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Production of Mammastatin, a Tissue-Specific Growth Inhibitor, by Normal Human Mammary Cells

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The growth of human mammary cells may be regulated by a balance between growth stimulatory and growth inhibitory pathways. Polypeptides of 47 and 65 kilodaltons (mammastatin) were isolated from conditioned medium of normal human mammary cells. Monoclonal antibodies against mammastatin were generated that blocked its activity and were used for purification and further characterization of the protein. Mammastatin inhibited the growth of 5 transformed human mammary cell lines, but had no effect on the growth of 11 transformed human cell lines derived from nonmammary tissues. Mammastatin appeared to be a heat-labile protein distinct from transforming growth factor–β (TGF-β). By immunoperoxidase staining it was detected in cultured normal human mammary cells, but was decreased in transformed mammary cells.

MAMMARY EPITHELIAL CELLS have provided a particularly useful model to study growth control mechanisms. Both in vitro and in vivo mammary epithelial growth is regulated by steroid hormones and polypeptide growth factors (*1*). Steroid hormones such as estrogen may influence cell proliferation by stimulating the autocrine or paracrine release of positive growth regulators such as TGF-α and insulin-like growth factor 1, as well as by inhibiting the production of negative growth regulators such as TGF-β (*2, 3*). The polypeptide growth factors involved in these pathways do not have cell type specificity, but are capable of affecting the growth of a wide variety of cell types in culture (*4, 5*). In contrast to these nonspecific inhibitors, we have postulated that mammary tissue–specific polypeptide growth factors should exist that are analogous to mullerian inhibitory substance, which selectively inhibits the growth of the mullerian duct in the male embryo (*6, 7*).

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Normal human mammary cells (NHMC), characterized as epithelial by keratin expression, were derived from reduction mammoplasties and serially passaged in low calcium medium (<60 n*M*) (*8*). Media conditioned by these cells was found to inhibit the growth of the transformed human mammary carcinoma cell line, MCF-7, in a time and dose-dependent manner (Fig. 1). The addition of 10% conditioned medium (concentrated ten times by ultrafiltration) to these cultures resulted in a 75% inhibition of cell growth at 10 days compared to cultures supplemented with 10% nonconditioned medium.

To purify and further characterize the factor, or factors, from conditioned medium responsible for growth inhibition, we used a combination of ion-exchange and molecular sieve chromatography (Table 1). Fractions were analyzed for growth inhibitory activity on MCF-7 cells. By ion-exchange chromatography on a DEAE column eluted with a

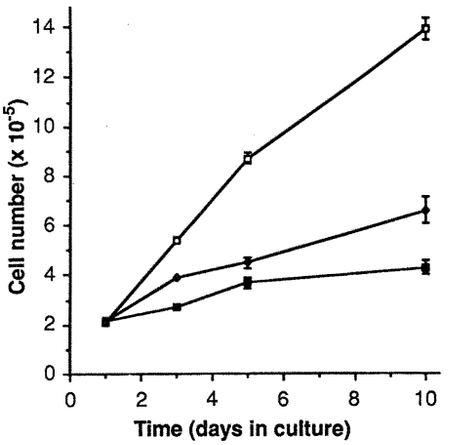

Fig. 1. Effect of conditioned medium on MCF-7 gorwth. MCF-7 cells were plated at 2×10^5 cells per T25 flask in Dulbecco's minimum essential medium (DMEM) with 10% fetal calf serum and insulin (10 μg/ml) and allowed to attach overnight. Medium was then replaced with indicated concentration of conditioned medium produced by incubating confluent cultures of NHMC for 4 days in DMEM–F-12, at 40 n*M* $CaCl_2$ with 5% chelex-treated bovine serum. Conditioned or nonconditioned medium was concentrated 10× by Amicon ultrafiltration. Cell number was determined at indicated times by Coulter counting. □, Ten percent nonconditioned medium; ◆, 5% conditioned medium; ■, 10% conditioned medium. Values represent mean ± SEM of triplicate determinations repeated twice.

