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Identification of Macrophage Colony-Stimulating Factor in Human Milk and Mammary Gland Epithelial Cells

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ABSTRACT

Human milk contains cellular and soluble host-protective components. Macrophages in human milk constitute a major cellular component in contrast to those in the peripheral blood. We have investigated a mechanism of local expansion of macrophages in human milk. First, biologically active macrophage colony-stimulating factor (M-CSF) was identified in human milk. The maximal concentrations of M-CSF in human milk were 10- to 100-fold higher than those in the serum. The concentrations of M-CSF in the milk had no correlation with those in the serum. M-CSF was immunohistochemically detected in the epithelial cells of the ducts and alveoli of the mammary gland. *In situ* hybridization study confirmed the local synthesis of M-CSF in the mammary gland epithelial cells. A possible role of female sex steroids was discussed in the regulation of M-CSF production by mammary gland epithelial cells. (***Pediatr Res* 37: 437–443, 1995**)

Abbreviations

M-CSF, macrophage colony-stimulating factor
DEAE, diethylaminoethyl
TPA, 12-*o*-tetradecanoyl phorbol-13-acetate

Human milk contains a variety of cellular and soluble components that may protect infants from infections (1, 2). Macrophages constitute a major cellular component (1, 2) and can survive under conditions simulating the newborn infant's gastrointestinal tract (3, 4). Furthermore, milk macrophages phagocytized infectious pathogens *in vitro* (5) and were found to release carrying IgA in association with phagocytosis or surface membrane stimulation (6, 7). Therefore, it is suggested that milk macrophages play a role in the host defense.

In the present study, we have investigated a mechanism of local expansion of macrophages in human milk. Because M-CSF is a cytokine that induces proliferation and differentiation of macrophages (8), we first studied M-CSF levels in human milk. The maximal M-CSF levels in the colostrum were 10- to 100-fold higher than those in the serum. M-CSF is also known to play an essential role in female reproduction (9–12), and the local production of M-CSF in female reproductive organs, placenta and uterus, is regulated by female sex steroids (13, 14). Because the mammary gland is closely involved in reproduction and the development of the milk-secreting apparatus of the mammary gland is also stimulated by female sex steroids (15), we have investigated the local production of M-CSF in the mammary glands by immunohistochemical staining and *in situ* hybridization. Here, we have shown that the M-CSF protein and mRNA were detected in the mammary gland epithelial cells. A role of female sex steroids in the regulation of M-CSF production by mammary gland epithelial cells was assessed by the study with human breast cancer cell lines.

METHODS

Milk samples. Human milk samples were obtained from 16 healthy women between 19 and 31 y of age who had delivered mature healthy infants after full-term pregnancy (range: 38–41 wk). Informed consent was obtained from each mother. Milk samples were collected on d 2–6 postpartum and 1 mo after delivery. The specimens were centrifuged at 3000 rpm for 10 min. The lipid layer was removed and the aqueous layer was collected and stored at −20°C until use. The aqueous milk specimens were delipidized with cold ether before use. This step removed a part of inhibitory activity in milk samples.

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Cellular pellets were resuspended in saline, counted, and stained for nonspecific esterase to identify macrophages. Serum samples were collected from the lactating women on d 3 postpartum.

ELISA. M-CSF concentrations in serum, culture supernatants or milk were determined by the ELISA specific for human M-CSF, as described previously (16). First, wells of a flat-bottomed, 96-well microtiter plate were incubated with horse anti-human M-CSF Ig in PBS for 24 h at 4°C. Each well was washed three times with 0.05% Tween 20 in PBS using a microtiter washer (Microwasher 120, Flow Laboratories, McLean, VA). Each well was then filled with blocking solution (10% normal horse serum, 0.25% BSA, 0.02% NaN_3 in PBS) overnight at 4°C. Each test sample or human M-CSF standard solution was added to a prewashed coated well and incubated for 2 h at 37°C. After a wash, rabbit anti-human M-CSF Ig in blocking solution was then added to each well. After incubation for 2 h at 37°C, each well was washed and filled with horseradish peroxidase–conjugated goat Ig G against rabbit Ig in blocking solution without NaN_3 for 2 h at room temperature. After five washes, to each well was added the reaction solution (0.015% H_2O_2, 0.8 g/L *o*-phenylenediamine dihydrochloride in 0.1 mol/L citrate buffer, pH 5.0). After incubation for 30 min at room temperature in the dark, the reaction was stopped by adding 2N H_2SO_4 and the color was read on an automatic microtiter plate reader (Twin Reader, Flow Laboratories) using dual beam wavelengths of 492 and 690 nm.

