Lactoferrin Inhibits Hepatitis C Virus Viremia in Patients with Chronic Hepatitis C: A Pilot Study

Katsuaki Tanaka,1,4 Masanori Ikeda,1,2 Akito Nozaki,1,2 Nobuyuki Kato,2 Hiroyuki Tsuda,3 Satoru Saito1 and Hisahiko Sekihara1

1Third Department of Internal Medicine, Yokohama City University School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004 and 2Virology Division, 3Experimental Pathology and Chemotherapy Division, National Cancer Center Research Institute, Tokyo 104-0045

Hepatitis C virus (HCV) is associated with the development of cirrhosis and hepatocellular carcinoma. We recently found that bovine lactoferrin, a milk protein belonging to the iron transporter family, effectively prevented HCV infection in cultured human hepatocytes (PH5CH8). We tested the hypothesis that lactoferrin inhibits HCV viremia in patients with chronic hepatitis C. Eleven patients with chronic hepatitis C received an 8-week course of bovine lactoferrin (1.8 or 3.6 g/day). At the end of lactoferrin treatment, a decrease in serum alanine transaminase and HCV RNA concentrations was apparent in 3 (75%) of 4 patients with low pretreatment serum concentrations of HCV RNA. However, 7 patients with high pretreatment concentrations showed no significant changes in these indices. This pilot study suggests that lactoferrin is one potential candidate as an anti-HCV reagent that may be effective for the treatment of patients with chronic hepatitis.

Key words: Lactoferrin — Chronic hepatitis — Hepatitis C virus

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis1,2 and progresses to liver cirrhosis and hepatocellular carcinoma.3,4 To date, there have been no vaccines and no anti-HCV agents other than interferon (IFN). IFN is effective at reducing increased serum concentrations of aminotransferase and improving liver histology in individuals with chronic hepatitis C.5,6 However, only about 50% of patients respond to IFN therapy, and half of those in whom the serum concentration of aminotransferase is restored to normal relapse after the cessation of treatment.7,8 In Japan, IFN’s effectiveness is limited to about 30% of cases.9 Furthermore, IFN’s side effects are sometimes severe enough to warrant cessation of treatment.

We recently established an HCV replication system using a non-neoplastic human hepatocyte line, PH5CH10,11 and its clonal cell line, PH5CH8,12 which were immortalized with simian virus 40 large T antigen. This culture system allows us to evaluate the antiviral activities of several promising reagents. One such reagent is lactoferrin, a milk protein belonging to the iron transporter family. Lactoferrin is especially rich in colostrum, existing at a maximum level of 3%,13 and is known to be a primary defense protein against pathogenic microorganisms,14,15 including viruses. To date, it has been reported that lactoferrin has an antiviral effect against human herpes simplex virus-1,13 human immunodeficiency virus-1 and human cytomegalovirus,16 and simian rotavirus17 in cultured cells. For this reason, we examined the anti-HCV effects of bovine lactoferrin using a culture system which permits HCV replication. We found that bovine lactoferrin effectively prevents HCV infection in human hepatocytes, and demonstrated that the anti-HCV activity of bovine lactoferrin was due to the interaction between bovine lactoferrin and HCV.18 These observations prompted us to undertake a study evaluating the effect of bovine lactoferrin on HCV viremia in patients with chronic hepatitis C. This is the first report describing the anti-HCV activity of lactoferrin in patients with chronic hepatitis C.

Eleven Japanese patients (5 males, 6 females, 35 to 66 years old, mean age: 52 years) with chronic hepatitis C from the outpatient Hepatitis Clinic at Yokohama City University Hospital were randomly enrolled in the trial between June 1997 and September 1998. All patients had elevated levels of serum alanine transaminase (ALT) and HCV RNA at 6 months of follow-up or later. Eight patients had previously received IFN treatment but had not responded. The remaining 3 patients had not received IFN treatment because of advanced age or refusal to be treated. Liver histology revealed features of chronic active hepatitis 2A in 5 patients and chronic active hepatitis 2B in 6 patients. HCV genotype analysis indicated genotype...
1b in 6 patients, genotype 2a in 4 patients, and genotype 2b in 1 patient. HCV RNA concentrations ranged from 2 to 962 kcopy/ml (mean, 302 kcopy/ml). None had previously received corticosteroid or immunosuppressive treatment. None had a history of alcohol or drug abuse or evidence of metabolic or autoimmune disorders. All patients tested positive for HCV antibodies by enzyme-linked immunosorbent assay (Ortho-HCV ELISA; Ortho Diagnostics, Tokyo). The study was performed according to the guidelines in the Helsinki Declaration. Informed consent was obtained from all patients.

