ORIGINAL PAPER

Eur. J. Histochem.
46: 101-104, 2002

Simultaneous staining of cytoplasmic iron and collagen matrix in human liver biopsy specimens

F. Grizzi[1,2], G. Ceva-Grimaldi[3], B. Franceschini[1,2], M. Roncalli[3], M. Chiriva-Internati[4], and N. Dioguardi[1,2]

[1]Scientific Direction and [3]Pathologic Unit, Istituto Clinico Humanitas, Rozzano, Milan, Italy; [2]Fondazione "Michele Rodriguez". Istituto Scientifico per le Misure Quantitative in Medicina, Milan, Italy; [4]Center for Immunology & Microbial Disease, Albany Medical College, Albany, New York, USA

Accepted: 19/11/01

Key words: liver, extra-cellular matrix, collagen, iron, histochemistry

SUMMARY

One of the major liver fibrogenic activators is the cellular iron overload that can severely damage parenchymal and non-parenchymal cells.

The aim of this study was to investigate a histochemical staining technique that allows the simultaneous detection of the irregular deposition of matrix collagen and both the amount and distribution of iron in liver cells on the same histological section.

The method was evaluated using 3-μm histological sections obtained from ten standard liver biopsy specimens taken from patients with hepatitis C virus-related diseases and simultaneous liver cell iron overload.

The results indicate that the double-staining technique is simple, sensitive and rapid, and can be routinely applied to liver biopsy specimens for diagnostic purposes. Furthermore, it may also facilitate the study of the complex relationship between hepatic fibrosis and iron overload during common genetic or secondary liver metabolic disorders.

INTRODUCTION

Fibrosis is the name given to the excessive accumulation of extra-cellular matrix (ECM) components in the liver caused by their markedly increased synthesis and unbalanced degradation. Hepatic fibrosis is the most common response to a chronic liver insult which, regardless of its nature (viral, toxic, immune or metabolic) leads to severe cell damage and/or necrosis. One of the major liver fibrogenetic activators is iron or copper overload in parenchymal and non-parenchymal cells. Although its molecular and cellular mechanisms are still unknown, the fibrotic liver disease associated with iron overload represents the prototype of oxidative stress-related fibrotic liver disease (Andrews N.C., and Pietrangelo A.).

The growing interest in iron overload and hepatic chronic disease, and its key-role in abnormal ECM synthesis, prompted us to investigate a double-staining histochemical method that allows the simultaneous detection of the collagen extra-cellular network and iron accumulation in human liver tissue. The aims of the study were: *a)* to assess the

Correspondence to: F. Grizzi
E-mail: fabio.grizzi@humanitatis.it

usefulness of the technique in routinely and accurately assessing liver biopsy specimens for tissue damage and the amount of cell iron storage; and *b)* to discuss the advantages of the method.

MATERIALS AND METHODS

Standard liver biopsy specimens were obtained from ten patients with primary genetic hemochromatosis (HC) or secondary hemosiderosis (HS) as part of the patients' routine clinical evaluation. The histological samples (≥10mm) were fixed in 10% formaldehyde in phosphate buffered saline (pH 7.2) and embedded in paraffin. Three-micrometre sections were cut, mounted on glass slides, de-waxed in xylene and re-hydrated using graded alcohol/water baths. They were then rinsed in distilled water for five minutes and incubated for thirty minutes at room temperature with a freshly made staining solution consisting of the following reagents:

1. 1 g potassium ferrocyanide in 50 ml distilled water [2% (w/v)];
2. 1 ml concentrated hydrochloric acid in 49 ml distilled water [2% (v/v)];
3. 0.1 g Direct-red 80 (C. I. 35780, Sigma Chem. Co., MO, USA) in 100 ml saturated aqueous picric acid.

