BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **240,** 104–107 (1997)
ARTICLE NO. RC977617

EGF Inhibits Expression of WDNM1 and Sulfated Glycoprotein-2 Genes in Mammary Epithelial Cells

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Received September 26, 1997

We have previously shown that expressions of ferritin heavy chain (FHC), WDNM1, and sulfated glycoprotein-2 (SGP-2) genes are induced at an involution stage of mammary gland. Here we studied the effect of lactogenic hormones and EGF on the expression of involution-induced genes in HC11 mammary epithelial cells. Insulin, dexamethasone, prolactin, and its combinations did not affect expression of the genes. When cells were cultured in growth medium containing EGF, expression of WDNM1 and SGP-2 genes was strongly inhibited in a dose- and time- dependent manner, whereas expression of FHC gene was not influenced by EGF. Results demonstrate that EGF inhibits expression of WDNM1 and SGP-2 genes in mammary epithelial cells.

After completion of lactation, mammary gland undergoes involution during the weaning period, regressing to a state resembling that of a virgin animal. This phase of mammary gland development is characterized by dramatic epithelial cell death and tissue remodelling. Recent studies suggest that the involution of mammary gland requires active gene expression. Previously, we isolated involution-induced genes by differential screening of cDNA library, and we found that expressions of ferritin heavy chain gene (FHC), WDNM1 and sulfated glycoprotein-2 (SGP-2) genes were induced during involution of mammary gland (1, 2). Involution of the mammary gland is presumed to be mediated by a decrease in serum prolactin level induced by weaning, but may also involve changes in paracrine or autocrine growth factors. In this study, effects of lactogenic hormones and EGF on the expression of the involution-induced genes were examined. We demonstrate here that insulin, dexamethasone, and prolactin do not affect expression of the genes. However, EGF strongly inhibits the expression of WDNM1 and SGP-2 genes in mammary epithelial cells.

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Abbreviations: FHC, ferritin heavy chain; SGP-2, sulfated glycoprotein-2.

MATERIALS AND RESULTS

Cell culture. HC11 cells were cultured in growth medium containing RPMI1640 (Gibco BRL, USA), 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL), 5 μg/ml insulin (Sigma, USA), 10 ηg/ml EGF (Sigma), and 50 μg/ml gentamicin (Sigma) as described (3, 4). Medium was changed every 2 days. Hormonal and EGF treatments were carried out as described in the figure legends.

Northern analysis. Total RNA was extracted by the acid/guanidinium thiocyanate/phenol chloroform method (5). Twenty micrograms of total RNA were electrophoresed on a 1% agarose gel containing formaldehyde, and blotted onto a membrane. FHC (1.0 kb), WDNM1 (450 bp) and SGP-2 (1.9 kb) cDNAs have been previously cloned by differential screening of mouse mammary gland cDNA library (1, 2). pBluescript containing the cDNA was digested with EcoR I and Xho I, and the insert was obtained after low melting agarose gel electrophoresis. The insert of cDNA clone was labeled using a Prime-It Random Primer Labeling Kit (Stratagene). The membrane was hybridized with the labeled insert of the indicated cDNA clone. The equal amount of RNA loading was confirmed by the intensities of 28S and 18S band, and the efficiency of transfer was monitored by ethidium bromide staining.

RESULTS AND DISCUSSION

We have previously shown that expressions of several genes including ferritin heavy chain (FHC), WDNM1 and sulfated glycoprotein-2 (SGP-2) are induced at involution stages of mammary gland (1, 2). In this experiment, we confirmed the induction of FHC, WDNM1 and SGP-2 genes at involution stage of mam-

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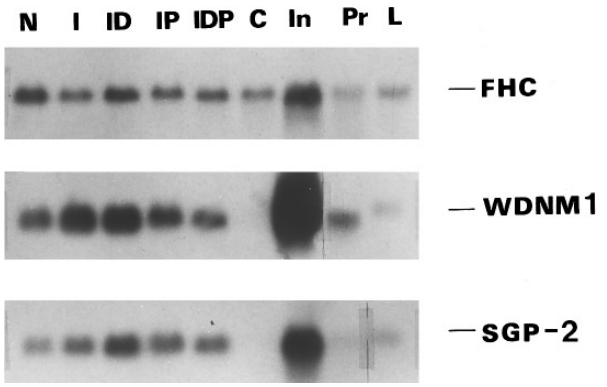

