

Role of Hyperbilirubinemia in the Impairment of Osteoblast Proliferation Associated with Cholestatic Jaundice

Christine H. Janes,[‡] E. Rolland Dickson,[‡] Ryo Okazaki,* Susan Bonde,* Antony F. McDonagh,[§] and B. Lawrence Riggs*

*Endocrine Research Unit and [‡]Division of Gastroenterology, Department of Internal Medicine, Mayo Clinic and Mayo Foundation, Rochester, Minnesota 55905; and [§]Department of Medicine and the Liver Center, University of California Medical Center, San Francisco, California 94143

Abstract

Because the osteoporosis occurring in chronic cholestatic liver disease (CCLD) is associated with decreased bone formation and is reversible by liver transplantation, substances retained in plasma during cholestasis may impair osteoblast function. This hypothesis was tested using a new bioassay that measures plasma mitogenic activity (PMA) for normal human osteoblast-like (hOB) cells. In 29 jaundiced patients, mean PMA was 56.4% ($P < 0.001$) of that in 29 age- and sex-matched normal subjects, and the decrease in PMA was similar in the 14 with CCLD and the 15 with other causes of jaundice. Bile acids and bilirubin are the two major groups of products retained during cholestasis. The common conjugated bile acids and bilirubin were added to normal human plasma in concentrations simulating those found in patients with CCLD. Various bile salts had no effect on PMA whereas unconjugated bilirubin decreased PMA in a dose-dependent fashion ($r = -0.98$, $P < 0.0001$) without affecting cell viability. Relatively selective removal of bilirubin from the plasma by photobleaching normalized the decreased PMA in five jaundiced patients but produced no apparent change in five normal subjects. These data support the hypothesis that hyperbilirubinemia or possibly other photolabile substances impair osteoblast proliferative capacity and thus may play a major role in the pathogenesis of the osteoporosis associated with CCLD. (*J. Clin. Invest.* 1995; 95:2581–2586.) Key words: bilirubin • primary biliary cirrhosis • cholestasis • osteoporosis • liver disease

Introduction

Primary biliary cirrhosis (PBC)¹ and primary sclerosing cholangitis (PSC) are forms of chronic cholestatic liver disease (CCLD) that mainly affect young and middle-aged adults (1). The clinical manifestations of the osteoporosis that occurs with

these diseases include low bone density and fractures of the vertebrae and peripheral skeleton (2–6).

The causes of bone disease in CCLD are not well understood. In Great Britain, a mixture of osteoporosis and osteomalacia commonly occurs due to associated vitamin D deficiency aggravated by steatorrhea. In contrast, in the United States, because of fortification of food with vitamin D, osteoporosis, not osteomalacia, is the major form of the bone disease (3, 6–9).

In American and Australian patients with severe liver disease, bone formation is decreased. Hodgson et al. (10) found that serum osteocalcin, a biochemical marker for bone formation, was significantly decreased whereas urinary hydroxyproline excretion, a biochemical marker for bone resorption, was normal. Decreased bone formation in patients with CCLD has been found by histomorphometric studies of iliac biopsy samples (10–13). Also, it has been demonstrated that successful liver transplantation is associated with substantial increases in bone density in these patients (5).

These observations led us to hypothesize that substances retained in plasma during cholestasis impair osteoblast function. To test this hypothesis, we developed a new bioassay for plasma mitogenic activity (PMA), based on proliferation of bone cells in vitro, and we used this assay to study the antiproliferative effects of bile acids and bile pigments.

