



Inducible nitric oxide synthase and histopathological correlation in chronic viral hepatitis

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Summary

Background: Chronic liver disorders represent a serious health problem. Nitric oxide (NO) synthesized by inducible nitric oxide synthase (iNOS) can function as an antimicrobial agent able to kill or reduce replication of microorganisms, and plays an important role in immune regulation. This study was undertaken to evaluate the expression of iNOS in chronic viral hepatitis and its relation to histopathology.

Methods: This study included 56 patients with chronic viral hepatitis (38 hepatitis B, 18 hepatitis C). There were 35 men and 21 women with a mean age of 38.6 ± 21.731 years. A modified form of the histology activity index (HAI) designed by Ishak and colleagues was used to assess grading and staging of chronic viral hepatitis. The needle biopsy specimens were fixed in 10% formalin and routinely processed. Routine hematoxylin–eosin, periodic acid–Schiff, and reticulin staining, and iNOS immunoperoxidase technique were performed on paraffin-embedded tissues.

Results: We demonstrated that all liver samples had a marked iNOS expression, with a diffuse distribution pattern. iNOS consistently labeled mononuclear cells infiltrating portal tracts in all samples. Statistical evaluation of data showed that the iNOS expression correlated with the HAI and fibrosis. Furthermore a correlation between iNOS and severity of disease was detected ($r = 0.772$, $p = 0.000$).

Conclusions: Further investigations are required to determine whether iNOS-related treatment protocols could be useful in reducing disease severity.

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Introduction

Chronic liver disorders represent a serious health problem, especially considering that 300 million people worldwide are hepatitis B virus carriers and that in the USA alone, 8000–10 000 patients die every year as a result of liver

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failure caused by hepatitis C.^{1,2} An epidemiological and clinical association between either hepatitis B virus (HBV) or hepatitis C virus (HCV) infections and the development of chronic hepatitis and the appearance of hepatocellular carcinoma is currently evident.^{3–5} The role of prolonged cellular damage such as in viral or bacterial infection-related chronic inflammation has become widely recognized.^{3–5} In humans hepatocellular carcinomas are usually associated with viral hepatitis B and C and cirrhosis resulting from chronic hepatitis.⁵ Much less is known about nitric oxide synthase (NOS) function in the human liver; human inducible nitric oxide synthase (iNOS) was first cloned from human hepatocytes in culture.¹

One of the major mediators in chronic inflammatory processes is nitric oxide (NO), which is produced by liver parenchymal and non-parenchymal cells from L-arginine via NOS.^{3,5,6} NO is considered to exert a hepatoprotective action against tissue injury and cytotoxic effects due to invading microorganisms, parasites and tumor cells.^{2,5} However any situation that causes uncontrolled, prolonged and/or massive production of NO by iNOS may result in liver damage, leading to inflammation and even tumor development.⁵

Mihm et al. have found that the hepatic expression of iNOS in chronic hepatitis C is positively related to the hepatic viral load and liver injury.⁷ A long-term overproduction of iNOS-generated NO may mediate important pathogenic events in the course of chronic viral hepatitis (CVH).³ We aimed to demonstrate the histopathological correlation with immunorexpression of iNOS in chronic viral hepatitis.

Materials and methods

This study included 56 patients with CVH (38 hepatitis B, 18 hepatitis C). A modified form of the histology activity index (HAI) designed by Ishak and colleagues was used to assess grading and staging of the CVH.⁸ There were 35 men and 21 women with a mean age of 38.6 ± 21.731 years (age range 3–75).

Liver biopsy specimens from CVH patients were obtained using a Menghini needle by percutaneous route. The needle biopsy specimens were fixed in 10% formalin and routinely processed. Routine hematoxylin–eosin, periodic acid–Schiff, and reticulin staining were performed on paraffin-embedded tissues. These case slides were reviewed, and the immunoperoxidase technique was performed with iNOS monoclonal antibody to selected sections from each case.

Immunohistochemical staining was carried out by deparaffinization, dehydration, and incubation in citrate buffer. Single antigen staining was performed with iNOS protein (monoclonal, biotinylated goat anti-rabbit) and Ultravision Polyvalent, HRP-AEC kit (Neomarkers-Biogen, Lab Vision, USA). A labeled streptavidin–biotin–peroxidase (immunoenzymatic) antigen detection system and AEC chromogen were used. For the evaluation of iNOS, positive stained cells were counted in five different areas at a magnification $\times 40$ using an Olympus BX51 microscope in each case. The mean values were calculated. iNOS expression was independently recorded by two pathologists and the results were averaged.

