Detection of Interleukin-1 and Interleukin-6 on Cryopreserved Bovine Mammary Epithelial Cells in Vitro

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ABSTRACT. This investigation was performed to determine whether primary cultures of mammary cells from lactating cows would sustain production of interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF), and express mRNA for cytokines interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, interferon (INF)-τ, TNF-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF) by the reverse transcription-polymerase chain reaction (RT-PCR) in vitro. Cryopreserved mammary epithelial cells collected from cows at 1 week post calving were plated in collagen-coated 24-well culture plates (250,000 cells/well). IL-1 and IL-6 productions were measured using a α was not detected in medium alone. TNF-like activity was not detectable in any experiments. This study also demonstrated the expression of multiple cytokines in the mammary gland suggest a pathophysiological role in host defense to infection and response to tissue injury [17, 22]. It is still unclear whether these cytokines originate in part from either mononuclear cells in milk or mammary epithelial cells. The production of IL-6 and IL-8 has been demonstrated in human mammary epithelial cells in vitro [1, 17]. However, precise information on the expression of cytokines in bovine mammary epithelial cells is not available. The purpose of the present study was to measure the production of IL-1 and IL-6 and to detect cytokine mRNA using RT-PCR in cryopreserved bovine mammary epithelial cells from lactating cows in vitro.

MATERIALS AND METHODS

Cell culture: Mammary tissues were collected from three lactating Holstein dairy cows at 1 week after parturition. The mammary tissue was enzymatically digested to release glandular acini as described previously [16]. The dispersed cells were suspended in M199 medium (Gibco BRL, Grand Island, NY, U.S.A.) with 20% fetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO) at 1–1.5 × 10^7 cells/ml, frozen at -80°C overnight, and stored in liquid nitrogen. For culture, cryopreserved cells were quickly thawed at 37°C and washed with M199 medium containing 10% FBS. The mammary epithelial acini were separated by a preformed 45% Percoll density gradient (Pharmacia, Uppsala, Sweden) centrifugation at 20,000 × g for 1 hr as described [8, 19]. Mammary cells recovered from the Percoll density fraction higher than 1.055 g/ml were resuspended with M199 containing 15% FBS, insulin (5 µg/ml, Sigma, St. Louis, MO, U.S.A.), hydrocortisone (5 µg/ml, Sigma), ovine prolactin (1 µg/ml, Sigma), gentamicin (50 µg/ml), and amphotericin B (2.5 µg/ml) and plated at a concentration of 2.5 × 10^5 cells/0.5 ml onto collagen-coated plastic 24-well cell culture plates (Iwaki Grass Co., Chiba, Japan). Rat type I collagen gel was prepared as originally described [3]. The medium was changed at two-day intervals until day 14. At day 6 (preconfluent), the medium was replaced with M199 alone to avoid the effects of supplements. After a 12-hr incubation, the medium was replaced with M199 alone, and the medium collected at 4, 8, 24, and 48 hr after. The medium was stored at -20°C until assayed for IL-1, IL-6, and TNF bioactivity. Cell numbers were determined by trypsin blue dye exclusion and hemocytometer enumeration and by colorimetric assay using 2,2,3,5-diphenyltetrazolium bromide (MTT) [15].

Immunocytochemistry: At day 6, cells grown on glass coverslips were stained immunohistochemically with monoclonal mouse anti-cytokeratin IgG (clones AE1/AE3,
Bioassay for IL-1: IL-1-like activity in medium samples was measured by cytolytic assay using A375 human melanoma cell line. Briefly, the cells (5 \times 10^4/ml) were cultured with samples, recombinant human (rh) IL-1\(\alpha\) or rhIL-1\(\beta\) (final concentration 0.01-10 U/ml, Boehringer Mannheim) in minimum essential medium (MEM) supplemented with 10% FBS, gentamicin (50 \mu g/ml), and amphotericin B (2.5 \mu g/ml) in a 96-well culture plate in triplicate. After 96 hr incubation, 50 \mu l of 0.05% neutral-red dye was added to each well and plates were incubated for further 6 hr at 37°C. After washing each well with PBS, 50 ml of 50% ethanol in 0.05 M NaH\(_2\)PO\(_4\) was added to each well for extraction of dye incorporated by viable cells. The colored reaction product was read at a wavelength of 540 nm by a microplate reader (Immunoreader Model N12000, Nikon Intermed Co., Tokyo, Japan).

