Ethanol Activates the Interleukin-6 Promoter in a Human Bone Marrow Stromal Cell Line

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Chronic ethanol consumption is associated with the development of osteoporosis. The pro-inflammatory cytokine interleukin-6 (IL-6) plays a role in the development of osteoporosis through stimulation of osteoclastic activity. We hypothesized that ethanol promotes osteoporosis, in part, by increasing IL-6 production in the bone microenvironment. Accordingly, we evaluated ethanol's effect on IL-6 production in the Saka human bone marrow stromal cell line and in the HOBIT human osteoblast-like cell line. We found that ethanol increased IL-6 protein levels in the culture supernatant from Saka, but not HOBIT, cells. In addition, we observed that ethanol increased steady-state IL-6 mRNA levels and activated an IL-6 promoter-driven reporter vector in Saka cells. We conclude that ethanol stimulates IL-6 expression in the Saka bone marrow stromal cell line by enhancing transcriptional activity of the IL-6 gene. Our findings support the contention that ethanol may contribute to the pathogenesis of osteoporosis, in part, by increasing IL-6 expression in the bone microenvironment.

osteoporosis, an imbalance between bone synthesis and bone resorption, represents a highly prevalent, debilitating, and expensive disorder. Excessive consumption of alcoholic beverages is clearly associated with an increased risk for developing osteoporosis (Seeman et al., 1983). In one study, approximately 50% of ambulatory chronic alcoholics demonstrated radiographic evidence of extensive bone loss (Spencer et al., 1986). In contrast, the effects of moderate consumption of alcohol on bone are not well delineated. In fact, several reports suggest that moderate consumption may have a protective effect on bone. Both increased bone mineral density (Laitinen et al., 1991c; Holbrook et al., 1993) and increased plasma estradiol levels (Gavaler et al., 1992; Tivis et al., 1994) are observed in postmenopausal women consuming moderate levels of alcohol. In contrast, a negative correlation between moderate alcohol intake and axial bone mineral density in premenopausal (Stevenson et al., 1989) and postmenopausal (Hernandez-Avila et al., 1991) women has been demonstrated. It is possible that the apparent protective effects are due to confounding factors such as diet and exercise (Barrett et al., 1995). Carefully designed epidemiological studies are required to determine if moderate alcohol consumption is protective or not. However, that alcohol, either directly or indirectly, affects bone metabolism is well established.

The mechanisms by which alcohol alters bone metabolism are currently not well understood, but there is evidence for both direct and indirect actions. Acute alcohol intake alters systemic markers of bone homeostasis including a transitory hypoparathyroidism followed by rebound hyperparathyroidism, hypocalcemia, hypercalcicuria, hypermagnesemia, hypermagnesuria, and decreased osteocalcin levels (Kalbfleisch et al., 1963; Avery et al., 1983; Laitinen et al., 1991a). Moderate to chronic alcohol consumption results in similar alterations as acute intake; however, hyperparathyroidism is more pronounced, hypomagnesemia may be present, and vitamin D serum levels are low (Bjorneboe et al., 1988; Laitinen and Valimaki, 1991b). The observation of decreased osteocalcin levels may indicate a direct toxic effect of ethanol on bone formation (Rico et al., 1987; Diamond et al., 1989; Laitinen and Valimaki, 1991b). The molecular mechanisms by which alcohol achieves its metabolic effect on bone have not been elucidated.

Interleukin-6 (IL-6), a multifunctional cytokine produced by a great diversity of cells, is recognized as an important regulator of the immune and hematopoietic systems [reviewed by Ershler (1993)]. Recently, IL-6 has joined ranks with other cytokines/growth factors such as IL-1α, IL-1β, and tumor necrosis factor α (TNF-α) as demonstrably contributing to the process of bone resorption [reviewed by Lorenzo (1991) and Manolagas (1995)]. Although earlier studies did not document an influence of IL-6 on bone (Al-Humidan et al., 1991; Barton and Mayer, 1990; Littlewood et al., 1991; Löwik et al., 1989), the bulk of research currently supports IL-6's role in bone resorption. That IL-6-deficient mice (generated by targeted gene disruption) are protected from estrogen-deficit (Poli et al., 1994) and androgen-deficit (Bellido et al., 1995) associated bone loss especially supports the fact that IL-6 plays an important role in bone resorption.