All patients received an 8-week course of lactoferrin treatment. The initial 7 patients received 1.8 g of bovine lactoferrin daily for 8 weeks (total dose: 100.8 g) and the following 4 patients received 3.6 g of bovine lactoferrin daily for 8 weeks (total dose: 201.6 g). Bovine lactoferrin tablets (450 mg) were kindly provided by Morinaga Milk Industry Co. (Tokyo). At the end of lactoferrin treatment, a response to therapy was defined as a decrease of at least 50% in serum ALT and/or HCV RNA concentration compared to the pretreatment value. Serum ALT and HCV viremia were measured immediately before treatment and every 2 weeks thereafter for the next 12 weeks. Serum ALT concentration was determined with a sequential multiple autoanalyzer. Serum HCV RNA concentration was quantitated using an Amplicor HCV Monitor (Nihon Roche, Tokyo). Serum levels of HCV RNA are expressed as copy number per milliliter of serum.

At the end of lactoferrin treatment, a decrease in serum ALT concentration and/or a decrease or absence of serum HCV RNA concentration were apparent in 3 patients (cases 1, 2 and 4); however, the remaining 8 patients (cases 3 and 5 to 11) showed no significant changes in these indices (Table I). Pretreatment serum HCV RNA concentrations of the 3 patients who responded to lactoferrin were less than 100 kcopy/ml. When the response rates were evaluated with regard to the pretreatment HCV RNA concentrations, 3 (75%) of 4 patients whose pretreatment serum HCV RNA concentrations were less than 100 kcopy/ml responded to lactoferrin, while patients whose pretreatment serum HCV RNA concentrations were more than 100 kcopy/ml did not respond to these doses of lactoferrin.

During the follow-up period, 2 (cases 1 and 4) of the 3 patients who responded to lactoferrin relapsed 2 weeks after cessation of the treatment. In case 1, re-treatment with lactoferrin was instituted (Fig. 1). Two weeks after cessation of the initial course of lactoferrin, the serum HCV RNA concentration was markedly elevated (105 kcopy/ml) and serum transaminase levels started to increase (48 U/liter). Four weeks after cessation of lactoferrin, she had an ALT level of 170 U/liter. A second course of lactoferrin (1.8 g/day) was instituted, following which an improvement in serum aminotransferase activities and a decrease in serum HCV RNA concentration were again noted. In case 4, 2 weeks after cessation of the

| Table I. Characteristics of Patients at the Start of the Study and Responses to an 8-Week Course of Lactoferrin Treatment |
| --- |
| **Case No.** | **Age (yr)/ Sex** | **Histology** | **Genotype** | **Serum alanine transaminase (U/liter)** | **Serum HCV RNA concentration (kcopy/ml)** |
|  |  |  |  | **During treatment (weeks)** | **End** | **During treatment (weeks)** | **End** |
|  |  |  |  | Before | 2 w | 4 w | 6 w | End | 2 w | 4 w | Before | 2 w | 4 w | 6 w | End |
| Low pretreatment serum HCV RNA concentration (less than 100 kcopy/ml) group |
| 1. | 58/female | 2A/2a | 139 | 107 | 70 | 22 | 26 | 48 | 170 | 68 | 56 | 4 | <1 | 5 | 105 | 81 |
| 2. | 66/female | 2B/2a | 56 | 17 | 26 | 24 | 27 | 24 | 26 | 2 | <1 | <1 | <1 | <1 | <1 | 1 |
| 3. | 51/female | 2A/1b | 30 | 43 | 46 | 48 | 50 | 31 | 33 | 77 | 64 | 80 | 85 | 98 | 94 | 72 |
| 4. | 43/male | 2B/2a | 254 | 225 | 243 | 250 | 116 | 74 | 55<sup>c</sup> | 23 | <1 | 7 | 9 | 12 | 69 | <1<sup>c</sup> |
| High pretreatment serum HCV RNA concentration (more than 100 kcopy/ml) group |
| 5. | 62/male | 2B/2b | 185 | 177 | 160 | 193 | 204 | 119<sup>c</sup> | 93<sup>c</sup> | 316 | 222 | 215 | 349 | 287 | 385<sup>c</sup> | 509<sup>c</sup> |
| 6. | 53/female | 2A/1b | 96 | 83 | 81 | 78 | 76 | 66 | 90 | 962 | 793 | 1231 | 503 | 1155 | 1031 | 585 |
| 7. | 49/male | 2B/1b | 101 | 97 | 84 | 89 | 107 | 97 | 79 | 368 | 454 | 660 | 864 | 510 | 352 | 789 |
| 8. | 49/female | 2B/1b | 79 | 105 | 82 | 82 | 75 | 60 | 78 | 488 | 569 | 154 | 322 | 378 | 477 | 537 |
| 9. | 35/male | 2A/2a | 32 | 19 | 25 | 25 | 20 | 22 | 25 | 368 | 304 | 267 | 450 | 378 | 225 | 376 |
| 10. | 50/male | 2A/1b | 90 | 72 | 72 | 57 | 74 | 37 | 23 | 121 | 102 | 206 | 140 | 139 | 65 | 6 |
| 11. | 58/female | 2B/1b | 34 | 40 | 31 | 39 | 39 | 75 | 42 | 529 | 478 | 476 | 585 | 484 | 420 | 653 |

