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Pseudouridine for Monitoring Interferon Treatment of Patients with Chronic Hepatitis C

Alfredo Colonna[1], *Vincenzo Guadagnino*[2], *Aldo Maiorano*[2], *Elvira Stamile*[2] and *Chiara Costa*[2]

[1] Dipartimento di Farmacologia Sperimentale, Università di Napoli Federico II, Napoli, Italy
[2] Dipartimento di Medicina Sperimentale e Clinica, Università di Reggio Calabria, Catanzaro, Italy

Summary: Pseudouridine is a modified nucleoside derived from RNA catabolism; the concentration of this nucleoside is elevated in body fluids of both tumour-bearing and human immunodeficiency virus (HIV) infected patients. We used an HPLC procedure to evaluate the serum pseudouridine concentration in patients with chronic hepatitis C in an attempt to determine whether the nucleoside serum concentration was related to the response to α-interferon treatment.

We found that:

a) pseudouridine serum concentration was increased significantly in 76% (29/39) of patients with chronic hepatitis C at the time of diagnosis and before any therapeutic treatment;

b) pseudouridine excretion was higher in patients affected by chronic hepatitis C with cirrhosis;

c) there was a positive correlation between response to therapy and pseudouridine serum concentration in patients undergoing treatment with α-interferon;

d) during one year of α-interferon treatment, the pseudouridine serum concentration remained within the normal range in responder patients.

These results indicate that serum pseudouridine might be useful as a valuable biochemical marker with which to monitor chronic hepatitis C patients treated with α-interferon.

Introduction

Hepatitis C virus is the most important causative agent of non-A, non-B hepatitis (1); the infection tends to become chronic and to progress to cirrhosis and/or hepatocellular carcinoma (2). The virus contains a positive-stranded RNA genome with only one open reading frame encoding for a single polyprotein precursor of 3011 amino acids that is cleaved into three structural proteins and four non-structural enzymatic proteins (3).

Interferons are a heterogeneous family of glycoproteins with antiviral and antiproliferative activity (4); α-interferon has been shown to normalize aminotransferase levels and reduce disease activity in a significant number of patients affected by chronic hepatitis C (5). Interferon treatment is a long-term therapy and can cause adverse effects. Therefore, it is critical to monitor the response to therapy in order to adjust therapeutic protocols.

Pseudouridine, a modified nucleoside derived from RNA degradation, mainly tRNA, is detectable in mammalian body fluids (6). It is not reutilized by the cell or further degraded, but is excreted as an end product of RNA catabolism (7); consequently, pseudouridine is excreted unchanged and is thus a faithful index of RNA turnover (8). Numerous studies have shown that when RNA turnover is increased, as during neoplastic growth or HIV infection, the concentration of pseudouridine in body fluids increases (9–16).

We evaluated serum pseudouridine concentrations in patients with chronic hepatitis C to determine whether viral RNA metabolism (duplication and/or translation) causes changes in pseudouridine concentration, and whether such changes reflect the progression of the infection, i. e. we determined the potential value of pseudouridine as a biochemical marker in the monitoring of chronic hepatitis C patients treated with interferon.

Materials and Methods

Controls

Twenty-six apparently healthy volunteers of both sexes, from the Clinical Biochemistry Department of the School of Medicine, Catanzaro, Italy, were enrolled as controls. They were not taking any drugs, their serum values for various biochemical quantities were normal, and they were negative for hepatitis C virus RNA.

Patients

Sixty-five consecutive subjects (32 males and 33 females, mean age 51.9 ± 13.3; age range 14–80) affected by active chronic hepatitis C of different histologically determined severity, attending the Infectious Disease Out-Patient Unit of the School of Medicine, Catanzaro, were enrolled in the study. Twenty-one of these patients received α-interferon treatment; 38 patients without cirrhosis and 6 with active hepatitis C virus cirrhosis, all at the time of diagnosis, were untreated.

The disease was diagnosed from histological examination of percutaneous liver biopsy specimens according to conventional criteria. Anti-hepatitis C virus antibody was detected by second generation enzyme-linked immunosorbent assay, and serum viral RNA was detected with the polymerase chain reaction technique. Patients with autoimmune hepatitis, alcoholic or drug-related liver disease, haemochromatosis, *Wilson*'s disease or α_1-antiproteinase deficiency were excluded. The biochemical tests also used for exclusion, were: bilirubin > 51.3 μmol/l, albumin < 30 g/l, prothrombin time > 3 s longer than that of the control, serum creatinine > 150 μmol/l, platelet count < 100 × 10^9/l, granulocyte count < 1.5 × 10^9/l. The study protocols were approved by the local ethics committee and informed consent to the study was obtained from each patient.

Sample collection

Venous blood was collected into vacutainers tube (Becton Dickinson, Milan, Italy) and centrifuged within 2 h of withdrawal; alanine and aspartate aminotransferase were analysed thereafter. Aliquots of serum samples were stored at −80 °C until pseudouridine analysis. Alanine and aspartate aminotransferase were assayed with a Cobas Mira S analyser (Roche Diagnostic Systems, Milan, Italy) using reagents from Roche.