FIG. 1. Hormonal regulation of ferritin heavy chain (FHC), WDNM1, and sulfated glycoprotein-2 (SGP-2) genes in HC11 cells. Cells were grown to confluency in RPMI1640 medium supplemented with 10 ηg/ml EGF, 5 μg/ml insulin, and 10% fetal bovine serum (FBS), kept for 2 days in medium containing 2% FBS and insulin but no EGF, and incubated for 2 days in the medium containing 2% FBS with 5 μg/ml insulin (I), insulin plus 0.1 μM dexamethasone (ID), insulin plus 5 μg/ml prolactin (IP), insulin plus dexamethasone and prolactin (IDP), or no hormone (N). Total RNA was prepared from each treatment, confluent cells (C) cultured in growth medium containing EGF and 10% serum, and from mouse mammary tissues of involution day 2 (In), pregnant day 19 (Pr), and lactation day 10 (L). The membrane was hybridized with the [^{32}P] labeled clone.

mary gland (Fig. 1). The higher levels of FHC, WDNM1 and SGP-2 mRNA at involution stage were observed compared to pregnant- and lactating-stages. Lactation is under multi-hormonal control including prolactin. Lactogenic hormones prolactin and glucocorticoid are responsible for the induction of milk protein β-casein gene expression in HC11 cells (4). The involution of the mammary gland is presumed to be mediated by a decrease in serum prolactin level induced by weaning, but may also involve changes in paracrine or autocrine growth factors.

To examine the effects of lactogenic hormones on the expression of the involution-induced genes, the HC11 mammary epithelial cells were cultured to confluency in growth medium containing EGF and 10% serum, and maintained for 2 days in medium containing 2% FBS but no EGF. Cells were treated with hormones for 2 days. From three independent experiments including Fig. 1 data, insulin, dexamethasone, prolactin and its combinations did not affect the expression of the FHC, WDNM1 and SGP-2 genes. When cells were cultured in the growth medium containing EGF and 10% serum, expressions of WDNM1 and SGP-2 genes were however strongly inhibited. Growth medium did not affect the expression of FHC gene.

Since growth medium contained EGF and high (10%) serum and media for hormonal treatment contained no EGF and low (2%) serum, we postulate that EGF or serum inhibits expression of WDNM1 and SGP-2 genes. To examine whether EGF or serum inhibits expression of the WDNM1 and SGP-2 genes, cells were grown to confluency, maintained in medium containing 2% serum but no EGF for 2 days, and then treated with EGF, serum or its combination for 2 days. When either EGF or EGF plus serum was added in the medium, expression of both WDNM1 and SGP-2 genes was strongly inhibited (Fig. 2), while expression of FHC gene was not influenced by EGF and EGF plus serum. In contrast, the addition of 10% serum increased the expression of FHC, WDNM1, and SGP-2 genes. Which factor(s) in the serum induces the expression of the genes is presently not known. Results suggest that EGF strongly inhibits expression of WDNM1 and SGP-2 genes in mammary epithelial cells. On the other hand, EGF did not affect the expression of FHC gene.

The inhibitory effect of EGF on the expression of WDNM1 and SGP-2 genes was confirmed with the increase in EGF amount from 0.01 ηg/ml to 10 ηg/ml (Fig. 3). Addition of 0.1 ηg/ml EGF caused 20% and 50% decrease in mRNA levels of WDNM1 and SGP-2, respectively. Addition of 10 ηg/ml EGF further decreased expression of WDNM1 and SGP-2 genes, 70% and 88% inhibition, respectively. However, the expression of FHC genes was not influenced by EGF addition. Time-dependent expressions of WDNM1 and SGP-2 genes