<sup>a</sup> 2A, chronic active hepatitis 2A; 2B, chronic active hepatitis 2B.

<sup>b</sup> 3.6 g of bovine lactoferrin administered.

<sup>c</sup> IFN treatment administered.

<sup>d</sup> Stronger neo minophagen C administered.
Lactoferrin treatment, the serum HCV RNA concentration was elevated (69 kcopy/ml) and IFN treatment was instituted, following which an improvement in serum aminotransferase activities and a decrease in serum HCV RNA concentration were noted.

No serious complications occurred during or after the treatment. No increases in the serum alkaline phosphatase, lactate dehydrogenase, or total bilirubin levels were observed in any patient. Iron studies were normal in all patients.

Lactoferrin is a multifunctional milk protein associated with many biological activities, including primary nonspecific defense against pathogenic microorganisms. Recently, we found that bovine lactoferrin effectively prevents HCV infection in human PH5CH8 hepatocytes, and demonstrated that the anti-HCV activity of bovine lactoferrin was due to an interaction between bovine lactoferrin and HCV. In this paper, we have demonstrated for the first time that treatment with lactoferrin (1.8 or 3.6 g/day) may be associated with a dramatic decrease in serum HCV RNA concentration and/or a decrease in serum ALT concentration in 3 patients with low pretreatment serum concentrations of HCV RNA. These results strongly suggest that lactoferrin has antiviral activity in patients with chronic HCV infection.

Bovine lactoferrin is thought to reduce serum HCV RNA concentration in hepatitis C patients via the following two distinct mechanisms. The first mechanism, seen in human herpes simplex virus-1, human cytomegalovirus, and human immunodeficiency virus-l infections, involves the direct interaction of lactoferrin with the cells. However, in rotavirus infection, a second mechanism involves an interaction between lactoferrin and the virus. These results suggest that lactoferrin can bind not only cellular viral receptors, but also viral envelope proteins, to prevent viral infection. More recently, we found that, in the case of HCV, lactoferrin showed no antiviral activity after adsorption and/or internalization of HCV into human PH5CH8 hepatocytes, and that the interaction between lactoferrin and HCV occurred immediately after mixing lactoferrin and serum containing HCV, resulting in the inhibition of adsorption of the HCV-lactoferrin complex into human PH5CH8 hepatocytes (unpublished results). Furthermore, since it has recently been reported that bovine lactoferrin binds to HCV E1 and E2 proteins in vitro, our results suggest that the antiviral activity of bovine lactoferrin is due to the direct binding of bovine lactoferrin to the HCV virion. These results indicate that the anti-HCV activity of lactoferrin is due to a neutralizing activity, which prevents the adsorption of HCV virion into hepatocytes.

We have shown in case 1 that the time course of serum ALT concentration paralleled that of serum HCV RNA during and after the lactoferrin therapy. Furthermore, after cessation of the initial course of lactoferrin, the increase in serum HCV RNA concentration preceded the increase in serum ALT concentration. It has been shown that the reappearance of serum HCV RNA always precedes the...
increase in serum ALT concentration in patients who relapse. Therefore, we believe that the effect of lactoferrin is most likely related to an antiviral mechanism of action.

It has been reported that rats fed a 2% bovine lactoferrin diet displayed no significant side effects. Human lactoferrin is especially rich in colostrum, with the maximum concentration estimated to be about 30 mg/ml; therefore, lactoferrin absorbed by newborns accounts for about 3% of their total nutrition. Furthermore, an intact form of human lactoferrin of maternal origin has been isolated from the urine of preterm infants fed human milk. Therefore, the present findings may encourage the clinical use of lactoferrin in patients with C-type chronic hepatitis.