The local medical ethics committee approval for the study was obtained, and all patients included in the study gave informed consent.

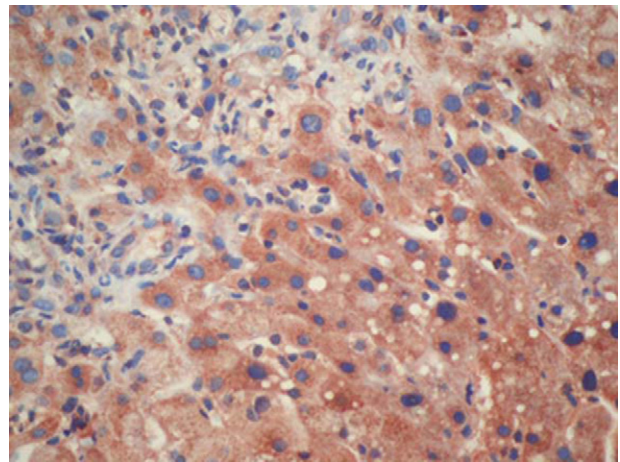


Figure 1 iNOS positive stained hepatocytes with steatosis and interface hepatitis in a case of severe chronic hepatitis (iNOS $\times 400$).

Statistical procedures were carried out using the software of Epi INFO version 3.3.2 (CDC, The Centers for Disease Control and Prevention, USA) and a p -value < 0.05 was considered statistically significant. For comparison of the findings, Spearman's correlation test was performed.

Results

The chronic hepatitis cases were classified as mild chronic hepatitis (1), moderate chronic hepatitis (36), and severe chronic hepatitis (19), according to the Ishak scoring system. We demonstrated that all liver samples from patients with either chronic hepatitis B (CHB) or chronic hepatitis C (CHC) had a marked iNOS expression, with a diffuse distribution pattern throughout the hepatic lobules, which is consistent with the literature.^{3,4,9} Hepatocellular positive iNOS staining was mainly cytoplasmic (Figures 1 and 2). The mean immunorexpression value of iNOS was 45.17 ± 19.702 . The distribution of iNOS in CHB exhibited an almost identical pattern to that in CHC. Furthermore iNOS consistently labeled

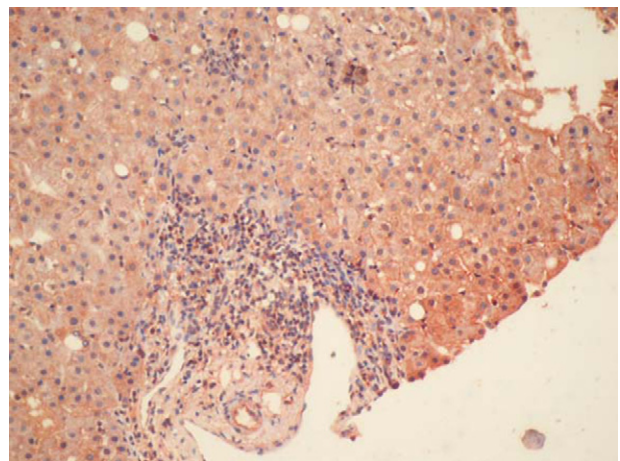


Figure 2 iNOS immunoexpression with a diffuse distribution pattern throughout the hepatic lobules and monocytes (iNOS $\times 200$).

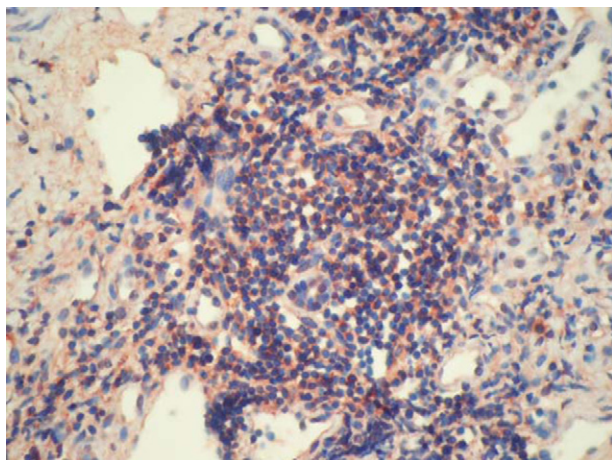


Figure 3 Collections of inflammatory cells positive stained by iNOS in a portal area (iNOS $\times 400$).

mononuclear cells infiltrating portal tracts in all samples (Figure 3). No iNOS induction was observed in normal liver samples.