Methods

Subjects

Plasma was obtained from 29 fasting, jaundiced patients and from 29 fasting, normal control subjects who were pair-matched by age and sex. Of the patients, 14 had CCLD (PBC in 3 and PSC in 11), 5 had obstructive jaundice secondary to cholangiocarcinoma or pancreatic carcinoma, and 10 had primary hepatic dysfunction (alcoholic liver disease in 6, chronic autoimmune hepatitis in 3, and metastatic carcinoid in 1). Only the three patients with chronic autoimmune hepatitis were under treatment with glucocorticoids. No patient was receiving cholestyramine or supplementation with calcium or vitamin D and all had normal serum calcium concentrations. There were 16 men and 13 women in each group. The mean (\pm SEM) total serum bilirubin concentration measured by standard colorimetric assay after reaction with sulfonilic acid to form isomers of azobilirubin in the jaundiced patients was 0.228 ± 0.146 mM; range, 0.255–0.592 (13.4 \pm 8.6 mg/dl [range, 1.5–34.8 mg/dl]).² The median ages of the jaundiced patients and the control subjects were 51 yr (range, 34–68 yr). The protocol was reviewed and approved by the Mayo Institutional Review Board, and all subjects gave informed consent.

Bioassay of PMA

Bone cell culture. We used normal human osteoblast-like (hOB) cells as surrogates for normal osteoblasts. These hOB cells were obtained

Address correspondence to Dr. B. L. Riggs, North 6 Plummer, Mayo Clinic, Rochester, MN 55905. Phone: 507-284-3961; FAX: 507-284-8271.

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1. Abbreviations used in this paper: CCLD, chronic cholestatic liver disease; hOB cells, human osteoblast-like cells; PBC, primary biliary cirrhosis; PMA, plasma mitogenic activity; PSC, primary sclerosing cholangitis.

from trabecular bone discarded as waste material during orthopedic surgery and were cultured as described (14, 15). After removal of fibrous tissue, the bone samples were washed, diced into small (2–4 mm in diameter) fragments, and digested with crude bacterial collagenase (1 mg/ml in DME) for 2 h at 37°C in a shaking water bath. The bone fragments were then cultured in a growth medium consisting of low-calcium (0.2 mM) DME/Ham's F-12 medium containing penicillin and streptomycin (100 U/ml; 100 µg/ml) and 10% (vol/vol) normal human plasma. Cells grown from explants were passaged once and grown to confluence. At this time, the cells are nearly homogeneous because > 95% of them contain osteocalcin, a protein that is specific for cells of the osteoblast lineage (16). Extensive characterization (15) has shown that the cells display the complete phenotype of mature osteoblasts (17). The confluent cells were trypsinized, subcultured on 24-well plates in medium supplemented with 10% normal human plasma at a seeding density of 10,000/cm², and were used for assay of test plasmas. To minimize variability, each well was seeded with a mixture of hOB cells from six donors.

Assay of test plasma or putative inhibitors

Citrated plasma samples were obtained and stored at –70°C until assay. Plasma samples were mixed with an equal volume of basal medium, a 1:1 (vol/vol) mixture of DME and Ham's F-12 medium, to create the proliferative 50% test plasma. After 48 h of subculture in medium supplemented with 10% normal human plasma to establish cell attachment, the medium was replaced with fresh medium that contained 0.5% normal human plasma for 48 h to induce proliferative quiescence. The hOB cells were then exposed to the proliferative test plasma mixture for a final 48 h of incubation. [³H]Thymidine (2 µCi/ml) was added for the final 24 h to measure DNA synthesis (18). [³H]Thymidine incorporation was assessed by liquid scintillation counting of the trichloroacetic acid–precipitable material. All assay results represent the mean of measurements from triplicate wells. Because of wide variability among strains of hOB cells, all experiments were made using six cell strains. For each experiment the same cell strains were used, and the results are reported as mean±SEM of these strains. In some experiments, proliferation of hOB cells was also assessed by direct cell counting in a Coulter counter (Coulter Corp., Hialeah, FL). Cell viability of hOB cells was assessed by their ability to exclude trypan blue after its addition to the culture medium.

To avoid photodegradation, all bilirubin-containing plasma samples were prepared in red light (19), and all cell culture plates were enclosed in aluminum foil to avoid exposure to white light. Each assay included samples of plasma from paired jaundiced and control subjects and a standard reference plasma (pooled plasma from six normal subjects). Based on the results of 12 normal subjects assessed in 2 different cell preparations, the coefficient of variation for PMA was 22%.