Bioassay for IL-6: IL-6-like bioactivity was quantified using IL-6 dependent 7TD1 hybridoma proliferation assay. Briefly, 7TD1 cells were cultured in RPMI-1640 medium supplemented with L-glutamine (2 mM), N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES; 25 mM), 2-mercaptoethanol (2-ME; 5 \times 10^{-4} M), gentamicin (50 \mu g/ml), amphotericin B (2.5 \mu g/ml) and 10% FBS. IL-6 assay was performed in triplicate for each sample or standard (final concentration 0.01–25 U/ml, Gibco BRL) in 96-well culture plates. 7TD1 cells were seeded at a density of 5 \times 10^4 cells per well and cultured in the described medium in either the presence or absence of IL-6 containing samples. After 72 hr, 25 \mu l of MTT (5 mg/ml, Sigma) was added to each well and plates were incubated for 6 hr at 37°C. Then 100 \mu l of sodium dodecyl sulfate (SDS; 3.5 M) in 50% N-N dimethylsulfamide was added, and a further 16 hr incubation at 37°C was conducted. The dark blue-colored reaction product was read at a wavelength of 540 nm by a microplate reader. Assays were conducted in triplicate.

RESULTS

Morphology: Within 24 hr after plating most cells attached to the collagen gels and began to grow outward from the attached organoids or cell clumps as evaluated by phase-contrast microscopy. Elongated cells and large polygonal cells spread and grew from the organoids and formed sheets of cells. Elongated cells were present at the periphery of the epithelial cell sheets. The cells formed preconfluent monolayers by day 6 (Fig. 1). Almost all cells grown in these conditions stained strongly positive for cytokeratin but were negative for \(\alpha\)-smooth muscle actin and vimentin (data not shown).

Bioactivity for IL-1, IL-6, and TNF: IL-1-like activity was not detected during the experiment in which the epithelial cells were culture medium supplemented with FBS. After replacement with unsupplemented M199, there was a significant increase (P<0.01) in IL-1 production and
the production continued to increase up to 48 hr (Fig. 2).

IL-6 production in the conditioned medium increased after day 2 and was produced at a steady level (1.3–1.8 U/ml) until day 14 (Fig. 3). When IL-6 production was adjusted for cell number among day 4 and day 14, production of IL-6 in mammary epithelial cells decreased from 0.49 U/10^5 cells on day 4 to 0.25 U/10^5 cells on day 6 (P<0.05) and was relatively stable after confluence (approximately 0.28–0.35 U/10^5 cells). However, IL-6 production increased to 0.48 U/10^5 cells on day 14 again. On the other hand, IL-6-like bioactivity produced by mammary epithelial cells was not detected in unsupplemented M199.

RT-PCR: Reverse transcription-PCR amplification of mRNA from bovine mammary gland cultures demonstrated cDNAs of predicted size for IL-α, IL-β, IL-6, IL-10, TNF-α, and GM-CSF (Fig. 4). However, expression of IL-10 and GM-CSF was less compared to IL-α, IL-β, IL-6, and TNF-α. After extraction of the IL-10 cDNA from the agarose gel and reamplification for an additional 20 cycles, the amplified cDNA of IL-10 was strongly detected (data not shown). Transcripts for IL-2, IL-4, and INF-γ were not identified in any bovine mammary cell cultures. Each fragment was cleaved with appropriate restriction enzymes and the fragment size confirmed with the previously sequenced data of each cytokine as described [11, 12] (data not shown).

DISCUSSION

It is difficult to determine whether cytokines present in milk originated from inflammatory cells or from mammary cells in vivo, because many somatic cells such as macrophages and neutrophils are present in milk and lymphocytes infiltrate into the interstitial tissue of the mammary gland. High concentrations of IL-1 and IL-6, or TNF were detected in milk of cows in which lipopolysaccharide (LPS) was infused into the mammary gland [22] or Escherichia coli was infused to induce mastitis [23]. It has been demonstrated that macrophages from milk can produce IL-1 and TNF-α [5] and that bovine macrophages secrete IL-6 in response to LPS stimulation [13].