Colony-stimulating activity. Colony-stimulating activity was determined by a monolayer agar culture system containing mouse unfractionated bone marrow cells according to the method described previously (17). One unit was defined as the amount of M-CSF needed to form a colony.

For a neutralization experiment, preincubation was carried out with control rabbit IgG or IgG antibody specific for human M-CSF for 1 h at room temperature before colony assay. Rabbit anti–recombinant human M-CSF IgG (1 mL) neutralizes 120×10^4 units of human M-CSF and does not react with other human colony stimulating factors (16).

Purification and immunoblotting of milk M-CSF. Approximately 100 mL of colostrum were delipidized with cold ether, concentrated, and desalted. The starting material contained 134.7×10^4 units of M-CSF (100%). The sample was first applied to a DEAE Sepharose column preequilibrated with 0.02 mol/L sodium phosphate buffer (pH 7.0). After wash with the same buffer, the sample was eluted with the phosphate buffer containing 0.3 mol/L NaCl (pH 7.0). The eluent was concentrated and desalted. The 0.3 mol/L NaCl-eluted fraction from DEAE contained 127.6×10^4 units (94.7%) and the DEAE pass-through fraction contained 0.6×10^4 units (0.4%). The eluent was further applied to a horse anti–M-CSF Ig-coupled immunoaffinity column, as described previously (18). After washing with PBS containing 0.3 mol/L NaCl, M-CSF was eluted with 3.5 mol/L potassium thiocyanate in 0.1 mol/L phosphate buffer. The eluent was concentrated and subjected to SDS-PAGE. After electrophoresis, the proteins on the gel were transferred to a nylon membrane in blotting buffer with an electroblotting apparatus (Funa-Blotting Set, Funakoshi, Tokyo, Japan). The membrane was blocked with 3% BSA in PBS and probed with rabbit anti–recombinant human M-CSF antibody for 12 h. After washing the membrane with PBS containing 0.05% Tween 20, the membrane was treated with peroxidase-conjugated goat IgG against rabbit IgG, followed by H_2O_2 and *o*-phenylenediamine dihydrochloride (18).

Immunohistochemical staining of M-CSF. Mammary gland specimens were obtained from patients with breast tumors (fibroadenoma, fibrocystic disease, cancer) including four lactating women aged 25 to 34 y (3 wk to 6 mo after delivery), six nonlactating premenoposal women aged 20 to 40 y, and three postmenoposal women aged 68 to 72 y. No mammary gland specimens were available from women in the last trimester of pregnancy or immediately after delivery. Immunohistochemical staining of M-CSF was performed with rabbit anti–recombinant human M-CSF IgG or control rabbit IgG and Universal DACO LSAB Kit K680 (Dako Japan Co. Ltd., Kyoto, Japan). The specimen was first incubated for 5 min with 3% hydrogen peroxide, followed by a 5-min incubation with blocking serum. The specimen was then incubated with rabbit anti–recombinant human M-CSF IgG or control rabbit IgG for 10 min. Each incubation was followed by a wash with PBS. The specimen was incubated with biotinylated second antibody for 10 min and with peroxidase-labeled streptavidin for 10 min. Staining was completed after a 10-min incubation with 3% 3-amino-9-ethylcarbazole.

In situ hybridization. Human 1.8-kb M-CSF cDNA (kindly provided by the Genetics Institute, Boston, MA) was inserted into Bluescript. Using a digoxigenin RNA labeling kit (Boehringer Mannheim, Mannheim, Germany), sense RNA and antisense RNA probes were prepared from 1 μg of temperate DNA fragment. Frozen sections (4 μm in thickness) were mounted on gelatin-coated glass slides. After air drying, the sections were immersed in a fixative (4% paraformaldehyde in PBS, pH 7.4) for 5 min and were subsequently washed in PBS. The slides were then incubated for 10 min in a solution containing 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 10 μg/mL of proteinase K (Boehringer Mannheim) at 37°C. These slides were incubated for 10 min in 0.25% acetic anhydride in 0.1 M triethanolamine buffer (pH 8.0) at room temperature. After washing in PBS and dehydration in ethanol, the slides were subjected to an overnight hybridization at 43°C in a buffer that contained 3 μg/mL of digoxigenin-labeled RNA, 50% formamide, 0.3 M NaCl, 20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 10 mM NaH_2PO_4 (pH 8.0), 10% dextran sulfate, 1× Denhardt's solution, 10 mM DTT, 250 μg/mL of salmon sperm DNA, and yeast RNA. After hybridization, the slides were washed in 2 × SSC (1 × SSC: 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0) for 30 min at 60°C, followed by a 30-min incubation in 2 × SSC, 50% formamide solution at 60°C and a subsequent 5-min incubation in 0.1 × SSC solution at 40°C. The color of the probe was then developed using a Dig detection kit (Boehringer Mannheim) (19).

