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Molecular determination of liver fibrosis by synchrotron infrared microspectroscopy

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Abstract

Liver fibrosis is an adaptive response to various injuries and may eventually progress to cirrhosis. Although there are several non-invasive methods available to monitor the progression of liver fibrogenesis, they cannot reliably detect fibrosis in its early stages, when the process can be stopped or reversed by removing or eliminating the underlying etiological agent that cause the hepatic injury. In this study, early fibrosis alterations were characterized biochemically, morphologically, and spectroscopically in a rat bile duct ligation (BDL) model. Progressive elevations in serum alanine transaminase (ALT), aspartate transaminase (AST), and bilirubin levels in the BDL rats were found indicating the dynamic deterioration of hepatocellular function. Immunofluorescence microscopy using monoclonal anti-collagen III antibody further revealed abnormal intertwined networks of collagen fibres surrounding the portal areas and extending into the lobules towards the central veins in all BDL samples starting from week one. Synchrotron infrared microspectroscopy of liver sections was exploited to generate false color spectral maps based upon a unique and strong collagen absorption at 1340 cm^{-1}, revealing a collagen distribution that correlated very well with corresponding images provided by immunofluorescence imaging. We therefore suggest that infrared microspectroscopy may provide an additional and sensitive means for the early detection of liver fibrosis.

Keywords: Liver fibrosis; Cirrhosis; Infrared; Mapping; Collagen

1. Introduction

Liver fibrosis and cirrhosis are common sequelae of chronic liver injuries, emerging through wound-healing processes in the liver. Progressive formation of a fibrillar extracellular matrix (ECM) in the liver is the consequence of repeated liver injuries in response to a variety of insults, including chronic viral infection (commonly hepatitis B and C), toxins (e.g., alcohol), and both metabolic and autoimmune disorders [1]. Liver fibrosis results from a dynamic process in which fibrillar ECM formation is associated with ECM degradation and remodelling. This is a balanced process that may either progress to an advanced stage defined as 'cirrhosis' or be reversed to restore normal hepatic structure and function [2]. Cirrhosis is characterized by the formation of regenerative nodules of liver parenchyma that are separated by and encapsulated in fibrotic septa and is associated with major angio-architectural changes. It is generally considered that liver fibrosis is reversible while cirrhosis is irreversible unless an effective treatment for the underlying insult is available.

The need for rapid, safe and reliable methods to detect and monitor the liver fibrogenic progression has become an urgent clinical reality due to increased knowledge regarding the mechanisms responsible for hepatic fibrogenesis and hence the introduction of potentially more effective therapeutic strategies [3]. The concept of a 'fibrosis progression rate' has implications for risk stratification, prognosis and for the evaluation of therapy [4]. Although there has been considerable effort to identify serum markers as non-invasive measures of hepatic fibrosis, e.g., alterations in

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Table 1
Serum markers for hepatic functions in sham and BDL-treated group

		0 week	2 weeks	4 weeks
ALT	Sham	78.0±3.2	63.0±3.5	61.0±1.0
	BDL-treated	68.6±5.1	176.0±15.1*	165.5±18.6*
AST	Sham	109.6±2.9	98.3±14.9	145.5±44.5
	BDL-treated	112.3±24.1	645.5±126.9*	763.7±97.1*
BIL	Sham	0.5±0.2	0.9±0.3	1.1±0.4
	BDL-treated	0.7±0.3	8.2±1.5*	8.2±1.2*

ALT and AST are in units/liter and bilirubin in mg/dl. The data were obtained from twelve rats for each group and are depicted as Mean±SE.
* indicate significant differences ($P<0.05$).

hepatic TIMP and MMP expression and serum hydroxyproline and hydroxylysine levels, the accuracy and predictive value of these measures are insufficient to provide the basis for clinical decision-making [5,6]. From the clinical standpoint, liver biopsy is still considered the 'gold standard' to confirm the clinical diagnosis, to assess the severity of necro-inflammation and fibrosis, to evaluate possible concomitant disease processes, and to guide therapeutic intervention. However, needle liver biopsy is associated with potential morbidity and mortality and has a high rate of sampling error in patients with diffuse parenchymal liver diseases [7]. For instance, needle liver biopsy sampling error may occur despite an adequate sample size and a satisfactory sampling of portal tracts, when evaluating the severity of inflammation [8], the degree of fibrosis [9], and presence of cirrhosis [7]. The present approach, if successful, might permit routine, accurate, non-subjective diagnostic assessment of very small-bore needle biopsies, minimizing morbidity and at the same time providing clear guidance in the evaluation of treatment options.