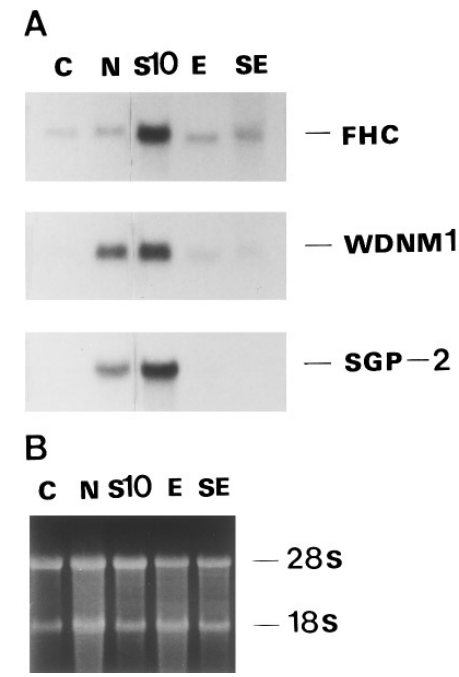

FIG. 2. Effects of serum and EGF on the expression of ferritin heavy chain (FHC), WDNM1, and sulfated glycoprotein-2 (SGP-2) genes in HC11 cells. Cells pretreated as described in Fig. 1 were incubated for 2 days in medium containing insulin but neither FBS nor EGF with the addition of 10 ηg/ml EGF (E), 10% serum (S10), EGF and 10% serum (SE), or neither treatment (N). (A) Total RNA was prepared from each treatment and from confluent cells (C) cultured in growth medium containing EGF and 10% FBS. The membrane was hybridized with the [^{32}P] labeled clone. (B) Ethidium bromide staining of 18S and 28S RNA was used as a loading standard.

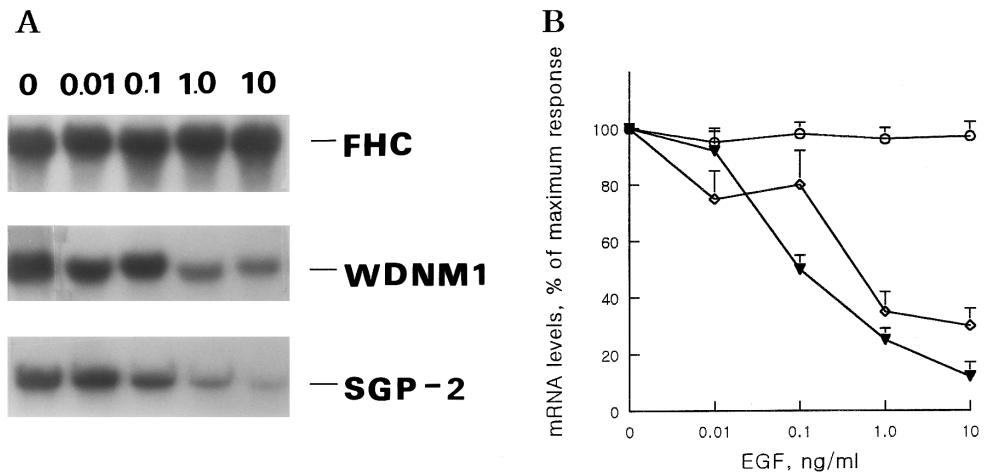

FIG. 3. Effects of varying dose of EGF on the expression of ferritin heavy chain (FHC), WDNM1, and sulfated glycoprotein-2 (SGP-2) genes in HC11 cells. Cells pretreated as described in Fig. 1 were incubated for 2 days in medium containing insulin but neither FBS nor EGF with the addition of various amounts of EGF (0.01, 0.1, 1.0, and 10 ηg/ml). (A) Total RNA was prepared from each treatment. The membrane was hybridized with the [^{32}P] labeled clone. (B) The levels of FHC (○), WDNM1 (◇), and SGP-2 (▼) mRNA were quantitated by a phosphoimage analyzer. Values are means, expressed as the percentage of maximum mRNA levels in each experiment. Bars indicate standard deviations of mean of three experiments including A data.

were determined after addition of EGF (Fig. 4). Within 1h after EGF addition, over 50% inhibition of WDNM1 and SGP-2 expression was observed. Over 80% of WDNM1 and SGP-2 mRNA levels were decreased at 6h after EGF treatment. But, mRNA levels of FHC were constant throughout the experimental period. Previously, it was shown that about 60-80% of EGF were internalized within 5 min upon stimulation of endocytosis in HC11 cells (6).