To our knowledge, lactoferrin is the first physiological substance other than IFN found to show anti-HCV activity. Lactoferrin is one potential candidate in the search for anti-HCV reagents with a low risk of severe clinical side effects. In the present study, we introduced 1.8 g of bovine lactoferrin daily in the initial 7 patients and found that, in patients with high pretreatment serum concentrations of HCV RNA, this dose of lactoferrin was not effective. Therefore, in the following 4 patients, we introduced 3.6 g of bovine lactoferrin daily and found that, in 1 patient (case 10) with high pretreatment serum concentrations of HCV RNA, serum ALT and serum HCV RNA levels dramatically decreased after treatment. The reason for this decrease in these indices after cessation of lactoferrin treatment remains unclear. Since lactoferrin has been shown to be a multifunctional immunoregulatory protein, high doses of lactoferrin may modulate inflammatory and immune responses. Therefore, in patients with high pretreatment serum concentrations of HCV RNA, a high dose of lactoferrin may be preferable.

In conclusion, this pilot study has shown that lactoferrin is effective in chronic hepatitis patients with low serum concentrations of HCV RNA. Further clinical trials should clarify whether lactoferrin is a potential anti-HCV reagent that will be effective in the treatment of patients with chronic hepatitis.

REFERENCES

1) Choo, Q. L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W. and Houghton, M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science*, 244, 359–362 (1989).

2) Kuo, G., Choo, Q. L., Alter, H. J., Gitnick, G. L., Redwker, A. G., Purcell, R. H., Miyamura, T., Dienstag, J. L., Alter, M. J., Stevens, C. E., Tegtmeier, G. E., Bonino, F., Colo, M. W. S., Lee, W. S., Kuo, C., Berger, K., Shuster, J. R., Overby, L. R., Bradley, D. W. and Houghton, M. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. *Science*, 244, 362–364 (1989).

3) Ohtsuki, S., Kojima, H., Tawaraya, H., Miyajima, T., Kamimura, T., Asakura, H., Satoh, A., Hirose, S., Hijikata, M., Kato, N. and Shimotohno, K. Prevalence of antibody against non-A, non-B hepatitis virus in Japanese patients with hepatocellular carcinoma. *Jpn. J. Cancer Res.*, 81, 550–553 (1990).

4) Saito, I., Miyamura, T., Ohbayashi, A., Harada, H., Katayama, T., Kikuchi, S., Watanabe, Y., Koi, S., Onji, M., Ohta, Y., Choo, Q. L., Houghton, M. and Kuo, G. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc. Natl. Acad. Sci. USA*, 87, 6547–6549 (1990).

5) Hoofnagle, J. H., Mullen, K. D., Jones, D. B., Rustgi, V., Di Bisceglie, A., Peters, M., Waggoner, J. G., Park, Y. and Jones, E. A. Treatment of chronic non-A, non-B hepatitis with recombinant human alpha interferon: a preliminary report. *N. Engl. J. Med.*, 315, 1575–1578 (1986).

6) Di Bisceglie, A. M., Martin, P., Kassianides, C., Lisker-Melman, M., Murray, L., Waggoner, J., Goodman, Z., Banks, S. M. and Hoofnagle, J. H. Recombinant interferon alpha therapy for chronic hepatitis C: a randomized, double-blind, placebo-controlled trial. *N. Engl. J. Med.*, 321, 1506–1510 (1989).

7) Tine, F., Margin, S., Craxi, A. and Pagliaro, L. Interferon for non-A, non-B chronic hepatitis: a meta-analysis of randomized clinical trials. *J. Hepatol.*, 13, 192–199 (1991).

8) Marcellin, P., Boyer, N., Gloor, E., Degott, C., Courouce, A. M., Degos, F., Coppere, H., Cales, P., Couzigou, P. and Benhamou, J.-P. Recombinant human alpha-interferon in patients with chronic non-A, non-B hepatitis: a multicenter randomized controlled trial from France. *Hepatology*, 13, 393–397 (1991).

9) Shiratori, Y., Kato, N., Yokosuka, O., Imazeki, F., Hashimoto, E., Hayashi, N., Nakamura, A., Asada, M., Kuroda, H., Tanaka, N., Arakawa, Y. and Omata, M. for the Tokyo-Chiba Hepatitis Research Group. Predictors of the efficacy of interferon therapy in chronic hepatitis C virus infection. *Gastroenterology*, 113, 558–566 (1997).