Trypan blue exclusion was used as an index of cell viability. To assess the effect of graded doses of bilirubin in 50% plasma and 50% nutrient (vol/vol) media at the end of 48 h of treatment, media were removed, and the cells were rinsed and then stained with trypan blue. The proportion of dead cells (blue staining) was assessed by grid intersection in two representative fields per well in each of the six replicate wells. No less than 120 cells were counted. Results were expressed as a percentage of total cells counted.

To investigate putative inhibitors of osteoblast proliferation, bile acids or unconjugated bilirubin was added to the standard reference plasma, and the effect on [³H]thymidine incorporation was measured. Unconjugated bilirubin (Porphyrin Products, Logan, UT) was mixed with bioassay buffer containing albumin, and the mixture was brought to pH 10 with 2 N NaOH to dissolve the bilirubin. The solution was then adjusted to pH 7.4 and mixed with normal human plasma to create a 50% (vol/vol) normal human plasma stock solution containing albumin at 20 mg/ml and having a bilirubin concentration of 250 µM. This stock solution was diluted with 50% normal human plasma medium to obtain concentrations of bilirubin ranging from those found in plasma of normal subjects (10 µM) to those of severely jaundiced subjects (250 µM). The

Table I. Effect of Normal Human Plasma and Fetal Calf Serum on [³H]Thymidine Uptake by Normal hOB Cells

Plasma or serum in medium	[³ H]Thymidine uptake	
	Human plasma added	Fetal calf serum added
%	dpm/well/24 h	
1	7,100	1,450
30	19,540	9,690
50	23,095	Not done
90	13,240	Not done

bilirubin dose–response curve was determined at albumin concentrations of 20 and 40 mg/ml.

The bile acids taurocholic acid, glycocholic acid, taurochenodeoxycholic acid, and glycochenodeoxycholic acid (Sigma Chemical Co., St. Louis, MO) were combined in equimolar concentrations and in individual concentrations to simulate plasma concentrations observed in patients with cholestasis (20–22). An initial stock solution that contained a bile acid concentration approximating the maximal plasma concentration (400 µM) seen during cholestasis was serially diluted to a minimal total concentration (2 µM). Each individual bile acid was serially diluted in a similar manner simulating its plasma concentration during cholestasis (0.5–200 µM). The PMAs of these proliferative test media were then measured by [³H]thymidine incorporation.

Statistical analysis

All statistical comparisons were made using the two-tailed Student's *t* test.

Results

Establishment of assay. Because it has been believed that addition of fetal calf serum to an incubation medium is required to induce proliferation of human bone cells, we first compared the effects of different concentrations of human plasma and fetal calf serum on induction of hOB cell proliferation. Normal human plasma promoted greater ($P < 0.05$) proliferation of hOB cells (Table I) than did equivalent concentrations of fetal calf serum; stimulation was maximal at 50% plasma. Thus, full proliferative capacity of the hOB cells was maintained in vitro, which permitted different plasma samples to be assayed for mitogenic activity.

Bioassay of plasma. Stimulation of hOB cell proliferation by plasma samples from the 29 jaundiced patients was 56.4%±4.0% that of plasma samples from the age- and sex-matched controls ($P < 0.0001$) (Fig. 1). The degree of suppression of hOB cell proliferation of plasma from the 14 patients with CCLD was similar to that of plasma from the 15 patients with other causes of jaundice (mean±SEM, 57.2%±6.9% vs 55.6%±5.4%, respectively, NS). Among plasma samples from individual subjects, there was no correlation between PMA and plasma bilirubin concentration or plasma albumin concentration.