We proposed that ethanol contributes to the pathogenesis of osteoporosis by influencing bone resorption through increasing IL-6 levels in the bone microenvironment. The localized increase of IL-6 would then shift the balance of bone remodeling in favor of bone resorption. To begin exploration of this hypothesis, we examined whether ethanol influences IL-6 expression in a human osteoblast and a human bone marrow stromal cell line. In this study we report that ethanol increases IL-6 protein and mRNA expression levels in both cell lines.

References


levels from the Saka bone marrow stromal cell line but not the HOBIT osteoblast-like cell line. In addition, we describe experiments which demonstrate that ethanol increases IL-6 promoter activity in the Saka cell line.

**METHODS**

**Cell culture and treatments.** — The Saka cell line (generously provided by G. D. Roodman, University of Texas, San Antonio) was established from human bone marrow stromal cells by simian virus 40 (SV40) infection (Takahashi et al., 1995). These fibroblast-like cells are devoid of myeloid, lymphoid, or osteoclast surface antigens. They do not express osteoblast alkaline phosphatase activity or endothelial factor VIII. They do produce fibronectin and vimentin and secrete a pattern of cytokines similar to that observed to be produced by human marrow stromal cells in vivo. They also express SV40 large T antigen and thus are not identical to normal marrow stromal cells. Saka cells were maintained in a minimal essential medium (MEM) supplemented with 20% fetal bovine serum (FBS). The HOBIT cell line (generously provided by B. L. Riggs, Mayo Foundation, Rochester, MN) was established by transfecting normal adult human osteoblast-like cells with pSV3 neo, a plasmid encoding SV40 small and large T antigen (Keeting et al., 1992). HOBIT cells approximate the phenotype of mature osteoblasts including (a) 1,25-dihydroxy vitamin D-inducible expression of osteocalcin and alkaline phosphatase; (b) expression of α(I)procollagen, osteopontin la, transforming growth factor β, IL-1β, IL-6, androgen receptor and estrogen receptor; and (c) production of a mineralizable matrix. HOBIT cells were maintained in F12/Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum (FBS).

As indicated in the results, the cells were incubated at various cellular densities with 0, 50, or 100 mM ethanol, which are physiologically attainable levels (Haeckel and Bucklitsch, 1987; Winck and Esposito, 1987). To minimize loss of ethanol from the culture dishes, they were wrapped with parafilm and taped closed. Cell viability, as assessed by a combination of cell count, trypan blue exclusion, and tetrazolium salt (MTS) conversion (Cell Titer Aqueous, Promega, Madison, WI), revealed that these levels of ethanol were not toxic to cells (data not shown).

**Measurement of IL-6 protein and mRNA levels.** — Supernatants were collected at various time intervals and frozen at –80 °C until assayed. Supernatant IL-6 protein levels were measured in duplicate by ELISA (Quantikine Kit, R&D Systems, Minneapolis, MN). IL-6 mRNA levels were semiquantified by competitive polymerase chain reaction (PCR) because the HOBIT and Saka cells tend to grow slowly; therefore, it is difficult to obtain adequate levels of mRNA for other mRNA quantitation methods such as Northern blot analysis. Competitive PCR was performed as described (Keller and Ershler, 1995), with modifications. Briefly, total RNA, obtained by guanidinium thiocyanate single-step isolation (Chomczynski et al., 1987), was reverse transcribed with an oligo(dT) primer. Identical aliquots of the cDNA were placed in a series of PCR. Initially, 10-fold dilutions of an IL-6 PCR mimic (Clontech, Palo Alto, CA) were added to a series of PCR. Once the quantity of cDNA was determined within a 10-fold range, the process was repeated with 2-fold dilutions of the mimic which flank the 10-fold range. This set of reactions was spiked with [32P]dCTP. The reactions were subjected to electrophoresis and the resulting bands were imaged and quantified on a PhosphorImager system (Molecular Dynamics, Sunnyvale, CA). Linear regression was then used to determine the point of equivalency for the IL-6 mimic PCR product cpm and the IL-6 cDNA PCR product cpm. From this we determined the moles of input IL-6 cDNA because we know how many moles of mimic were used for input. Competitive PCR of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed to normalize the IL-6 values for differences in reverse transcription efficiency.

**Plasmids.** — To create an IL-6 promoter reporter plasmid, a plasmid containing the human IL-6 gene (Yasukawa et al., 1987) (kindly provided by T. Kishimoto, Osaka University) was double digested with XhoI/BamHI, and the resulting 1200-bp IL-6 promoter region was gel-purified and directionally subcloned into a promoterless luciferase reporter vector (pGL2-basic; Promega), resulting in the plasmid pGL2-IL6(-1200). The integrity of the construct was confirmed by restriction digests, testing activity in transient transfections, and partial sequencing. Plasmids were purified on a DEAE cellulose column (Qiagen, Chatsworth, CA).

