BASIC—LIVER, PANCREAS, AND BILIARY TRACT

Silymarin Inhibits In Vitro T-Cell Proliferation and Cytokine Production in Hepatitis C Virus Infection

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BACKGROUND & AIMS: Silymarin, an extract from the seeds of the milk thistle plant *Silybum marianum*, has been used for centuries for the treatment of chronic liver diseases. Despite common use by patients with hepatitis C in the United States, its clinical efficacy remains uncertain. The goal of this study was to determine whether silymarin has in vitro effects on immune function that might have implications for its potential effect on hepatitis C virus (HCV)-induced liver disease. **METHODS:** Freshly isolated peripheral blood mononuclear cells (PBMC) and T cells from HCV-infected and uninfected subjects were tested in vitro for responses to nonspecific and antigenic stimulation in the presence and absence of a standardized preparation of silymarin (MK001). **RESULTS:** Minimal MK001 toxicity on PBMC was found at concentrations between 5 and 40 μg/mL. MK001 dose dependently inhibited the proliferation and secretion of tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and interleukin (IL)-2 by PBMC stimulated with anti-CD3. In addition, MK001 inhibited proliferation by $CD4^+$ T cells to HCV, *Candida*, and tetanus protein antigens and by HLA-A2/HCV 1406–1415-specific $CD8^+$ T cells to allogeneic stimulation. MK001 inhibited T-cell TNF-α and IFN-γ cytokine secretion to tetanus and *Candida* protein antigens. Finally, MK001 inhibited nuclear factor-κB transcriptional activation after T-cell receptor-mediated stimulation of Jurkat T cells, consistent with its ability to inhibit Jurkat T-cell proliferation and secretion of IL-2. **CONCLUSIONS: Silymarin's ability to inhibit the proliferation and proinflammatory cytokine secretion of T cells, combined with its previously described antiviral effect, suggests a possible mechanism of action that could lead to clinical benefit during HCV infection.**

Chronic hepatitis C afflicts approximately 3 million individuals and is the leading cause of end-stage liver disease requiring transplantation in the United States. Liver fibrosis progression, the major consequence of chronic infection, generally occurs over decades in immunocompetent individuals.[1]

The current standard treatment for hepatitis C virus (HCV) infection, pegylated interferon and ribavirin, results in an overall sustained virologic response (SVR) rate of approximately 55%, which, in the vast majority of cases, represents durable viral eradication.[2,3] However, significant numbers of patients do not achieve SVR or are intolerant/have contraindications to therapy. Complications of hepatitis C-induced liver fibrosis including liver failure and cancer are predicted to increase significantly over the next 20 years, and no other treatment options are currently available.[4] Thus, many individuals have turned to herbal remedies. Two recent studies have reported that as many as 13% to 23% of US patients with chronic liver disease are using herbal remedies, with silymarin being the most common preparation ingested for such purpose.[5,6]

Silymarin, an extract from the seeds of the milk thistle plant *Silybum marianum*, has been used for centuries as a "hepatoprotectant." Silymarin is a mixture of 7 flavonolignans (silybin A, silybin B, isosilybin A, isosilybin B, silychristin, silydianin, and isosilychristin) and 1 fla-

Abbreviations used in this paper: CFSE, carboxyfluoroscein succinimidyl ester; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; HCV, hepatitis C virus; IFN-γ, interferon-γ; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; PMA, phorbol myristate acetate; rh, recombinant human; TNF-α, tumor necrosis factor-α.

0016-5085/10/$36.00
doi:10.1053/j.gastro.2009.09.021

vonoid (taxifolin). Although its mechanism of action is incompletely understood, silymarin has been described to possess antioxidant, immunomodulatory, antifibrotic, antiproliferative, and antiviral activities.[7,8] Silymarin's clinical efficacy in chronic liver disease has not yet been demonstrated[9,10] because results have been inconsistent. Problems with the studies have included insufficient power, use of varying doses (including those that may be too low to have an effect), and the use of different nonstandardized preparations of silymarin, making it difficult to compare results among studies. Moreover, many reports have utilized silibinin or chemically modified silibinin, an ~50:50 mixture of silybin A and silybin B, rather than silymarin.

HCV is generally believed to be a noncytopathic virus, and hepatic fibrosis in hepatitis C is thought to be the end result of long-standing inflammation, characterized by the accumulation of $CD4^+$ and $CD8^+$ T cells in the infected liver. A substantial amount of data suggests that HCV-specific T-cell cytokine and cytolytic responses as well as "bystander" effects contribute to immune-mediated liver injury.[11] As an important component of the inflammatory response to HCV infection, T cells are stimulated to proliferate, secrete cytokines, and kill infected cells. In this study, we investigated whether a standardized preparation of silymarin (MK001) can affect proinflammatory immune responses, such as T-cell proliferation and cytokine secretion, as a means of providing hepatoprotection.