Addition of putative inhibitors. To identify the factor or factors in plasma from patients with cholestasis that inhibited hOB cell proliferation, we added varying amounts of bile acids and unconjugated bilirubin—substances that are retained during cholestasis—to the normal standard reference pool to produce plasma concentrations simulating those found in patients with

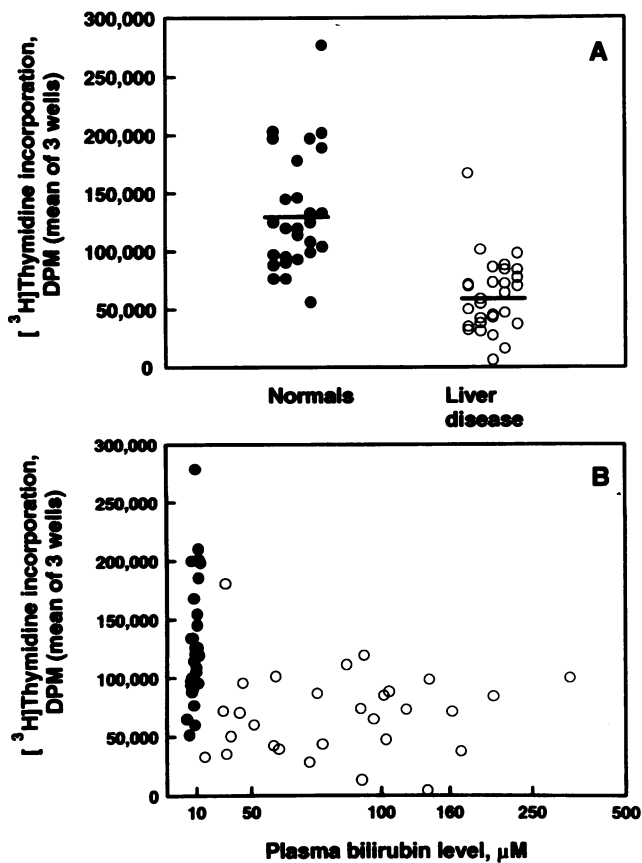


Figure 1. PMA of plasma samples from 29 jaundiced patients (○) and from 29 paired age- and sex-matched comparable normal subjects (●). (A) PMA in the plasma samples from jaundiced patients was only about half that in the normal subjects ($P < 0.0001$). (B) Relationship of PMA and plasma bilirubin levels.

CCLD. In six separate experiments, the addition of an equimolar mixture of bile acids (taurocholic, glycocholic, taurochenodeoxycholic, and glycochenodeoxycholic) over a range of plasma concentrations from 2 to 400 μM had no effect on hOB cell proliferation (Fig. 2). Nor was any effect noted when the indi-

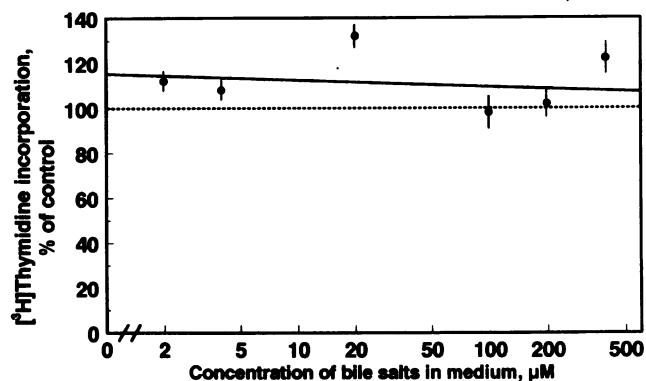


Figure 2. Effect of addition of a mixture of bile salts over the dose range of 2–400 μM on the PMA of a pool of normal plasma, expressed as percentage of the PMA of the same plasma pool without the addition. Closed circles and vertical lines represent mean \pm SEM of individual assays. No effect is apparent.