In vitro stimulation of breast cancer cells with hormones. Breast cancer cell lines, MCF-7 and ZR-75–1, were kindly provided by Dr. T. Kubota, Department of Surgery, Keio University, Tokyo, Japan. MRK-nu-1 and YMB-1 were provided by Japanese Cancer Research Resources Bank-Cell, Tokyo, Japan. All four cell lines carry estrogen receptors

(20–23). 17β-Estradiol and TPA were purchased from Sigma Chemical Co. (St. Louis, MO).

Cells were cultured in Eagle's minimum essential medium (Nissui Co. Ltd., Yuhki, Japan) supplemented with 10% FCS (Gibco Laboratories, Grand Island, NY), L-glutamine (0.292 g/L), and kanamicin (0.06 g/L). At 60–80% confluency, cells were cultured in the presence of various concentrations of estradiol or TPA. After 24 h, the supernatants were collected and stored at −80°C for cytokine assays.

Statistical analysis. Assessment of correlations between variables was made by Pearson correlation analysis. The significance of the relationships was tested by the *t* test.

RESULTS

Identification of M-CSF in human milk. We examined whether the ELISA system established for the quantitation of serum M-CSF (18) was applicable to the determination of milk M-CSF concentrations. First, the recovery of exogenous M-CSF in milk was studied. Various amounts (2000–10 000 U/mL) of exogenous M-CSF were added to defatted human milk specimens, and M-CSF concentrations were measured by the ELISA. The ratios of the measured and caliculated concentrations were 101.4 ± 7.5% (mean ± SD), which means that most of the added M-CSF was recovered by the ELISA without inhibition. At 20- to 100-fold dilutions, calculated M-CSF concentrations were almost constant as long as measured M-CSF concentrations were within the quantitative linear region (20–400 U/mL) of the standard curve (18). The within-run imprecision expressed as a coefficient of variation was below 10%.

Milk M-CSF concentrations were serially measured on d 2–6 postpartum. Maximal M-CSF concentrations were observed on d 2 postpartum in most cases with a great variation between 52 470 and 3740 U/mL, as shown in Figure 1. Then, milk M-CSF concentrations declined rapidly to less than 10 000 U/mL. One month after delivery, milk M-CSF concentrations were 1150 ± 120 U/mL (mean ± SD). At this time, seven samples were collected because of a little individual variation.

Molecular form of milk M-CSF. Milk M-CSF was purified by a DEAE Sepharose and anti–M-CSF antibody-coupled immunoaffinity column, as described in Methods. Purified M-CSF was subjected to SDS-PAGE and Western blotting analysis. As shown in Figure 2, the molecular mass of milk M-CSF was approximately 80 kD under nonreduced conditions and 40 kD under reduced conditions. Thus, the molecular structure of milk M-CSF was an 80-kD disulfide-liked homodimer similar to that of serum or urine M-CSF.

Biologic activity of milk M-CSF. Determination of colony-stimulating activity by a monolayer agar culture showed a significant positive correlation between colony-stimulating activity and ELISA units in milk with a correlation coefficient of 0.977 ($p < 0.01$), as shown in Table 1.

To exclude the possibility that the colony-stimulating activity observed was due to other contaminants in the milk, neutralization experiments with anti–M-CSF antibody were performed. The colony-stimulating activity was strongly inhibited by preincubation with anti–M-CSF IgG (mean percent inhibition ± SD: 84.3 ± 5.9%, $n = 4$) but not with control IgG (2.9 ± 2.5%, $n = 4$).