Table 1. Purification of mammastatin. The inhibitory activity in NHMC-conditioned medium was purified by ion-exchange chromatography on DEAE Sephacryl followed by affinity chromatography on a 3C6 monoclonal antibody affinity column. One liter of conditioned medium was loaded on a DEAE column in 50 m*M* NaCl and eluted with a step gradient between 0.05 and 1.0*M* NaCl. Dialyzed fractions were assayed for MCF-7 growth inhibitory activity. Fractions exhibiting inhibitory activity, which eluted between 0.1 and 0.5*M* NaCl, were passed over an affinity column produced by binding 22 mg of 3C6 monoclonal antibody to 1.5 ml of Bio-Rad Affi-Gel Beads. The affinity column was washed with 500 column volumes of PBS, and bound protein was eluted with 0.5*M* glycine, *p*H 2.35. The eluent was neutralized with tris base and dialyzed against PBS. Specific activity is defined in units per milligram, where one unit is the amount of protein necessary to produce 50% inhibition of MCF-7 cell growth. Concentration of the inhibitory protein was determined by comparison with albumin standards on SDS-PAGE gels visualized by silver stain. Final purity of the inhibitory proteins was determined to be greater than 50%.

Sample	Total protein (mg)	Specific activity	Purification (fold)	Yield (%)
Conditioned media	2,150	1.16	1	100
0.5*M* DEAE fraction	20.3	47.7	41	40.3
Affinity purified	0.0012	106,000.0	91,380	5.3

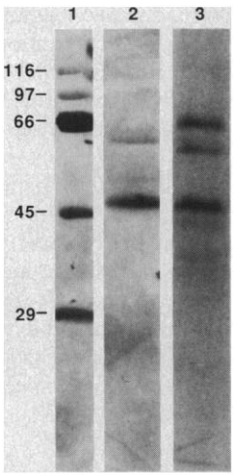

Fig. 2. SDS-PAGE analysis of affinity-purified mammastatin. Mammastatin purified as in Table 1 was analyzed on 10% SDS-PAGE gels. Lane 1, Molecular size standards, in kilodaltons; lane 2, affinity-purified mammastatin, silver stain; lane 3, fluorogram of [^{35}S]methionine-labeled NHMC conditioned medium purified by affinity chromatography. Methionine-free DMEM medium (5 ml) supplemented with [^{35}S]methionine (200 μCi/ml) were incubated with a confluent T-75 flask of NHMC for 24 hours. Medium was purified on a 3C6 monoclonal antibody affinity column as in Table 1.

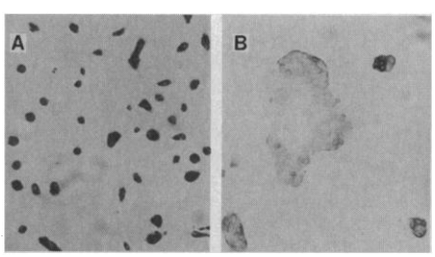

Fig. 3. Immunoperoxidase staining of human mammary cells. NHMC or MCF-7 cells were cultured for 3 days on chamber slides (as in Fig. 1), rinsed with PBS, and fixed in 2% paraformaldehyde for 10 min at 4°C. Subsequent steps were done at room temperature. Cells were permeabilized with 0.1% Triton X-100 and blocked with 1% bovine serum albumin (BSA) for 30 min, and then incubated for 60 min with a 1:100 dilution of monoclonal antibody 3C6. They were then washed and incubated in a 1:250 solution of peroxidase-conjugated goat anti-mouse IgG antibody in PBS + 2.5% nonfat dry milk for 60 min and developed for 10 min with 3-amino, 9-ethylcarbazole aquamount (Lerner Laboratories) solution. Slides were mounted with aquamount and photographed (**A**) Normal human mammary cells (×100); (**B**) MCF-7 cells (×100).

linear gradient of NaCl, the inhibitory activity from conditioned medium eluted at 0.25*M* NaCl. When this peak was fractionated on an S-200 Sephacryl molecular sieve column, two peaks of inhibitory activity were found at approximately 45 and 65 kD (*9*). The 45-kD peak was further purified by preparative SDS–polyacrylamide gel electrophoresis (PAGE) and used to immunize mice and generate monoclonal antibodies as previously described (*10*).

