Effects of Isoprothiolane on Cell Growth of Cultured Bovine Mammary Epithelial Cells

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ABSTRACT. This study was performed to investigate the effects of isoprothiolane on cell growth and the production of interleukin (IL)-1 and IL-6 by bovine mammary epithelial cells in vitro. Isoprothiolane increased proliferation of mammary epithelial cells in a dose-dependent manner at the concentration of 0.05 to 5 µM when cultured either with or without serum-supplemented medium. In contrast, isoprothiolane (0.0005–5 µM) significantly inhibited the production of IL-1 and IL-6 by mammary epithelial cells. Moreover, the cytokines, IL-1α, IL-1β, IL-6, and tumor necrosis factor (TNF)-α tended to inhibit the proliferation of mammary epithelial cells in a dose-dependent manner. These results indicated that isoprothiolane regulated mammary epithelial cell growth in vitro possibly by modulating the production of cytokines.—KEY WORDS: cytokine, isoprothiolane, mammary gland.


It has been reported that isoprothiolane (diisopropyl 1,3-dithilan-2-yldene malonate) is effective in treating fat necrosis in Japanese Black cattle [11] and experimentally-induced fatty liver in Holstein cattle [6] and rats [1]. After treatment with isoprothiolane, the serum concentrations of phospholipid and total lipid were decreased, whereas serum non-esterified fatty acids (NEFA) concentration was increased [4]. Isoprothiolane may influence lipid metabolism by accelerating fatty acid desaturation in adipose tissue [4] and may effect protein synthesis as well as lipoprotein secretion in fatty livers [1, 6].

Isoprothiolane is also useful to reduce the number of mammary somatic cells in milk from dairy cows with mastitis [3]. However, it still is unknown whether isoprothiolane has direct effect on mammary tissue. Recently we have shown that bovine mammary epithelial cells produce interleukin (IL)-1 and IL-6, and express multiple cytokine transcripts including IL-1α, IL-1β, IL-6, IL-10, tumor necrosis factor (TNF)-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF) in vitro [12]. However, little is known about the regulation of cytokines produced by mammary epithelial cells. Both IL-1 and IL-6 are known to play a pathophysiological role in the immune response and tissue repair [18]. The purpose of this study was to investigate the effects of isoprothiolane and inflammatory cytokines on cell growth and production of IL-1 and IL-6 in cryopreserved primary cultures of bovine mammary epithelial cells.

Mammary epithelial acini were prepared as described previously [12, 13]. Briefly, cryopreserved cells were washed and separated by a preformed 45% Percoll density gradient (Pharmacia, Uppsala, Sweden) as described previously [12]. Immunohistochemically, the predominant cells recovered form the Percoll density gradient centrifugation were mammary epithelial cells that stained strongly positive for cytokeratin but were negative for α-smooth muscle actin and vimentin [12]. A suspension of cells with >70% viability was usually obtained by using trypan-blue dye-exclusion test. Cells were plated at a density of 1 × 10^6 cells/well in plastic 6-well culture plates (Corning Inc., Corning, NY, U.S.A.) coated with rat type I collagen [12]. The M199 medium (Gibco BRL, Grand Island, NY, U.S.A.) was supplemented with 15% fetal bovine serum (FBS, Boehringer Mannheim, Indianapolis, IN, U.S.A.), 5 µg/ml insulin (Sigma Chemical Co., St. Louis, MO, U.S.A.), 5 µg/ml hydrocortisone (Sigma), and 1 µg/ml ovine prolactin (Sigma). The medium was refreshed at two-day intervals until confluence.

After confluence, mammary epithelial cells were washed with serum-free M199, trypsinized, and replated at first passage into 96-well culture plates (Corning) in 100 µl of serum-free M199 (5 × 10^4 cells/well). Isoprothiolane (0.001–5 µM) were added to 100 µl of M199 either supplemented with or without FBS (final concentration 0, 5, 10, or 15%). Cells were incubated for a further 24 or 48 hr. After incubation, the medium was collected and stored at -20°C until assayed for IL-1 and IL-6 activity as described previously [12].