Materials and Methods

Subjects

Healthy subjects and those with chronic HCV infection were recruited at Harborview Medical Center after written informed consent was obtained through a University of Washington (UW) Institutional Review Board-approved protocol.

Silymarin

A standardized preparation of silymarin (MK001)[8] was used in all experiments. Using high-performance liquid chromatography analyses with detection at 254 nm, all peaks more than 0.7% in quantity were assigned, and the total flavonolignan content was 92%. MK001 was solubilized in dimethyl sulfoxide (DMSO), 95% ethanol, or methanol. Control samples were treated with the same concentration of DMSO, 95% ethanol, or methanol as used in the MK001-treated samples. Silybin A, silybin B, and silibinin were purified as previously described in detail.[12]

Peripheral Blood Mononuclear Cell Isolation and Stimulation

All peripheral blood mononuclear cells (PBMC) were freshly isolated using standard Ficoll-Hypaque centrifugation within 24 hours of venipuncture and immediately applied to the assays described. PBMC were stimulated for varying periods of time at 37°C, 5% CO_2 with plate-bound anti-CD3 (UCHT1, 10 μg/mL; BD Biosciences, San Jose, CA), anti-CD3 and anti-CD28 Dynabeads (Invitrogen, Carlsbad, CA), phytohemagglutinin (PHA; 1.6 μg/mL; Remel, Lenexa, KS), or phorbol myristate acetate (PMA)/ionomycin (50 ng/mL of PMA and 1 μg/mL ionomycin; both from Sigma Aldrich, St. Louis, MO) in RPMI 1640 medium supplemented with 10% human serum (Gemini Bio-Products, Woodland, CA). Stimulation with tetanus and *Candida* protein antigens was performed as described under proliferation assays (below).

Cellular Proliferation Assays

Cellular proliferation detected by ^{3}H-thymidine incorporation into replicating DNA was measured by adding 1 μCi to each replicate well of 10^5 PBMC for 24 hours (for α-CD3 and PHA experiments) or 16–18 hours (for protein antigen experiments) before quantitative analysis using a Topcount Liquid Scintillation Counter (Perkin-Elmer, Waltham, MA). In general, data are presented as mean counts per minute (cpm) incorporated per condition tested. All experiments utilized 4 replicates per condition.

To measure HCV-specific proliferative responses, PBMC were incubated with superoxide dismutase (SOD)-recombinant HCV protein antigens (courtesy of Dr Michael Houghton and Kevin Crawford, Chiron Corp) at a final concentration of 10 μg/mL for 5 days prior to addition of ^{3}H-thymidine as described above.[13] Antigens included yeast-derived SOD (negative control), SOD-c22 (HCV amino acids [aa] 2–120), SOD-c100 (HCV aa 1569–1931), SOD-NS5 (HCV aa 2054–2995), and *Escherichia coli*-derived SOD-c33c (HCV aa 1192–1457). PHA (1.6 μg/mL; Remel), *Candida albicans* (20 μg/mL; Greer Laboratories, Lenoir, NC), and tetanus toxoid (12 Lf/mL, Wyeth-Ayerst Laboratories, Marietta, PA) were also tested. Assays were considered valid only when PHA responses were intact.

PBMC and T-cell proliferation was also measured using carboxyfluoroscein succinimidyl ester (CFSE) dilution. PBMC were exposed to 1 μmol/L CFSE (Invitrogen/Molecular Probes, Carlsbad, CA) for 10 minutes at 37°C then washed and cultured with or without stimulation and with or without MK001 (20 μg/mL) at 37°C, 5% CO_2. The next day, cells were labeled with the fluorescent surface antibodies α-CD5-PerCP-Cy5.5, α-CD8-PE, and α-CD45-APC (all BD Biosciences) and fixed with 1% paraformaldehyde and analyzed by flow cytometry as described below. For T-cell experiments, HCV 1406–1415-expanded T cells were cultured with an allogeneic B-lymphoblastoid cell line at a ratio of 10:1 (T cells:B-lymphoblastoid cells), with or without MK001 (20 μg/mL) for 3 days before labeling with α-CD8-PerCP, α-CD3-APC (both BD Biosciences), and HLA-A2 HCV 1406–1415 pentamer-PE (ProImmune, Bradenton, FL).