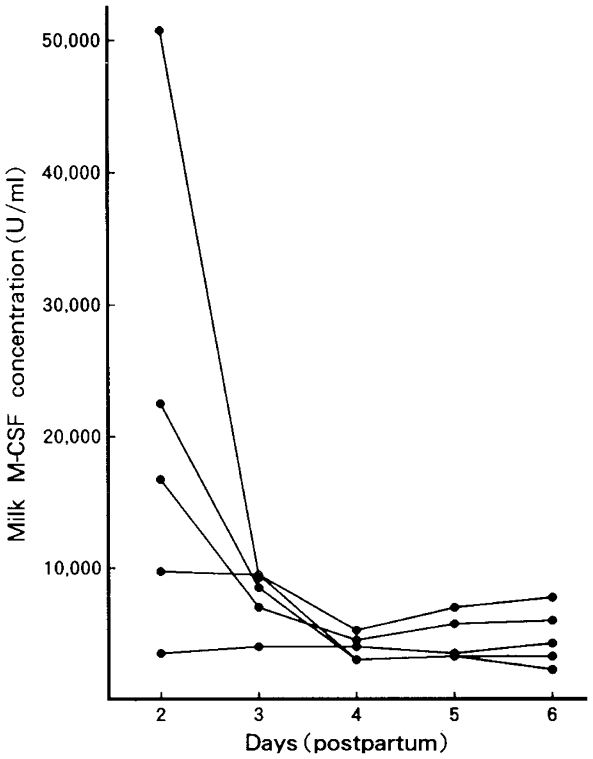

Figure 1. Serial determination of milk M-CSF concentrations.

Simultaneous comparison of milk and serum M-CSF concentrations. To determine whether milk M-CSF was derived from serum M-CSF, we examined milk and serum M-CSF concentrations simultaneously on d 3 postpartum. As shown in Figure 3, despite high milk M-CSF concentrations, serum M-CSF concentrations were consistently less than 1000 U/mL ($p > 0.1$). Thus, it is less likely that milk M-CSF was derived from serum.

Immunohistochemical localization of M-CSF in mammary gland specimens. M-CSF was immunohistochemically detected in the epithelial cells of the alveoli of the mammary gland obtained from lactating women (Fig. 4*A*) and in the epithelial cells of the ducts in nonlactating premenoposal (Fig. 4*C*) and postmenoposal women.

Detection of M-CSF mRNA in mammary gland tissue sections by in situ hybridization. M-CSF mRNA was mainly expressed in the epithelial cells of the ducts of the mammary gland (Fig. 5). No significant mRNA was detected in the mammary gland tissue sections when control sense probe was used.

Stimulation of human breast cancer cells with female sex steroids. To investigate whether female sex steroids have effects on the secretion of M-CSF by the mammary gland epithelial cells, cloned cell lines of human breast cancer were used. Four cell lines derived from the mammary gland were cultured in the presence of various concentrations of 17β-estradiol or TPA that induce differentiation of tumor cells (24). Estradiol and TPA did not affect cell viability at all. As shown in Figure 6, MRK-nu-1 cells constitutively produced M-CSF,

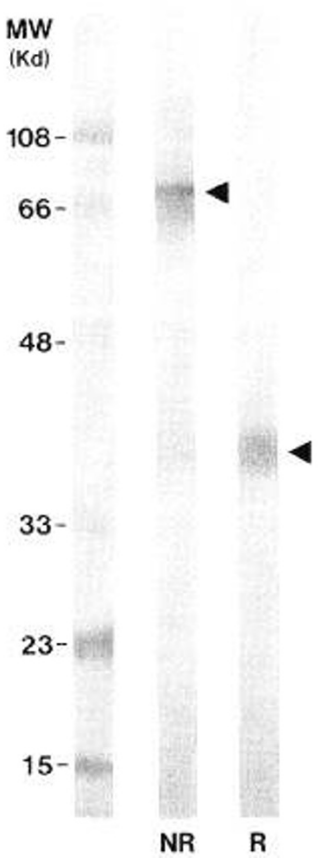

Figure 2. Molecular structure of milk M-CSF. *Arrowheads* indicate the molecular weights (*MW*) of milk M-CSF under nonreduced (*NR*) and reduced (*R*) conditions.