10) Kato, N., Ikeda, M., Mizutani, T., Sugiyama, K., Noguchi, M., Hirohashi, S. and Shimotohno, K. Replication of hepato-
Lactoferrin and Chronic Hepatitis C

10) Ikeda, M., Kato, N., Mizutani, T., Sugiyama, K., Tanaka, K. and Shimotohno, K. Analysis of cell tropism of HCV by using in vitro HCV-infected human lymphocytes and hepatocytes. *J. Hepatol.*, **27**, 445–454 (1997).

11) Ikeda, M., Sugiyama, K., Mizutani, T., Tanaka, T., Tanaka, K., Sekihara, H., Shimotohno, K. and Kato, N. Human hepatocyte clonal cell lines that support persistent replication of hepatitis C virus. *Virus Res.*, **56**, 157–167 (1998).

12) Hasegawa, K., Motsuchi, W., Tanaka, S. and Dosako, S. Inhibition with lactoferrin of in vitro infection with human herpes virus. *Jpn. J. Med. Sci. Biol.*, **47**, 73–85 (1994).

13) Kirkpatrick, C. H., Green, I., Rich, R. R. and Schade, A. L. Inhibition of growth of *Candida albicans* by iron-unsaturated lactoferrin: relation to host-defense mechanisms in chronic mucocutaneous candidiasis. *J. Infect. Dis.*, **124**, 539–544 (1971).

14) Yamanouchi, K., Tomita, M., Giehl, T. J. and Elison, R. T., III. Antibacterial activity of lactoferrin and a pepsin-derived lactoferrin peptide fragment. *Infect. Immun.*, **61**, 719–728 (1993).

15) Harmesen, M. C., Swart, P. J., De Bethune, M.-P., Pauwels, R., De Clercq, E., The, T. H. and Meijer, D. K. F. Antiviral effects of plasma milk proteins: lactoferrin shows potent activity against both human immunodeficiency virus and human cytomegalovirus replication in vitro. *J. Infect. Dis.*, **172**, 380–388 (1995).

16) Superti, F., Ammendolia, M. G., Valent, P. and Seganti, L. Antirotaviral activity of milk protein: lactoferrin prevents rotavirus infection in the enterocyte-like cell line HT-29. *Med. Microbiol. Immunol. (Berl.)*, **186**, 83–91 (1997).

17) Ikeda, M., Sugiyama, K., Tanaka, T., Tanaka, K., Sekihara, H., Shimotohno, K. and Kato, N. Lactoferrin markedly inhibits hepatitis C virus infection in cultured human hepatocytes. *Biochem. Biophys. Res. Commun.*, **245**, 549–553 (1998).

18) Brock, J. Lactoferrin: a multifunctional immunoregulatory protein? *Immunol. Today*, **16**, 417–419 (1995).

19) Levay, P. F. and Viljoen, M. Lactoferrin: a general review. *Haematologica*, **80**, 252–267 (1995).

20) Yi, M., Kaneko, S., Yu, D. Y. and Murakami, S. Hepatitis C virus envelope proteins bind lactoferrin. *J. Virol.*, **71**, 5997–6002 (1997).

21) Hagiwara, H., Hayashi, N., Mita, E., Takehara, T., Kasahara, A., Fusamato, H. and Kamada, T. Quantitative analysis of hepatitis C virus RNA in serum during interferon alpha therapy. *Gastroenterology*, **104**, 877–883 (1993).

22) Gil, B., Quian, C., Riezu-Boj, J. I., Civeira, M. P. and Prieto, J. Hepatic and extrahepatic HCV RNA strands in chronic hepatitis C: different patterns of response to IFN treatment. *Hepatology*, **18**, 1050–1054 (1993).

23) Sekine, K., Watanabe, E., Nakamura, J., Takasuka, N., Kim, D. J., Asamoto, M., Krutovskikh, V., Baba-Toriyama, H., Ota, T., Moore, M. A., Masuda, M., Sugimoto, H., Nishino, H., Kakizoe, T. and Tsuda, H. Inhibition of azoxymethane-initiated colon tumor by bovine lactoferrin administration in F344 rats. *Jpn. J. Cancer Res.*, **88**, 523–526 (1997).

24) Hutcheson, T. W., Henry, J. F. and Yip, T.-T. Structurally intact (78-kDa) forms of maternal lactoferrin purified from urine of preterm infants fed human milk: identification of a trypsin-like proteolytic cleavage event in vivo that does not result in fragment dissociation. *Proc. Natl. Acad. Sci. USA*, **88**, 2994–2998 (1991).