The sections were subsequently washed in running tap water for thirty minutes to remove excess staining, counter-stained in Mayer's hemalum solution for ten seconds, dehydrated, and mounted in Permount. The distribution of iron granules in parenchymal and non-parenchymal cells, as well as the collagen framework between the cells, were observed under light microscope (Zeiss Axiophot, Germany). The specificity of the double staining was tested by treating five histological sections with 5% aqueous oxalic acid for 12 hours in order to demonstrate the total absence of iron granules in the cell cytoplasm, whereas the collagen fibres were as strongly stained as in the non-treated histological sections.

RESULTS

The method was found to be rapid, inexpensive and easily applied to the routine clinical examination of liver biopsy specimens.

Under light microscopy, the iron can be seen as well-defined green or bluish-green granules (Fig.1a). The irregular distribution of iron in the cells and the amount of collagen fibres between them were dependent in terms of both *grade* (the extent of necro-inflammatory lesions) and *stage* (which reflects the disruption of normal hepatic architecture). During the initial disease phases, the iron granules were found around the portal spaces of the cytoplasm whereas, in the later phases, the

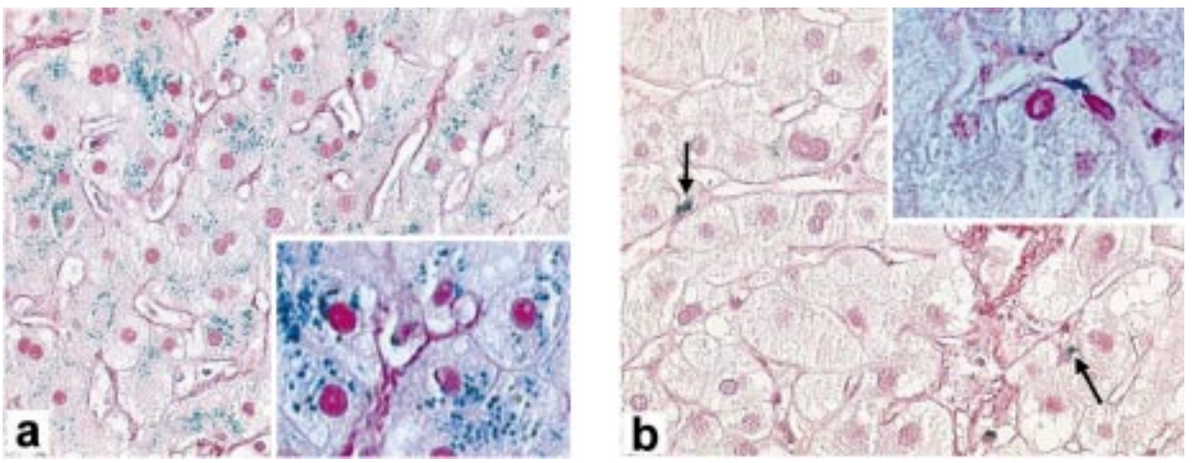

Fig. 1 - Liver histological sections taken from patients with chronic hepatitis C related-diseases and simultaneous liver cell iron overload. *A.* The iron deposited in parenchymal cells can be seen as well-defined green or bluish-green granules (objective magnification, 10x; *insert* 40x); *B.* Kupffer cell pigmentation is normally less marked but, during the late phases of iron overload hepatic diseases, are characterised by a higher amount of cytoplasmic iron (objective magnification 20x; *insert* 40x).

rest of the liver lobule became progressively involved. The pigmentation of the bile duct epithelium and Kupffer cells was normally less marked but, during the late phases of disease, they were characterised by a larger amount of iron inclusions in their cytoplasms (Fig.1b, arrows).

DISCUSSION

An increasing number of histochemical techniques have been developed and routinely applied to liver biopsy specimens in order to provide an accurate evaluation of tissue damage. The minimal advised techniques are hematoxylin-eosin and a reliable method for connective tissue. Reticulin, trichrome stains or elastic-van Gieson are the most common staining methods for the assessment of parenchymal changes and the detection of new collagen deposition. Additional routinely used stains provide extra details that are invisible or only poorly seen in routine hematoxylin-eosin stained sections.