Table 1. *Correlation between biologic activity and ELISA data of milk M-CSF*

Milk sample	Biologic activity (units)	ELISA data (units)
1	2300	3000
2	5700	5000
3	1000	2000
4	4500	5000
5	400	2000

Colony-stimulating activity was determined by a monolayer agar culture system containing mouse unfractionated bone marrow cells. One unit was defined as the amount of M-CSF needed to form a colony. A significant correlation was observed between biologic activity and ELISA data ($p < 0.01$).

and its production was significantly enhanced by TPA ($p < 0.01$) but not by estrogen. MCF-7 produced a small amount of M-CSF in response to 100 nmol/L of estradiol in the presence of TPA. ZR-75–1 produced M-CSF in response to 100 nmol/L of estradiol in the presence or absence of TPA. YMB-1 cells produced no detectable M-CSF to any stimulation.

DISCUSSION

Human milk contains cellular and soluble components with antiinfective properties (1, 2) and protects infants from infections (25). In the present study, we have investigated how macrophages are expanded as a major cellular component in human milk.

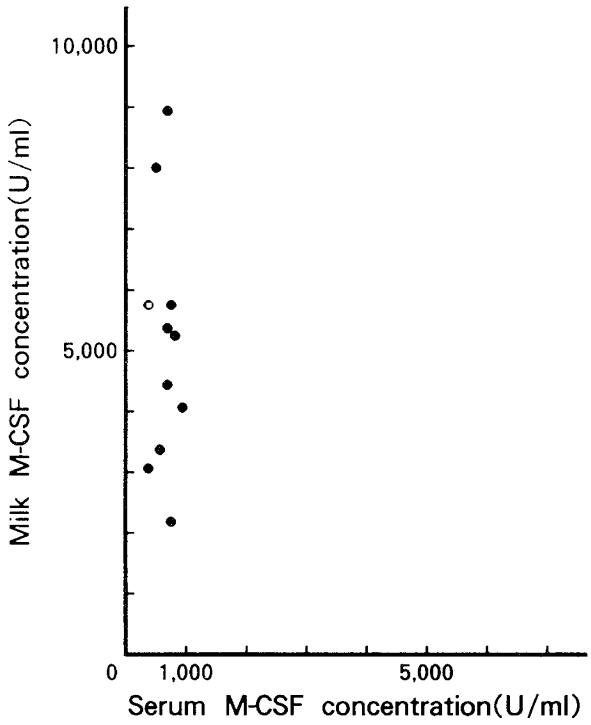

Figure 3. Simultaneous determination of milk and serum M-CSF concentrations.

We have found that human milk contained a large amount of biologically active M-CSF that could induce proliferation and differentiation of macrophages (8). Because concentrations of serum M-CSF were neither elevated nor correlated with those of milk M-CSF, it was suggested that the concentrating mechanism of M-CSF from the blood would not be a major one for high M-CSF levels of milk. With respect to cytokines, local production is common but no concentrating mechanism from the blood is known for local enrichment. Therefore, we assumed that milk M-CSF was produced locally in the mammary gland. It is conceivable that milk macrophages produced M-CSF in human milk because milk macrophages secrete most cytokines as peripheral blood monocytes do (26). However, production of M-CSF by milk macrophages themselves cannot explain an initial event of macrophage enrichment in human milk. Recent studies have shown that M-CSF is produced locally in the human placenta and uterus (11, 12). The present study has demonstrated that M-CSF was produced locally in the epithelial cells of the mammary gland. Because there was no great difference among the M-CSF–staining intensities of the mammary gland epithelial cells obtained during lactating, nonlactating premenoposal, and postmenoposal periods, it was suggested that mammary gland epithelial cells synthesize a certain amount of M-CSF at the three periods. However, because there is a great difference among the total cell numbers of the M-CSF–producing epithelial cells of the mammary gland at the three periods, female sex steroids might partly regulate M-CSF synthesis by controling the total cell number of M-CSF–producing epithelial cells of the mammary gland by inducing the ductal and lobuloalveolar development.

In addition, studies with cloned cell lines of mammary gland epithelial cells revealed that two cell lines, MCF-7 and ZR-