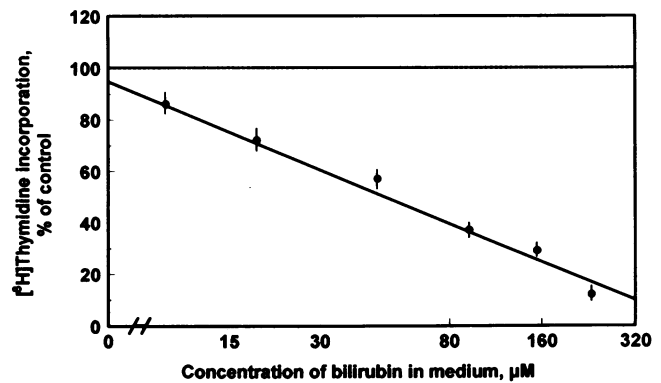


Figure 3. Effect of addition of unconjugated bilirubin to normal plasma over the range of 10–250 μM on the PMA, expressed as percentage of the PMA of the same plasma pool without the addition. Closed circles and vertical lines represent mean \pm SEM of individual assays. Note the dose-dependent inhibition of PMA ($r = -0.98$, $P < 0.0001$).

vidual bile acids were added at concentrations of 0.5–200 μM (data not shown). In contrast, in nine separate experiments, addition of unconjugated bilirubin over the range of 10–250 μM inhibited proliferation in each experiment in a dose-dependent fashion ($P < 0.001$) (Fig. 3). Varying the albumin concentration from 20 to 40 mg/ml did not alter this response (data not shown). Bilirubin's effect on cell proliferation was also assessed in three experiments by direct cell counting. The correlation between values obtained with [³H]thymidine incorporation and cell counting was high ($r = 0.91$, $P < 0.001$). Over the same range of bilirubin concentrations, cell viability was not affected as assessed by their ability to exclude trypan blue (Table II).

Effect of photobleaching. The plasma bilirubin in patients with CCLD is a complex mixture containing bilirubin, bilirubin monoglucuronides, bilirubin diglucuronide, and many isomers of the glucuronides, as well as covalent bilirubin–albumin complexes. In an attempt to study the combined effect of these bilirubin species on osteoblast mitogenic activity, plasma samples from patients with CCLD and from normal controls were tested before and after exposure to visible light at wavelengths that selectively destroy bile pigments. Plasma samples from five

Table II. Effect on Cell Viability of Addition of Unconjugated Bilirubin to Mixture of Nutrient Medium and 50% Normal Plasma Pool

Concentration of added bilirubin	Viable cells
μM	%
0	85 \pm 4
10	91 \pm 4
20	90 \pm 4
50	91 \pm 6
100	95 \pm 3
160	94 \pm 6
250	93 \pm 7

Cell viability was assessed by trypan blue exclusion and results are expressed as percentage of viable cells per total cells (mean \pm SD, $n = 6$).

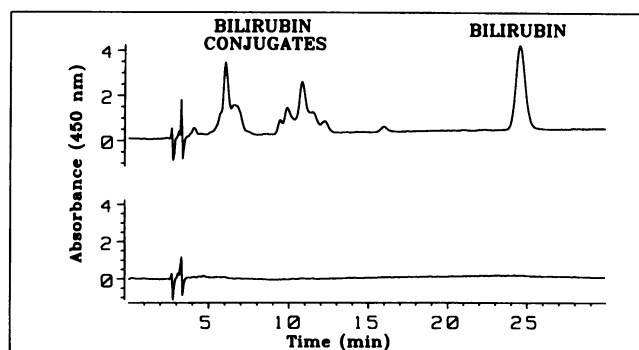


Figure 4. High-performance liquid chromatography (32) of plasma from a patient with PBC before photobleaching (*top*) and after photobleaching (*bottom*). Bleaching destroyed conjugated and unconjugated bilirubin. The bilirubin conjugates appear as broad groups of peaks because they include not only bilirubin diglucuronide, which elutes near 6 min, and the two bilirubin monoglucuronides, which elute at 11–12 min, but also numerous acyl-migration isomers of these.

jaundiced patients (total serum bilirubin concentration [mean±SD] of 14.1±1.3 mg/dl) and five age- and sex-matched normal controls were photobleached with narrow-spectrum blue light (450 nm) with peak emission close to the absorption maximum for bilirubin (19, 23). The plasma was irradiated at 0°C under 1 atm of oxygen through a filter that removed radiation below 400 nm (i.e., no exposure to ultraviolet light). After 16 h, the light was replaced with a broad-spectrum white light source and bleaching was continued for another 9 h. The plasma was centrifuged briefly to remove a slight turbidity and then was immediately frozen on dry ice and stored at -80°C until used in cell culture. Absorbance spectra and high-performance liquid chromatograms of plasma samples before and after bleaching demonstrated that this procedure effectively destroyed all measurable bilirubin species. This is shown in Fig. 4 for a single patient. Plasma samples were then assayed, as described previously, before and after bleaching.