**Transient transfection assays.** — Using standard calcium phosphate transfection techniques, the cells were cotransfected with a plasmid containing the SV40 promoter driving β-galactosidase (β-gal) cDNA and one of the three IL-6 promoter constructs or pGL2-basic or pGL2-control [contains the luciferase cDNA driven by the SV40 promoter (Promega)]. Briefly, 5 X 10^5 cells were plated on 60-mm plates; 24 h later plasmids (7 μg luciferase vector plus 1 μg β-gal vector/plate) were incubated with CaCl2 and Heps-buffered saline for 20 min. Subsequently the DNA-buffer mixture was added to cells in their growth media. The cells were incubated in 5% CO2 at 37 °C for an additional 16 h, washed with phosphate-buffered saline (PBS), and then incubated with fresh complete medium with or without ethanol. Cells were then collected at 24 and 48 h posttreatment by scraping into PBS. Total cellular extracts, obtained by three freeze–thaw cycles, were evaluated for β-gal and luciferase (luciferase assay reagent, Promega) activity on a luminometer. Luciferase activity was normalized by β-gal activity for each sample to account for differences in transfection efficiency.

**Statistical analysis.** — Statistical analysis was performed using StatView software (Abacus Concepts, Inc., Berkeley, CA). Results were considered significant at p ≤ .05. Data from the measurement of culture supernatant IL-6 levels from each cell line and time point were analyzed by one-way analysis of variance (ANOVA) using ethanol level as the main effect. Differences between the pattern of IL-6
production between HOBIT and Saka cells was evaluated by two-way ANOVA. When a significant main group effect was found by ANOVA, the Bonferroni/Dunn test was used for post hoc analysis to determine differences between individual groups. Data to semiquantitate IL-6 mRNA levels by competitive PCR were analyzed by linear regression. Data from the measurement of IL-6 promoter activation were analyzed for each plasmid by Student's t-test.

RESULTS

Ethanol increases IL-6 protein levels in culture supernatants from Saka, but not HOBIT, cell lines. — Our first goal was to examine ethanol’s effect on IL-6 expression by the HOBIT and Saka cells. To explore this issue, we incubated the cells with 0, 10, 50, or 100 mM ethanol for 24 or 48 h and then measured IL-6 in the supernatant by ELISA.

At 48 h, the levels of IL-6 in the culture supernatant from the Saka cells exposed to 100 mM ethanol significantly increased by 47% and 70% compared to the 0 mM and 10 mM ethanol-treated cells (Figure 1). Furthermore, the pattern of increased IL-6 levels observed with Saka cells was not observed in the HOBIT cells (two-way ANOVA; \( p = .001 \)) (Figure 1). These results demonstrate that ethanol increases IL-6 levels in a time-dependent manner in Saka cells.

Ethanol increase steady-state IL-6 mRNA levels in Saka cells. — Culture supernatant IL-6 levels may be increased due to increased secretion of previously formed IL-6, increased translation, or increased transcription. As many genes are controlled at the transcriptional level, we chose to evaluate ethanol’s effect on IL-6 mRNA levels. Saka cells were incubated with or without 100 mM ethanol for 48 h. Following the incubation period, RNA was isolated and reverse transcribed, and RNA levels were semiquantified by competitive PCR. Ethanol treatment increased steady-state RNA levels by approximately 40% (Figure 2).

Ethanol induces IL-6 promoter activity. — To explore the possibility that ethanol alters IL-6 mRNA levels through altered transcriptional activity, we transiently transfected Saka cells with an IL-6 promoter-driven luciferase reporter plasmid, pGL2-IL6(-1200). Upon ethanol treatment, IL-6 promoter-driven luciferase activity was increased by 275% (Figure 3). This finding suggests that ethanol is able to induce IL-6 promoter activity. Furthermore, ethanol did not enhance luciferase production in Saka cells transfected with pGL2-control, which contains the SV40 promoter, thus demonstrating that ethanol has some specificity for the IL-6 promoter. We have observed that ethanol stimulates the IL-6 promoter in HeLa cells, a cervical carcinoma cell line (unpublished observation). Thus, ethanol’s transactivation ability is not specific to Saka cells.

DISCUSSION

In the current study, we demonstrated that ethanol, at concentrations that do not affect viability of cells, increases IL-6 levels in culture supernatant of the Saka bone marrow stromal cell line, but not the HOBIT osteoblast-like cell line. Furthermore, we demonstrated that ethanol increases steady-state levels of IL-6 mRNA in Saka cells, and that this occurs, at least in part, through transcriptional activation of the IL-6 promoter.