We have shown that the expressions of FHC, WDNM1, and SGP-2 genes are induced at involution stage of mammary gland (1, 2). But, differential regulation of gene expression by addition of EGF was observed in this study. EGF strongly inhibited the expression of WDNM1 and SGP-2 genes, whereas EGF did not affect the expression of FHC gene. It was reported that circulating levels of EGF (7) and its receptor in mammary gland (8) are higher during pregnancy than non-pregnant states. These data indicate that levels of EGF and its receptor increase during the proliferative phase of mammary gland development. EGF also functions as a survival factor in preventing programmed

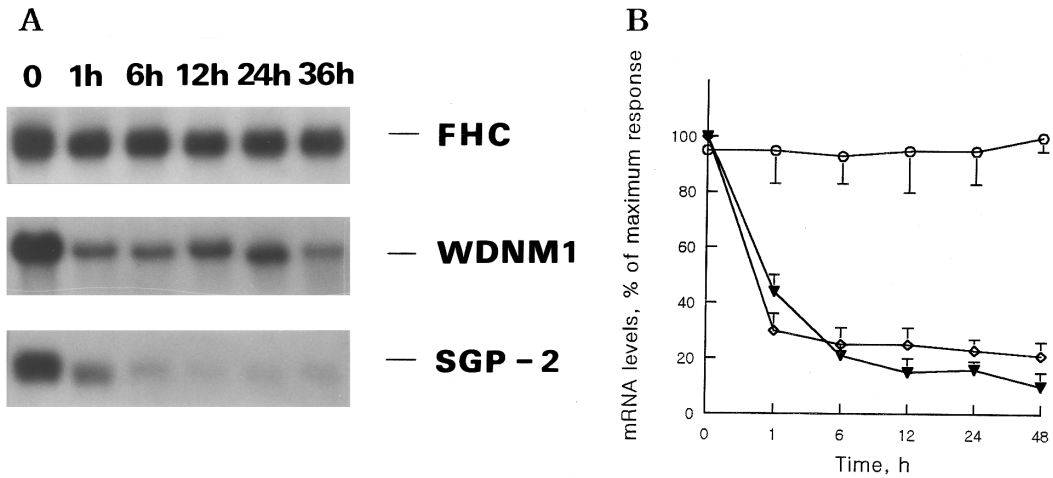

FIG. 4. Time course studies of EGF effect on expression of ferritin heavy chain (FHC), WDNM1, and sulfated glycoprotein-2 (SGP-2) genes in HC11 cells. Cells pretreated as described in Fig. 1 were incubated in medium containing insulin and 10 ηg/ml EGF but no FBS. (A) Total RNA was prepared at various times after incubation. The membrane was hybridized with the [^{32}P] labeled clone. (B) The levels of FHC (○), WDNM1 (◇), and SGP-2 (▼) mRNA were quantitated by a phosphoimage analyzer. Values are means, expressed as the percentage of maximum mRNA levels in each experiment. Bars indicate standard deviations of mean of three experiments including A data.

cell death of mammary epithelial cells (9). Addition of EGF and insulin together completely protected the HC11 cells from apoptosis (9). Presently, mechanisms that inhibit expression of WDNM1 and SGP-2 by EGF are not clear. Whether a drop in the local levels of EGF (or other members of the EGF family) occurs and plays a role in the involution of the mammary gland remains to be established. Role of WDNM1 and SGP-2 in regulation of mammary gland cell death through EGF signal should also be studied. Nass et al. (10) suggests a role for Bcl-X_L in the regulation of apoptosis by EGF and TGFβ in c-myc overexpressing mammary epithelial cells. Presently, little is known about the sequence of signals that lead to programmed cell death during mammary gland involution. Recent studies show that the two stages of mammary gland involution are controlled by progressive gain of death signals and loss of survival factors (11, 12). They suggest that the first stage of involution is controlled by local mammary-derived signals (12). Extracellular membrane and basement membrane structures maintained by glucocorticoid and other systemic hormones act as survival factors both during lactation and through the first stage of involution (12). Since EGF can act as a survival factor for mammary epithelial cells, whether removal or loss of EGF induces gain of death signal and leads to the first stage of involution should be established.

ACKNOWLEDGMENTS

This study was supported in part by the Genetic Engineering Research Program (96/97), Ministry of Education, Korea, a grant from KOSEF through the Hormone Research Center (HRC-97-0402), and by a grant (HMP-96-M-2-0030) of the '97 Good Health R&D Project, Ministry of Health & Welfare, R.O.K.

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