Results of the photobleaching experiment are given in Fig. 5. Before photobleaching, PMA in the five jaundiced patients was 52% lower than values in five age- and sex-matched normal controls. After photobleaching, PMA in the jaundiced patients increased to values that were virtually identical with those of the normal controls. Because of the small number of subjects, this increase was not significant by the paired *t* test but was significant ($P = 0.02$) by the sign test. In the normal controls, photobleaching had no apparent effect on PMA.

Discussion

Although normal hOB cells grow well in 10% fetal calf serum (14, 15), their ability to proliferate in normal human plasma has not been reported previously. We found that hOB cell proliferation in normal human plasma was greater than in fetal calf serum at an equal concentration. This observation formed the basis of a new bioassay for PMA that we used in this study.

Using this bioassay, we explored plasma factor(s) retained during cholestasis that might impair osteoblast proliferation. First, we found that PMA in patients with CCLD was approximately half that of age- and sex-matched normal controls. This finding was significant despite a wide distribution of PMA

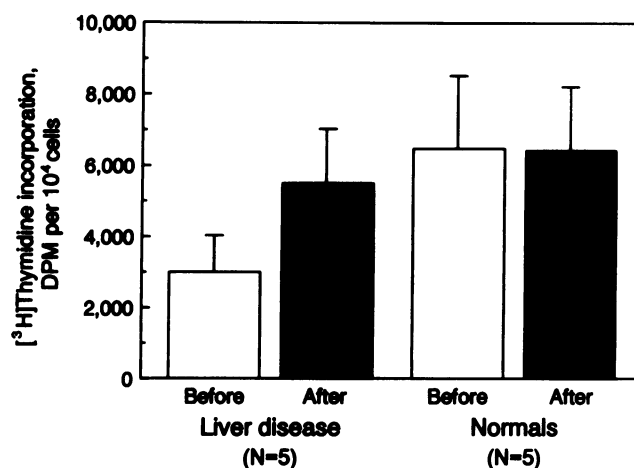


Figure 5. Effect of photobleaching on PMA of five jaundiced patients and five age- and sex-matched normal subjects, expressed as DPM per 10,000 cells before and after photobleaching (mean±SEM). Photobleaching appeared to increase PMA of jaundiced patients but had no apparent effect on that of normal subjects.

among normal subjects. This high variability may be related to the variability of circulating endogenous growth factors among subjects. Also, it may be related in part to release of growth factors that are mitogenic for osteoblasts from platelets, such as platelet-derived growth factor and transforming growth factor- β . Some degree of platelet degranulation is inevitable in collection of plasma using citrate. However, despite the wide variation of PMA in normal subjects, PMA fell below the mean for the normal controls in all except one of the CCLD patients, and 21 of the 29 of the CCLD patients had values for PMA that were lower than all but one of the normal controls. The reduced PMA for osteoblasts *in vitro* is consistent with the reduced bone formation in CCLD patients as documented by decreased levels of serum osteocalcin (10, 13) and by bone histomorphometry (8, 11–13).

We next assessed the effect on PMA of the addition of graded concentrations of major products retained during cholestasis—bile acids and bilirubin—to pooled normal plasma. Adding bile acids, alone or in combination, in concentrations spanning the range observed in the serum of patients with CCLD, had no effect on plasma mitogenic activity. However, when unconjugated bilirubin was added, we found a highly significant, reproducible, and dose-dependent reduction in osteoblast proliferation *in vitro*. Because of lack of an available preparation, we were unable to determine if conjugated bilirubin also decreased osteoblast proliferation and thus also could contribute to the decreased bone formation in patients with CCLD. However, there was no correlation between PMA and the total bilirubin concentration for the individual normal and CCLD plasma samples. We believe that the relationship between PMA and serum bilirubin concentration could be observed in the dose-response experiment because known amounts of a single bilirubin species were being added to a constant concentration of growth factors in the same plasma pool whereas for individual plasma samples it was not observed because of the wide variation in endogenous or platelet-released growth factor concentration among them in the presence of multiple bilirubin species in addition to unconjugated bilirubin.