Statistical evaluation of data showed that the iNOS expression correlated with the HAI and fibrosis (Table 1). There was a positive correlation with iNOS positivity and patient age ($r = 0.47$, $p < 0.001$), but there was no correlation between iNOS immunopositivity and gender. Furthermore a correlation between iNOS immunoexpression and severity of disease was detected ($r = 0.772$, $p = 0.000$).

Discussion

It is conceivable that in the intrahepatic microenvironment of CVH an important source of NO exists.^{3,9} Since a marked induction of iNOS expression is observed in the majority of hepatocytes from patients with CVH, it is likely that these liver cells are a powerful cellular source of large quantities of NO.^{1,3,9}

In CVH, it has been suggested that the viral infection itself, by mechanisms not yet fully understood, may be a triggering factor.⁹ It could be suggested that this nitration

process represents a non-specific pathogenic mechanism, common to different chronic inflammatory diseases and secondary to the elicitation of inflammation rather than its cause.^{3,9} Many studies have identified several risk factors such as age, gender, immune status, viral factors, alcohol consumption, cigarette smoking, exposure to aflatoxin, and viral superinfection as causes of liver disease progression.^{10,11} We also found a positive correlation with iNOS positivity and patient age ($r = 0.47$, $p < 0.001$), but there was no correlation between iNOS immunopositivity and gender in our study.

Smoking increases the production of pro-inflammatory cytokines (interleukin (IL)-1, IL-6 and tumor necrosis factor- α (TNF α)) that are involved in liver cell injury and affect both cell-mediated and humoral immune responses.¹² iNOS can also be induced by TNF α and interferon- γ (IFN γ) in many immune cells. These pathways may contribute to the pathobiology of Th1-mediated liver injuries.^{6,8}

Disturbance of the hepatocellular oxidant–antioxidant balance may contribute to the pathogenesis of progressive liver damage in chronic viral liver disease.^{3,9}

High concentrations of NO are generated by iNOS, which is expressed in activated macrophages and in hepatocytes.^{3,6} We demonstrated that all liver samples from patients with either CHB or CHC had a marked iNOS expression, with a diffuse distribution pattern throughout the hepatic lobules, consistent with the literature.^{3,4,9} Furthermore iNOS consistently labeled mononuclear cells infiltrating portal tracts in all our samples.

Intracellular pathogens are shielded from host extracellular defenses by the cell membrane. NO, a gaseous radical, readily diffuses across this membrane. It appears to play a significant role in the clearance of intracellular pathogens and may also be of potential importance in some viral infections.^{6,13} The protective function of NO in liver inflammation, probably related to its anti-apoptotic capacity, needs further investigation.⁹

NO, a small potent lipophilic gas with different biological activities, has important roles in modulating tissue injury and carcinogenesis.^{6,13} Three distinct forms of NOS catalyze the formation of NO.¹⁰ NO synthesized by iNOS can function as an antimicrobial agent able to kill or reduce replication of viruses, bacteria or protozoa, and plays an important role

Table 1 Bivariate correlations between iNOS, severity of disease, HAI, and fibrosis with Spearman's correlation analysis

		Severity of disease	HAI	Fibrosis	Age of patient
iNOS	Correlation coefficient	0.772 ^b	0.816 ^b	0.439 ^b	0.473 ^b
	Sig. (two-tailed)	0.000	0.000	0.001	0.000
	N	56	56	56	56
Severity of disease	Correlation coefficient		0.821 ^b	0.588 ^b	0.276 ^a
	Sig. (two-tailed)		0.000	0.000	0.040
	N		56	56	56
HAI	Correlation coefficient			0.582 ^b	0.384 ^b
	Sig. (two-tailed)			0.000	0.003
	N			56	56
Fibrosis	Correlation coefficient				0.281 ^a
	Sig. (two-tailed)				0.036
	N				56

iNOS, inducible nitric oxide synthase; HAI, histology activity index. N = number of the patients.

^a Correlation is significant at the 0.05 level (two-tailed).