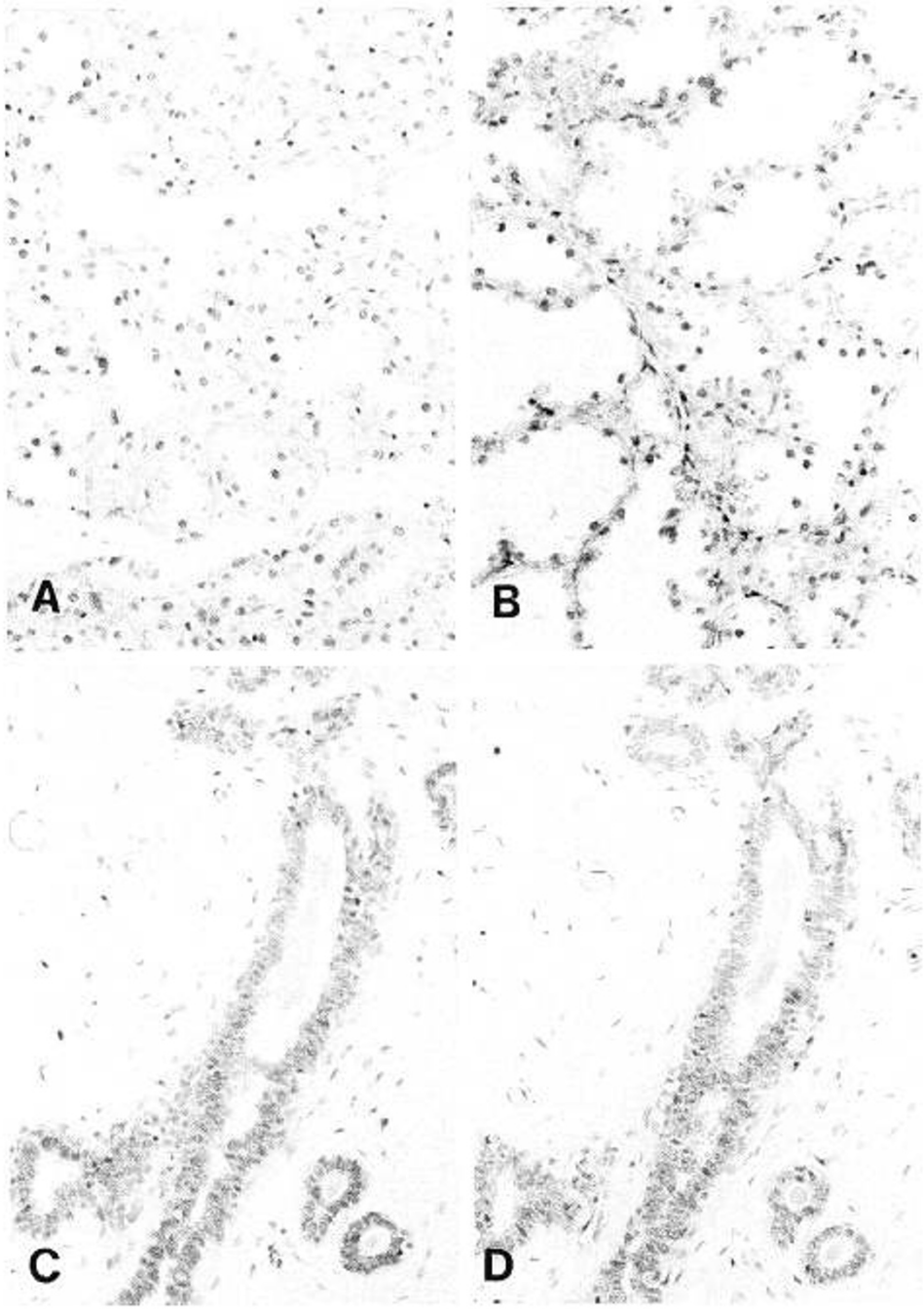

Figure 4. Immunohistochemical staining of the human mammary gland specimens. Epithelial cells of the alveoli of the mammary gland at the lactating period were stained with anti–M-CSF antibody (*A*) and control antibody (*B*). Epithelial cells of the ducts of the mammary gland at the nonlactating premenoposal period were stained with anti–M-CSF antibody (*C*) and control antibody (*D*).

75–1, produced M-CSF in response to 100 nmol/L of estrogen in the presence of TPA and to 100 nmol/L of estrogen with or without TPA, respectively. Although slight enhancement of cell multiplication was observed during the 24 h in the presence of 1–100 nmol/L of estradiol, M-CSF production was not simply attributable to cell multiplication because no M-CSF was produced at the optimal concentrations (1–10 nmol/L) for cell growth (27, 28). Although further confirmation using normal mammary gland epithelial cells is required before conclusions are made, it is possible that female sex steroids play a regulatory role in the production of M-CSF by the mammary gland epithelial cells at a single-cell level.