Finally, in a small subset of normal subjects and jaundiced patients, we assessed the effect of relatively selective removal of bilirubin and its metabolites by photobleaching of plasma with narrow spectrum blue light—a process that totally removes bilirubin but is unlikely to affect plasma proteins. Although the sample was too small for adequate statistical analysis, photobleaching appeared to restore the depressed plasma mitogenic activity in the jaundiced patients but had no apparent effect on plasma mitogenic activity in the normal subjects. Although the photobleaching also could have affected other plasma components that may regulate osteoblast activity, such as vitamin D sterols and retinoids, the lack of change in PMA after photobleaching in the five normal controls argues against this. However, we cannot exclude the possibility that photolabile substances accumulated with cholestasis other than bilirubin also affect osteoblast proliferation.

Our finding that unconjugated bilirubin inhibits the proliferation of normal hOB cells is consistent with results in other types of cells in which inhibition or toxic effects have been observed (24–30). However, in the dose–response experiment, we found no evidence of increased cell death as assessed by failure to exclude trypan blue at higher concentrations of bilirubin. Although the mechanism of bilirubin inhibition of osteoblastic proliferation is not clear, the saturable dose–response is consistent with binding to cell surface receptors.

Decreased bone mineral density in the lumbar spine has been reported to correlate with severity index for liver disease (as assessed by a score based on multiple risk factors) in 210 PBC (5) and 37 PSC patients (4). Diamond et al. (13) reported in 80 patients with various liver disorders including 20 with CCLD that serum albumin correlated weakly with histologically assessed bone formation rate whereas serum bilirubin did not. However, serum bilirubin did correlate inversely with serum osteocalcin, a biochemical marker of bone formation. Because serum albumin concentration is one of the major contributing factors to the severity index (31), the correlation indicates that the decrease in bone formation rate is associated with advanced stages of liver disease and that factors associated with advanced liver disease in addition to bilirubin may also contribute to the development of the osteopenia associated with CCLD. Potential contributing factors include malabsorption of calcium and vitamin D and retention of other toxic factors.

The association of osteoporosis and decreased bone formation with chronic liver disease has been reported chiefly in patients with CCLD. However, we found that plasma from patients with jaundice due to other causes also had a decrease in PMA to a similar degree for a comparable plasma concentration of bilirubin. This suggests that depressed osteoblast function may be related to the jaundice itself and is not a specific hallmark of CCLD. Possibly, severe jaundice develops at such a late stage in patients with hepatocellular disease that they do not survive long enough for osteoporosis to develop, whereas, in patients with CCLD, hepatic synthesis is well preserved, despite severe jaundice, until late in the course of the liver disease.

Of interest, osteoporosis has not been reported in patients with constitutional hyperbilirubinemia (Gilbert, Crigler-Najjar, Dubin-Johnson, and Rotor syndromes). However, patients with these syndromes have not been studied by sensitive techniques such as bone densitometry to assess bone loss. Also, patients with Gilbert's syndrome generally have lower serum bilirubin

concentrations, and our studies indicate that the toxic effect of bilirubin on osteoblastic function is dose related.

In conclusion, our study demonstrates the utility of a new bioassay for PMA and shows that PMA is depressed in patients with chronic liver disease. The results show that unconjugated bilirubin has a marked inhibitory effect on the PMA activity of plasma *in vitro* and provide strong experimental support for the hypothesis that hyperbilirubinemia, or accumulated photolabile substances, impairs osteoblast proliferative capacity and, thus, may play an important role in the pathogenesis of the osteoporosis associated with chronic jaundice.

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