All of these histochemical techniques are normally used to stain only one histological section each, which necessarily involves more *working time*, and requires the greater *use* and/or *availability* of the histological sample, because many preferably consecutive histological sections are needed to evaluate different variables in order to assess the possible causes of the progression of the liver disease. The continuing debate concerning these and other clinical questions prompted us to investigate the usefulness of a histochemical technique that allows the simultaneous detection of both natural and pathological extra-cellular collagen matrix and iron accumulation in liver parenchymal and non-parenchymal cells, including Kupffer cells. We also assessed the possibility of using it routinely to examine liver biopsies taken during the follow-up of patients at different institutions.

The importance of the proposed method lies in the fact that it allows the simultaneous assessment of the following histological parameters:

a. parenchymal, mesenchymal or mixed type iron overload and its distribution throughout the liver lobule or in the liver functional unit (Rappaport's acinus);
b. iron-related lesions (especially sideronecrosis) and iron-free foci;
c. the presence and extent of necro-inflammatory reactions;
d. the presence and irregular shape of abnormal collagen deposition;
e. the presence of micronodular, macronodular or other structural changes in parenchymal tissue;
f. other liver damage (i.e. fatty liver).

Although other stains, such as Acid Fuchsin have been used to evaluate ECM, Sirius red has become a popular stain for collagen because the color is rather fast in the stained sections. Moreover it renders the collagen fibers strongly anisotropic (Nielsen L.F., Moe D., Kirkeby S., and Garbarsch C.).

The triphenylmethane stain acid fuchsin (C.I. 42685) in the Gieson picrofuchsin stain for collagens has been replaced by several other stains and the technique has been modified to minimize fading of the collagen staining in the sections (Nielsen L.F., Moe D., Kirkeby S., and Garbarsch C.).

Our results suggest that the used technique can validly support the quantitative and semi-quantitative scoring systems so far used to quantify the amount and distribution of liver fibrosis, and grade the distribution and amount of iron overload (Basset M.L., Halliday J.W., Powell L.W., Faa G., Sciot R., Farci A.M.G., Pietrangelo A., *et al*.).

The relationship between iron overload and the response to therapeutic agents such as interferon suggests that this new staining technique may have even greater potential in terms of the routine examination of successive liver biopsy specimens taken during the follow-up of patients with chronic liver disease.

In conclusion, our results indicate that this simple, accurate and easily applied staining technique may be useful not only for diagnostic purposes, but also for providing new scientific explanations in both basic and applied pathological research.

ACKNOWLEDGEMENTS

The authors are very grateful to Patrizia Melodia and Corrado Filippone for their precious technical support.

REFERENCES

Andrews N.C. Disorders of iron metabolism. N. Engl. J. Med. *341*, 1986-1995, 1999.

Basset M.L., Halliday J.W., and Powell L.W. Value of hepatic iron measurements in early hemochromatosis and determination of the critical iron level associated with fibrosis. Hepatology *6*, 24-29, 1986.

Nielsen L.F., Moe D., Kirkeby S., and Garbarsch C. Sirius red and acid fuchsin Staining Mechanisms. Biotech Histochem. *73*, 71-77, 1997.

Faa G., Sciot R., Farci A.M.G., *et al*. Iron concentration and distribution in the newborn liver. Liver *14*, 193-199, 1994.

Finch C.A., and Huebers H. Perspectives in iron metabolism. N. Engl. J. Med. *306*, 1520-1528, 1982.

Pietrangelo A. Iron, oxidative stress and liver fibrogenesis. J. Hepatol. *28***,** 8-13, 1998.

Pietrangelo A. Iron, friend or foe? "Freedom" makes the difference. J Hepatol. *32*, 862-864, 2000.

Scheuer P.J., and Lefkowitch J.H. Liver biopsy interpretation. W.B. Saunders Company Ltd., 1992.