^b Correlation is significant at the 0.01 level (two-tailed).

in immune regulation.² The trigger mechanism for iNOS induction in CHC is not known.⁹ It has also been shown that hepatitis B surface antigen induces iNOS in cultured hepatocytes, which may play a role in hepatocarcinogenesis.⁵ iNOS may contribute to tumor promotion via NO production and the subsequent action of peroxynitrite.⁵ NO has a role in host defense in the normal liver,⁵ but may act in cancer promotion by stimulating aberrant differentiation of the cells and angiogenesis, and inducing tissue DNA damage.⁵

In this study, we have demonstrated that chronic liver disease is associated with an enhanced intrahepatic iNOS expression, which promotes increased synthesis of NO. Moreover, we have shown that NO-mediated nitration of hepatocellular proteins is substantially induced in the inflamed liver tissue from patients with CVH. This phenomenon correlated with the HAI and fibrosis. In chronic hepatitis infection the iNOS expression was found to correlate to the disease severity.

Further prospective investigations are required to determine whether iNOS-related treatment protocols could be useful in reducing disease severity and whether they could be beneficial in the antiviral treatment of some patients with CVH.

Conflict of interest: No conflict of interest to declare.

References

1. McNaughton L, Puttagunta L, Martinez-Cuesta MA, Kneteman N, Mayers I, Moqbel R, et al. Distribution of nitric oxide synthase in normal and cirrhotic human liver. *Proc Natl Acad Sci U S A* 2002;**99**:17161–6.
2. Chang WW, Su IJ, Lai MD, Chang WT, Huang W, Lei HY. The role of inducible nitric oxide synthase in a murine acute hepatitis B virus (HBV) infection model induced by hydrodynamics-based in vivo transfection of HBV-DNA. *J Hepatol* 2003;**39**:834–42.
3. Monzon CG, Majano PL, Zubia I, Sanz P, Apolinario A, Otero RM. Intrahepatic accumulation of nitrotyrosine in chronic viral hepatitis is associated with histological severity of liver disease. *J Hepatol* 2000;**32**:331–8.
4. Schweyer S, Mihm S, Radzun HJ, Hartmann H, Fayyazi A. Liver infiltrating T lymphocytes express interferon gamma and inducible nitric oxide synthase in chronic hepatitis C virus infection. *Gut* 2000;**46**:255–9.
5. Ahn B, Han BS, Kim DJ, Ohshima H. Immunohistochemical localization of inducible nitric oxide synthase and 3-nitrotyrosine in rat liver tumors induced by *N*-nitrosodiethylamine. *Carcinogenesis* 1999;**20**:1337–44.
6. Lake-Bakaar G, Sorbi D, Mazzocchi V. Nitric oxide and chronic HCV and HIV infections. *Dig Dis Sci* 2001;**46**:1072–6.
7. Mihm S, Fayyazi A, Ramadori G. Hepatic expression of inducible nitric oxide synthase transcripts in chronic hepatitis C virus infection: relation to hepatic viral load and liver injury. *Hepatology* 1997;**26**:451–8.
8. Ishak K, Baptista A, Bianchi L, Callea F, De Groote J, Gudat F, et al. Histological grading and staging of chronic hepatitis. *J Hepatol* 1995;**22**:696–9.
9. Sanz-Cameno P, Medina J, Garcia-Buey L, Garcia-Sanchez A, Borque MJ, Martin-Vilchez S, et al. Enhanced intrahepatic inducible nitric oxide synthase expression and nitrotyrosine accumulation in primary biliary cirrhosis and autoimmune hepatitis. *J Hepatol* 2002;**37**:723–9.
10. Wong LL, Limm WM, Tsai N, Severino R. Hepatitis B and alcohol affect survival of hepatocellular carcinoma patients. *World J Gastroenterol* 2005;**11**:3491–7.
11. Liaw YF, Sollano JD. Factors influencing liver disease progression in chronic hepatitis B. *Liver Int* 2006;**26**(Suppl 2):23–9.
12. El Zayadi AR. Heavy smoking and liver. *World J Gastroenterol* 2006;**12**:6098–101.
13. Rahman MA, Dhar DK, Yamaguchi E, Maruyama S, Sato T, Hayashi H, et al. Coexpression of inducible nitric oxide synthase and COX-2 in hepatocellular carcinoma and surrounding liver: possible involvement of COX-2 in the angiogenesis of hepatitis C virus-positive cases. *Clin Cancer Res* 2001;**7**:1325–32.