To determine the effect of bovine mammary epithelial cell growth, mammary epithelial cells (5 × 10^4 cells/well) were incubated in 96-well culture plates for 24 hr or 48 hr in M199 containing 5% FBS supplemented with various concentrations of recombinant human (rh) IL-1α (Boehringer Mannheim), IL-1β (Boehringer Mannheim), rhIL-6 (Gibco BRL), and rhTNF-α (Boehringer Mannheim).

Cell growth was estimated by colorimetric assay using 3(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT, Sigma) as described previously [12]. After collection of conditioned medium, the medium was replaced with 100 µl of serum-free M199 and 25 µl of MTT (5 mg/ml) was added to each well. The cells were incubated for 6 hr at 37°C. Then 100 µl of 3.5 M sodium dodecyl sulfate (SDS) in 50% N-N dimethylsulfoxide was added to each well incubated for 16 hr at 37°C. Absorption of the formazan product was measured at a wavelength of 570 nm by a microplate reader (BioRad, Tokyo, Japan). The number of living cells determined by trypsin blue dye exclusion and were counted using hemocytometer enumeration after
staining with trypan blue which was correlated well with colorimetric assay.

After 48 hr culture, total cellular RNA was isolated from mammary epithelial cells supplemented with 5% FBS and various concentrations of isoprothiolane (0.005–5 μM). A 424-bp fragment of the bovine IL-1α, a 251-bp fragment of the bovine IL-6, and a 552-bp fragment of the bovine IL-6 and IL-1β mRNA were amplified for a total of 35 cycles by reverse transcription-polymerase chain reaction (RT-PCR) as described previously [12]. IL-1α (5’-CTCTCTCAATCAGAAAG TCTTCTATG, 3’-CATGTCAAATTTTACTGCT CTTCC), IL-6 (5’-GACGGATGCTTCCAATCTG, 3’-ACCCACTGTTTTGAAGACTGCATCTT), and GAPDH (5’-TGCGAAATGGACATCGTCG, 3’-GCGGATG ATATTCGGGC) oligonucleotides were used as amplification primers. Photographs of ethidium bromide-stained agarose gels were scanned using Microsoft Photo Editor (Microsoft Corp., Santa Rosa, CA, U.S.A.) and imported into Scion Image (Scion Corp., Frederick, MD, U.S.A.) for semi-quantitation of PCR products.

Data were analyzed by one-way variance (ANOVA) using the statistical program InStat (GraphPad Software, San Diego, CA, U.S.A.) on an MS-DOS based computer and were expressed as a mean ± the standard error of the mean (SEM). The experiments were repeated at least twice.

Isoprothiolane stimulated mammary epithelial cell proliferation in a dose-dependent manner at 0.05–5 μM with or without the presence of various concentrations of FBS (P<0.05, Fig. 1). The greatest effect of isoprothiolane in promoting cell growth was detected at the concentration of 0.5 μM in the presence of 15% FBS (Fig. 1). FBS also enhanced the cell proliferation in a dose-dependent manner (Fig. 1).

IL-1-like biologic activity was detected in culture medium supplemented with 5% FBS and serum-free medium, but not in medium supplemented with 10% or 15% FBS. After replacement with serum-free M199, there was a significant increase (P<0.01) in IL-1 production and IL-1 production continued to increase for up to 48 hr as described previously [12]. IL-6 production in serum-supplemented medium increased after 48 hr and was produced at a steady level (1.3–1.8 U/ml) until day 14 [12]. In contrast, IL-6-like bioactivity produced by mammary epithelial cells was not detected in serum-free M199. After 48 hr of culture, the production of IL-1 by bovine mammary epithelial cells was significantly inhibited by isoprothiolane in a dose-dependent manner (0.0005–5 μM, Fig. 2). A significant decrease in IL-6 production was present in mammary epithelial cell cultures at 5 μM of isoprothiolane compared to control (Fig. 3). The ratio of IL-1α:GAPDH and IL-6:GAPDH mRNA expressions measured by image analysis decreased after 48 hr of culture (Fig. 4).

