA neonates, and absent expression in normal neonatal livers. Their mean ages were younger (aged 6-8 weeks) than our studied group (aged 7-11 weeks). Both studies were cross-sectional and not prospective. More insight is required to reveal the difference in reported results. In our study the predominantly absent stain for GSTPi might be due to absent substrate, genetically determined absence, age related enzyme immaturity, ontogenesis “normal” under-expression [27]. Suppression of expression by cholestasis [14] or CD14+ elevation in the course of endotoxemia that was reported to reduce microsomal GST activity [28]. Lipopolysaccharide toxin of E coli abundance is known to induce expression of CD 14+ monocytes, as CD 14+ monocytes are receptors of gram negative lipopolysaccharide endotoxin [21]. All our biopsies had unanimous elevated expression of CD 14+ monocytes.
and absent/mild expression of GSTPi and CD14+. Our results underscores role of sepsis and lipopolysaccharide endotoxin in pathogenesis of EHBA, but is challenged by the limited value of antibiotic prophylaxis in progression of cholangiopathy [29].

Predominantly absent or mild staining of GSTPi enzyme in EHBA liver biopsies makes liver in EHBA susceptible to consequences of impaired functions mediated by glutathione system, i.e. to reactive oxygen species and peroxides involved in leukotriene biosynthesis [24]. Role of sepsis in EHBA needs more elucidation.

It is hypothetically sound to incriminate sepsis in the absent/or under expressed GSTPi, that cascaded lower expression of p53. Yet it generates more question marks on the type of involvement of p53, whether it is a primary or secondary event? Is there a role of under expressed p53 in EHBA development and further “anomalous malformation”? Why was the sepsis picture masked in EHBA neonates? And will there be a future role of antibiotics in early management of EHBA?

In conclusion pathogenesis of intrahepatic cholangiopathy in EHBA includes involvement of neutrophil elastase damage, endotoxemia mediated CD 14+ monocyte response on bile ducts and portal tracts in the presence of disrupted p53 and GSTPi.

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References