Hepatitis C virus RNA detection

The serum of those patients positive for anti-hepatitis C virus antibody by second generation enzyme-linked immunosorbent assay (ELISA Ortho Diagnostic, Milan, Italy), was then tested for hepatitis C virus-RNA, using Amplicore HCV (Roche Diagnostic Systems, Milan, Italy).

Treatment with α-interferon

Twenty-one patients (10 males and 11 females) with active chronic hepatitis C, showing an increase of alanine aminotransferase of at least twice the upper limit of the reference range for more than 6 months, received 3 MU of recombinant interferon α-2b (Intron A) subcutaneously three times a week for 3 months. Patients who responded to treatment, i.e., return to normal serum alanine aminotransferase levels, received 3 MU of α-interferon for another 9 months and then 2 MU for 2 months and 1 MU for 1 month. Non-responder subjects, after the first three months of treatment, were given a second cycle of α-interferon at 6 MU for 6 months; if their serum alanine aminotransferase levels normalized, they underwent a protocol with decreasing doses. Therapy was stopped in non-responder subjects. Pseudouridine serum concentrations were measured at 1, 3, 6 and 12 months from the beginning of interferon therapy.

Pseudouridine determination

Serum pseudouridine was measured as previously described (17). Briefly, 0.5 ml of serum was deproteinized with an equal volume of cold acetonitrile, kept on ice for 15 min and centrifuged at 3000 *g* for 10 min. Nucleosides were purified by affinity chromatography on phenylboronic gel (Affigel 601, Bio Rad Laboratories, Segrate, Italy) equilibrated with 0.25 mol/l ammonium acetate, pH 8.5. Nucleosides were eluted with 15 ml of 0.1 mol/l formic acid; 2.5 nmol/250 μl of 2'-deoxyguanosine were added as internal standard, after which the samples were lyophilized. Aliquots of 25 μl were injected onto a C_{18}-μBondapack column (Waters Associates, USA) in an HPLC HP1050 system (Hewlett Packard, USA). The column was eluted with 10 mmol/l $NH_4H_2PO_4$ solution containing 60 ml of CH_3OH per litre at a flow-rate of 1 ml/min. Pseudouridine peaks were recorded and integrated with a 3396 HP system. All the reagents were of HPLC grade.

Statistics

The means and standard deviations were computed for each variable. The ANOVA "one-way" analysis of the variance (Epistat program) was used to evaluate significance.

Results

In the 26 controls the serum concentrations of pseudouridine, aspartate aminotransferase and alanine aminotransferase were 2.6 ± 0.2 mmol/l, 25 ± 13 U/l and 23 ± 16 U/l, respectively. This was in good agreement with the reference range of pseudouridine previously found in a healthy population from Italy and the United States (18). The pseudouridine cut-off level was set at the mean value plus 2 S.D. of normal controls (3.0 μmol/l).

The pseudouridine serum concentrations determined in the 38 patients with chronic hepatitis C at diagnosis and before any treatment are shown in table 1. Serum pseudouridine was higher in 29 (76%) of these 38 patients than in the control group, and serum aminotransferase levels were in the reference range.

Of the 21 patients treated with α-interferon, 12 responded to treatment and 9 did not. Table 2 shows the mean values of pseudouridine, alanine and aspartate aminotransferase in the 12 responders and in the 9 non-responders. In the 12 responder patients normalization of aminotransferase paralleled the decrease in pseudouridine concentration to the reference range. In contrast, pseudouridine concentration increased significantly in non-responder patients. The highest serum pseudouridine concentrations were found in the 6 patients with chronic C hepatitis which had evolved to liver cirrhosis.

Table 3 shows the profile of alanine aminotransferase and pseudouridine concentrations in the serum of seven responder patients from the time of diagnosis until 12 months after the start of treatment. Both analytes de-

Tab. 1 Aspartate aminotransferase, alanine aminotransferase and pseudouridine serum concentrations in 38 patients with chronic hepatitis C at the time of diagnosis and before α-interferon treatment.

Patient number	Aspartate amino-transferase (U/l ± S.D.)	Alanine amino-transferase (U/l ± S.D.)	Pseudo-uridine[a] (μmol/l ± S.D.)	%
26 (reference range)	25 ± 13	23 ± 16	2.6 ± 0.2	
9	61 ± 37	78 ± 53	2.7 ± 0.26	24
29	51 ± 27	60 ± 35	5.2 ± 1.4	76

[a] average of three determinations

creased gradually during treatment, although the greatest decrease occurred in the first month of α-interferon therapy. It is noteworthy that pseudouridine concentration preceded the normalization of alanine aminotransferase, in fact the pseudouridine concentrations had already decreased to reference values after the first month of treatment, while alanine aminotransferase was stationary.