All cytokines, IL-1α, IL-1β IL-6, and rhTNF-α tended to inhibit mammary epithelial cell proliferation after 24 hr in a dose-dependent manner (Fig. 5). Significant differences in cell growth were detected with IL-1α at greater than 6.25 ng/ml and IL-6 and IL-1β at greater than 12.5 ng/ml, as compared with controls (no cytokine and isoprothiolane) after 24 hr of culture (Fig. 5). No significant difference in cell growth was found when the cells treated with TNF-α after 24 hr (Fig. 5). After 48 hr of culture, isoprothiolane enhanced the cell proliferation when if cytokines such as IL-1β, IL-6, and TNF-α were supplemented in the medium (Figs. 6-8). However isoprothiolane did not enhance the cell proliferation after culture for 24 hr in the medium supplemented with IL-1β, IL-6, and TNF-α (data not shown).

Various cytokines, including IL-1β, IL-6, IL-8, TNF-α, and transforming growth factor (TGF)-β have been detected in milk [18]. Cytokines are produced by various types of cells constitutively or in response to external stimuli [14]. The biologic effects of cytokines differ and are dependent on the cell types and environmental conditions [14]. The production of IL-1, IL-6, and IL-8 has been demonstrated in
normal mammary epithelial cells in vitro [12, 15]. The physiological function of cytokines produced by mammary epithelial cells is still unclear. The presence of multiple cytokines in the mammary gland suggests a pathophysiological role in host defense against infection and in tissue injury in response to local, as well as systemic, inflammation [18].

The mechanism by which isoprothiolane stimulates mammary epithelial cell proliferation has not been clarified. It is unknown whether isoprothiolane induces mammary epithelial cells to produce other growth factors, such as epidermal growth factor or TGF-α, which act via activation of a protein kinase. Isoprothiolane has been reported to be effective in the treatment of fat necrosis in cattle [4]. The effect of isoprothiolane on fatty liver was to promote nucleic acid and protein synthesis [1]. Malotilate, an isoprothiolane analogue, exert a wound healing to accelerate granulation tissue formation and rapid re-epithelization in rat wound healing model [9]. The mechanism of wound healing effects of malotilate was found to stimulate the migration of fibroblasts and increase the collagenase activity [10]. It is suggested that malotilate stimulates the production of collagenase by fibroblasts [10]. Malotilate also inhibit the invasion and metastasis of rat mammary carcinoma [7, 8].

Cytokines used in this study inhibited in vitro cell proliferation. It is known that IL-1 is a mitogen for keratinocytes in culture and enhances keratinocyte proliferation [16]. In contrast, IL-6 has dual actions consisting of either enhancement of keratinocyte proliferation and inhibition of cell growth in cultures of breast carcinoma cell lines [5]. IL-6 might be a regulator of epithelial cell growth and cell-cell association [5]. It has been reported that TNF-α stimulates the cell growth of
normal rat mammary epithelial cells [2] and inhibits human breast cancer cells in culture [17]. TNF-α has been shown to up-regulate epidermal growth factor receptor (EGFR) expression, but EGFR is not necessary for TNF-α action in normal rat mammary epithelial cells [19]. It is unclear why cytokines used in this study inhibit the proliferation of cultured bovine mammary epithelial cells. Further study will be needed to clarify mode of action of isoprothiolane and cytokines on bovine mammary epithelial cells in vitro and mammary glands in vivo.

In conclusion, the present study demonstrated that isoprothiolane and inflammatory cytokines regulated mammary epithelial cell proliferation and isoprothiolane inhibited IL-1 and IL-6 production of bovine mammary epithelial cells in vitro.

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