The present study demonstrated that cryopreserved mammary epithelial cells from lactating cows produced IL-1 and IL-6 in vitro. The bioactivity of IL-6 was greatest in mammary cultures on day 2, then decreased, and remained at low levels for 14 days. High IL-6 activity in the conditioned medium at day 2 may have resulted from lysis of mammary epithelial cells. The production of IL-6 did not correlate with cell growth. Further investigations of this phenomenon may produce insight into cellular regulation of IL-6.

The cells secreted IL-1 under serum-free condition, whereas IL-1 bioactivity was not detected in the conditioned medium supplemented with FBS which contained IL-1 receptor antagonist. This is in contrast to cells in which IL-6 production was not demonstrated in cells cultured with unsupplemented M199 medium. It has been reported that both normal mammary epithelial cells and carcinoma cells constitutively produce IL-6 and IL-1α but not IL-1β and TNF-α in vitro [1, 2, 17]. Insulin, hydrocortisone, and PRL
are known as lactogenic hormones generally used to modulate milk proteins in primary cultured mammary epithelial cells. However, hydrocortisone [1] and PRL [17] did not influence on the production of IL-6 in the human mammary epithelial cell culture. Some growth factors (i.e. TGF-β and basic fibroblast growth factor) have been demonstrated in the mammary gland \textit{in vivo} [9]. Therefore, other lactogenic hormones, growth factors, and/or cytokines produced in the mammary gland may regulated the production of IL-1 and IL-6 by mammary epithelial cells [16].

RT-PCR is a sensitive method for detecting specific mRNAs from small samples [6, 10]. The present study detected multiple cytokine mRNAs such as IL-1α, IL-1β, IL-6, IL-10, TNF-α, and GM-CSF in cultured mammary epithelial cells from lactating cows with or without any stimuli \textit{in vitro}. Transcripts for IL-1α, IL-1β, IL-6, and TNF-α were strongly expressed, whereas those for IL-10 and GM-CSF were weakly expressed. Moreover, expression of cytokine mRNAs for IL-2, IL-4, and INF-τ were not detected in any of the cultures. Human mammary epithelial cells have been shown to express cytokine transcripts for IL-6, IL-8, and TNF-α but not for IL-1β [1]. The discrepancies of IL-1 production and mRNA expression between bovine and human mammary epithelial cells may be the due to differences of calcium concentrations (2.67 mM vs 0.04 mM) and/or sources of serum (bovine vs equine) in the culture medium.

A large number of cells including keratinocytes, endothelial cells, synovial cells, fibroblasts, vascular smooth muscle cells, and monocyte-macrophages throughout the body produce IL-1 and IL-6 [4, 24]. Recently, it has been demonstrated that normal human epidermal keratinocytes produce IL-1 and IL-6 [17]. Although cultured keratinocytes express mRNA for IL-1α and β, only IL-1α produced by keratinocytes is biologically active [18]. The expression of cytokine mRNAs has been reported in peripheral blood mononuclear cells (PBMCs) and alveolar macrophages stimulated with LPS or concanavalin A [11–13]. In the absence of mitogen stimulation, mRNA expression for IL-1α, IL-1β, IL-2, IL-6, INF-τ, and TNF-α was detected at a low level in PBMCs and macrophages [11, 12].

The physiological role of cytokines secreted by mammary epithelial cells is still unknown. It has been reported that IL-1α enhances wound re-epithelialization \textit{in vivo} [21]. Skin is the major barrier between the external environment and the body, therefore human skin and cultured keratinocytes produce large amounts of IL-1 constitutively without any causing effect [14]. Apparent production of IL-1 in cultured bovine mammary epithelial cells without any stimuli may provide the initial signal to the immune system in the mammary gland [14]. IL-1 and IL-6 may play a pathophysiological role in defense from infective organisms in response to both local and systemic inflammation in the mammary gland [17, 18, 22]. High production of IL-8 by mammary epithelial cells may be involved with the recruitment of neutrophils and T lymphocytes into milk [17].

The mammary cells cultured in the present study were positive for cytokeratin and did not stain for α-smooth muscle actin and vimentin. Under these conditions, immunohistochemical features demonstrated that the predominant cells recovered from the Percoll density gradient centrifugation were mammary epithelial cells but not cells with myoepithelial or fibroblastic characteristics [8].

Further investigations using this model of cryopreserved mammary cell culture will be useful to clarify the function and regulation of cytokines in mammary epithelial cells.

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REFERENCES