Discussion

Interferon is currently becoming the treatment of choice in chronic hepatitis C virus infection. However, the response to interferon treatment is heterogeneous, and is probably related to viral strain, genetic factors or host immunity (19, 20). Moreover, there is as yet no laboratory test giving an early prediction of the success or otherwise of interferon therapy. Currently, the response to treatment is evaluated from the normalization of serum alanine aminotransferase and the loss of virus from the serum as detected by quantitative polymerase chain reaction (PCR) of hepatitis C virus-RNA. However, PCR is still far from being a routine test, because of the lack of standardization, risk of contamination and poor automation. Other markers, such as the *Knodell* index, procollagen III and anti-hepatitis C virus antibodies, are under study and may serve as adjunctive markers of treatment response (21, 22).

We examined the relationship between pseudouridine serum concentration and the response to α-interferon treatment in patients with chronic hepatitis C to assess the possibility of using pseudouridine, a catabolic RNA index, as a biochemical marker of the replicative blockage of the viral cycle induced by interferon. We determined the serum pseudouridine concentration in patients with chronic hepatitis C at initial diagnosis and before any therapeutic treatment, to evaluate the correlation of the elevation of the nucleoside with the disease activity. Pseudouridine levels were significantly elevated in 76% of the patients, and even higher levels were found in six patients with liver cirrhosis. The elevation of the nucleoside in the 6 cirrhotics agrees with a study in which the pseudouridine concentration increased when cirrhosis evolved to hepatocellular carcinoma (23). Possibly these patients with advanced disease are in transition phase to hepatocellular carcinoma, or they could already have developed an undetectable small cancer.

A significant decrease in pseudouridine serum levels occurred only in cases of an effective response to α-interferon treatment (responder patients), thus revealing a good correlation between the response to the therapy and the nucleoside serum concentration. Long-term (12 months) therapy monitoring showed that the pseudouridine serum concentration decreased soon after the beginning of α-interferon therapy and much earlier than alanine aminotransferase normalization.

Elevated excretion of pseudouridine has been demonstrated in patients with various malignancies (8–16), although the biochemical basis of this phenomenon is unclear. That the increase in pseudouridine level is disease-related and not caused by tissue destruction is supported by the observation that in cancer patients the elevated excretion of pseudouridine and other modified nucleosides is the consequence of a higher turnover rate of tRNA in tumour tissue than in the corresponding normal tissue (24). It is not unreasonable to suppose that the excretion of pseudouridine in patients infected by hepa-

Tab. 2 Serum aminotransferase and pseudouridine concentrations in patients undergoing α-interferon treatment and in untreated cirrhotics.

Patients	N	Aspartate aminotransferase (U/l ± S.D.)	Alanine aminotransferase (U/l ± S.D.)	Pseudouridine (μmol/l ± S.D.)	P (vs controls)
Responders	12	28 ± 7	35 ± 8	2.8 ± 0.4	N.S.
Non-responders	9	69 ± 30	106 ± 79	5.6 ± 1.2	<0.003
Cirrhotic evolution[a]	6	108 ± 73	94 ± 40	9.3 ± 0.9	<0.001

[a] without α-interferon treatment
The results are the means of pseudouridine determinations at 1 and/or 3 months of α-interferon treatment.

Tab. 3 Alanine aminotransferase and pseudouridine concentrations in seven responder patients during a 12-month follow-up period.

	Interferon treatment (months[a])				
	Before	1	3	6	12
Alanine aminotransferase (U/l ± S.D.)	63.3 ± 29	61 ± 10	45 ± 8	38 ± 5	33 ± 2
Pseudouridine (μmol/l ± S.D.)	4.8 ± 0.9	1.8 ± 0.3	2.2 ± 0.2	2.3 ± 0.2	2.9 ± 0.6

[a] from the beginning of α-interferon treatment

titis C virus is increased. In fact, although the mechanisms of intracellular hepatitis C virus replication have not yet been clarified, it seems conceivable that, since hepatitis C virus is a positive-stranded RNA virus, early molecular events of the virus cycle such as the translation of the viral proteins, are accompanied by an enhanced turnover-rate of the RNA populations involved. Furthermore, the activity of enzymes involved in nucleoside metabolism appears to be increased in cirrhotic subjects (25) and HIV patients excrete elevated levels of pseudouridine (8, 16). Lastly, the structure of the genomic HCV region encoding the E_2 envelope protein is similar to that of HIV gp120 envelope protein (26).

In conclusion, given the correlation between serum pseudouridine levels and the effective responses to α-interferon treatment of patients with chronic hepatitis C, and given the simplicity and accuracy of the methodology, serum pseudouridine might be clinically useful as a marker of the response to interferon treatment in chronic hepatitis C. However, a more extended investigation on a greater number of patients is needed to verify this hypothesis and to evaluate the possible predictive value of pseudouridine for the response to α-interferon treatment.

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Corresponding author: Prof. Alfredo Colonna, Dipartimento di Farmacologia Sperimentale, Università di Napoli Federico II, Via D. Montesano 49, I-80131 Napoli, Italy