While biopsy samples are often stained with either hematoxylin and eosin (H&E) or specific connective tissue stains such as Masson's trichrome or reticulin silver impregnation [10], visualization of fibrosis is not easy and underestimation of the fibrillar ECM network is common [3]. Therefore, most pathologists routinely resort to more specific approaches such as picrosirius red staining [11] or immunofluorescence [12] that provide excellent definition of the distribution of fibrillar collagens (i.e., collagen type I and type III) [13]. These methods clearly reveal the connective tissue structure of liver tissue, including aspects of both perisinusoidal and pericellular fibrosis.

Infrared microspectroscopy offers the means to characterize the nature and distribution of tissue components by virtue of differences among the mid-IR spectra of those components. For example, IR microspectroscopy has been used to demonstrate differences between diseased and normal colon [14], cervical [15], and brain [16] tissues, and changes in composition with apoptosis and necrosis [17]. Of particular relevance here, IR microspectroscopy has been utilized previously to reveal the spatial distribution of collagen (and other components) within both cardiac and oral cancer tissues [18,19]. Collagens exhibit a series of unique IR absorption bands between 1000 and 1350 cm^{-1}, and these have been exploited in previous studies characterizing collagen tissue distribution [18–23]. For instance, an absorption at 1204 cm^{-1} was used as a marker band to characterize collagen distribution in heart tissue and oral cancers [18,19], while a band at 1338 cm^{-1} was shown to be directly related to the quantity of collagen in cartilage and exploited for a study of prostatic hyperplasia [21,22]. Liver tissue has been characterised by infrared microspectroscopy in two previous studies [23,24].

The spatial resolution of conventional infrared microspectroscopy is limited by diminishing signal-to-noise with small apertures. Therefore, fine tissue architectural features cannot be resolved with regular infrared microspectroscopy. Fortunately, this problem can be alleviated by employing synchrotron infrared radiation as a source, since it is 1000 times brighter than a standard black-body source. As a result, spectra with good signal-to-noise may be acquired with apertures at or near the diffraction limit [25,26]. By utilizing a synchrotron IR source, it is possible to resolve tissue architectural features at a spatial resolution near the diffraction limit (~10 μm at 1000 cm^{-1}), and to reveal structures that would not otherwise be resolved by conventional IR microspectroscopy.

Therefore, in the current study, we made use of synchrotron infrared microspectroscopy (at the National Synchrotron Light Source) to examine the distribution of liver fibrosis within a rat model, and compared the resulting maps to images produced from the same tissue section by immunofluorescence staining (monoclonal anti-collagen III antibody).

2. Material and methods

2.1. Animal model

A total of twenty-four adult male Sprague–Dawley rats were obtained from the Animal Facility of the University of Manitoba. All rats were maintained under 12-h light/dark cycles with food and water ad libitum. All animals received humane care in compliance with criteria set by the Canadian Council on Animal Care. These rats were divided into two groups, sham-operated and bile duct ligation (BDL), respectively. Since the BDL model represents the major features of liver fibrosis with significant modification of ECM and has been used most frequently for the investigation of liver fibrosis, we implemented this model according to a procedure described previously [27–30]. Briefly, the animal was subjected to mild anesthesia and the common bile duct was exposed by median laparotomy and occluded by double ligature with a nonresorbable suture (7-0 silk). The first tie was made below the junction of the hepatic ducts and the second was made above the entrance of the pancreatic duct. The common bile duct was then resected between the two ligatures, and the abdominal incision was closed. Liver specimens were then collected weekly (4 animals weekly; 24 animals total), up to 6 weeks following BDL by excision of the entire liver. These specimens were frozen in liquid nitrogen immediately following removal, and stored at −70 °C for further analysis.

2.2. Serum and liver biochemistry

Blood samples were obtained from the saphenous vein of rats at 0, 2, and 4 weeks intervals after sham or BDL procedures. The serum concentrations of alanine transaminase (ALT) and aspartate transaminase (AST) were determined using a Roche-Hitachi 917 Clinical Chemistry Analyzer with Roche application kit while bilirubin levels were measured using a bilirubin assay kit from Sigma (Sigma-Aldrich Inc, St. Louis, USA).

2.3. Liver histology

Frozen liver samples (approx. 0.5 cm^3) were randomly taken from the right, median and left lobes of each rat liver and sectioned consecutively at 5 μm using a cryostat at −18 °C. These sections were mounted on slides and air dried for at least 20 min followed by fixation in 10% Formalin for 30 s. Then these sections were stained with routine Haematoxylin and Eosin according to regular staining procedure such as hydration, staining, dehydration and clearing. The stained slides were finally covered with a cover-slip using a mounting medium Entellen.