In spite of the significant impact alcohol has on pathophysiology of bone and other organ systems, its effect on cytokine expression is largely unexplored. It is well docu-
Figure 2. Ethanol induces IL-6 mRNA expression in Saka cells. Saka cells (10^6) were plated in 100-mm plates and incubated in the absence or presence of 100 mM ethanol. After 24 h, RNA was isolated and IL-6 mRNA levels were evaluated by competitive PCR in reactions spiked with [α-32P]dCTP. (A) Agarose gel of 2-fold dilution PCR imaged by ethidium bromide staining. (B) Same gel as in A, but imaged by PhosphorImager. (C) Linear regression analysis of PCR product cpm. The triangles denote decreasing input (by 2-fold) of competitor template. The linear regression equation for the 0 mM ethanol treatment was $Y = .006X + .808$ ($R^2 = .93; p < .001$) and for the 100 mM ethanol group was $Y = .01X + .556$ ($R^2 = .98; p < .001$), where $X = 1/\text{attomol}$ and $Y = \text{ratio of IL6/mimic cpm}$.

Mentation that serum IL-1, IL-6, and TNF are elevated in chronic alcoholics (Bird et al., 1990; Deviere et al., 1989; Felver et al., 1990; Khoruts et al., 1991; McClain et al., 1986). However, these studies were performed in subjects who had hepatic cirrhosis, and the cytokine levels may have been increased in association with hepatocellular inflammation and not directly due to ethanol. The few studies that have explored this issue have demonstrated that in vivo ethanol consumption can influence cytokine expression independently of inflammation. For example, lipopolysaccharide (LPS)-stimulated IL-6 and IL-8 production from peripheral blood mononuclear (PBM) cells of alcoholics without liver disease was increased compared to nonalcoholics (Martinez et al., 1993). Interestingly, 30 days of...
chemotactic factor in rat hepatocytes (Shiratori et al., 1993). Although we did not examine for ethanol’s effect on other cytokines, our results document that ethanol induces increased expression of IL-6 protein from bone marrow stromal cells.

That both HOBIT and Saka cells produce IL-6, yet only Saka cells increased IL-6 supernatant levels in response to ethanol, was intriguing. This finding suggests that ethanol mediates activation of the IL-6 promoter in a cell-specific fashion. However, within the context of our assay, basal IL-6 supernatant levels were 10-fold higher in HOBIT cells compared to Saka cells (Figure 2). Perhaps HOBIT cells were already secreting IL-6 at threshold levels and thus were incapable of responding to ethanol. Furthermore, our observation that ethanol transactivates the IL-6 promoter in the HeLa cervical carcinoma cell line (unpublished observation) demonstrates that ethanol’s effect is not limited to Saka cells.

Our results suggest that IL-6 protein expression was increased, at least in part, due to increased steady-state levels of mRNA secondary to increased transcriptional activation of the IL-6 promoter. Ethanol has been documented to influence mRNA levels of a variety of genes (Miles et al., 1992, 1994; Wan et al., 1994). The mechanism by which ethanol activates the IL-6 promoter, or any promoter, is currently unknown. However, cis-acting elements are required for ethanol’s ability to induce the promoter of GRP78, a molecular chaperone (Hsieh et al., 1996).

Saka cells induce human marrow mononuclear cells to form osteoclast-like multinucleated cells that are capable of forming resorption lacunae on dentine (Takahashi et al., 1995). Our observation that ethanol induces increased IL-6 secretion from Saka cells suggests a mechanism by which ethanol may contribute to their osteoclastogenic activity. Indeed, IL-6, perhaps through stimulating release of IL-1β, induces differentiation of bone marrow mononuclear cells to osteoclasts in long-term human marrow cultures (Kurihara et al., 1990) and in cocultures of murine bone marrow and primary osteoblastic cells (Tamura et al., 1993). The observed effects may be due to a stress response secondary to the high ethanol concentrations used in the study. However, these concentrations are observed in chronic alcoholics, and thus may be relevant to in vivo pathophysiology.

In summary, ethanol can increase IL-6 supernatant levels produced by a bone marrow stromal cell line. This model suggests that ethanol may be capable of increasing IL-6 levels in the bone microenvironment, thus creating a milieu which favors osteoclastogenesis. The resultant increased osteoclasts could shift the balance of bone remodeling in favor of bone resorption that will clinically manifest as osteoporosis.

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