Thus, female sex steroids may play a role in the production of a major host-protective cellular component, macrophages, partly by stimulating M-CSF secretion by the mammary gland epithelial cells at a single-cell level and partly by controlling the total cell number of the epithelial cells of the mammary gland. Once milk macrophages are expanded by M-CSF derived from the epithelial cells of the mammary gland, it is likely that milk macrophages secrete M-CSF in an autocrine fashion. It remains to be determined whether other cytokines such as granulocyte macrophage–colony-stimulating factor, IL-3, and IL-4 are also involved in the production of milk macrophages.

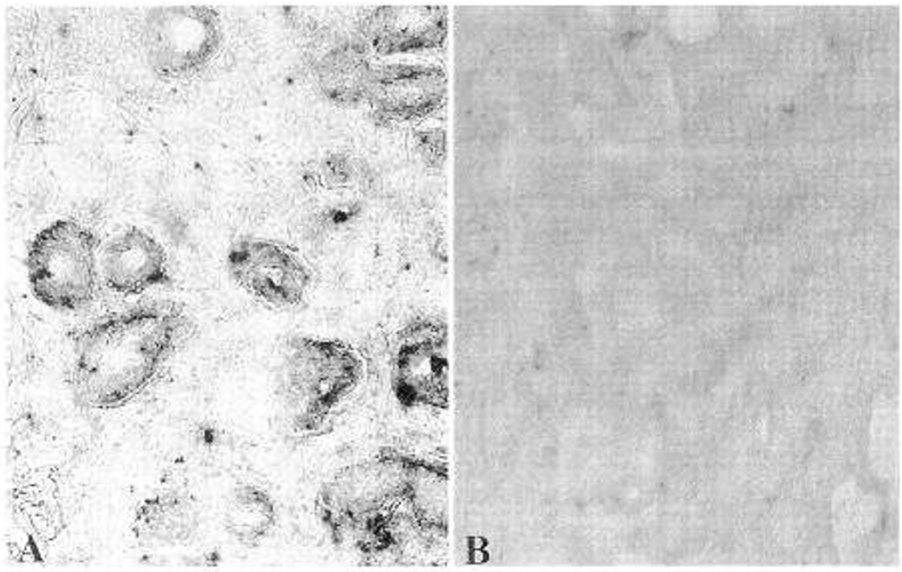

Figure 5. Localization of M-CSF mRNA in the mammary gland tissue sections. Antisense RNA probe for M-CSF (*A*) and control sense RNA probe for M-CSF (*B*). M-CSF mRNA was detected in the epithelial cells of the mammary gland.

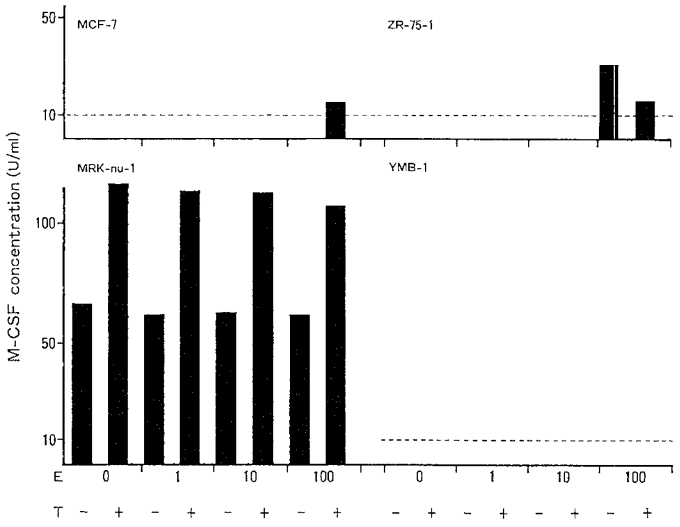

Figure 6. Effect of estrogen and TPA on the secretion of M-CSF by human breast cancer cells. Human breast cancer cell lines, MCF-7, ZR-75–1, MRK-nu-1, and YMB-1 were cultured in the presence of 0, 1, 10, or 100 nmol/L of 17β-estradiol (*E*) or TPA (*T*) (−: 0 μg/L; +: 100 μg/L) for 24 h. The culture supernatants were collected and assayed for M-CSF.

IL-1, IL-6, and tumor necrosis factor-α were detected in human milk (29–33). Because these cytokines were also produced in the female reproductive organs (34–36) and induced by estrogen and progesterone (36), there is a possibility that these cytokines are also derived from the mammary gland under the regulation of hormones.

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