Hybridoma supernatants were screened for their ability to recognize immunizing proteins and abrogate the inhibitory activity of conditioned medium on MCF-7 cells. One such hybridoma produced monoclonal antibody 3C6, an immunoglobulin M (IgM), that totally abrogated the inhibitory activity of NHMC-conditioned medium. The 3C6 monoclonal antibody was purified from mouse ascities and used to construct a monoclonal antibody affinity column to further purify NHMC-conditioned medium by affinity chromatography. By using a combination of ion-exchange and monoclonal antibody affinity chromatography, we achieved an approximately 90,000-fold purification of inhibitory activity from conditioned medium (Table 1). The purified material inhibited the growth of MCF-7 cells in a dose-dependent manner with 50% growth inhibition at an inhibitor concentration of 10 ng/ml and greater than a 90% inhibition of cell growth at 20 ng/ml. Inhibition of cell growth by either conditioned medium or purified inhibitor was abrogated by monoclonal antibody 3C6 (*11*).

SDS-PAGE followed by silver staining of material eluted from the antibody affinity column revealed two prominent bands at approximately 47 and 65 kD, as well as an inconsistent minor band at 63 kD (Fig. 2). When separated on an S-200 Sephacryl column, the material eluting at 47 and 65 kD both demonstrated inhibitory activity for MCF-7 cells (*12*). This suggests that the 47- and 65-kD species represent differentially processed or cleaved forms of the same protein, or that these represent two distinct inhibitory factors that share a common epitope. To demonstrate that mammastatin is synthesized by NHMC, we labeled cultures with [^{35}S]methionine and subjected supernatants to affinity chromatography on a 3C6 monoclonal antibody affinity column followed by SDS-PAGE and fluorography (Fig. 2).

Heating of mammastatin to 70°C for 60 min resulted in a 50% decrease in activity, and heating to 100°C for 60 min completely abrogated the inhibitory activity (*13*). This pattern of heat sensitivity distinguishes mammastatin from TGF-β, which is stable to 100°C heating (*14*). Trypsin digestion completely abrogated the inhibitory activity. These results suggest that mammastatin is a heat-labile protein.

The effects of mammastatin on the growth of a variety of transformed mammary and nonmammary human cell lines was tested. Mammastatin (10 ng/ml) produced significant inhibition of the growth of five transformed human mammary cell lines tested (Table 2). The growth of HBL-100, a nontransformed mammary cell line (*15*), showed inconsistent inhibition. Growth inhibition was seen in both estrogen-responsive (MCF-7 and ZR-75-1) and estrogen-nonresponsive [BT-20, MDA-MD-231, and evejos (*16*)] mammary cell lines. In contrast to the effect on mammary cells, the inhibitor had no effect on the growth of 11 transformed human cell lines derived from nonmammary tissues (Table 2). This tissue specificity of mammastatin further distinguishes it from TGF-β, which inhibits the growth of a wide variety of epithelial cell lines (*17*). In addition, antibodies to TGF-β did not detect mammastatin on immunoblots (*18*). Thus, by molecular size, heat sensitivity, tissue specificity, and antibody reactivity, mammastatin is distinct from TGF-β. Furthermore, the tissue specificity of inhibition distinguishes mammastatin from other smaller molecular size inhibitory proteins previously isolated from mammary cells (*19*).

Table 2. Effect of mammastatin on the growth of human cell lines. Cell lines were cultured in triplicate in RPMI + 10% fetal calf serum in 12-well plates at 2×10^4 cells per well and allowed to attach overnight. Cell number was determined at day 1 and cultures were then treated with mammastatin (10 ng/ml) or BSA (10 ng/ml). Percent inhibition was calculated as in Fig. 2. Negative numbers indicate growth stimulation.

Cell type	Designation	Percent inhibition
Mammary carcinoma	MCF-7	61 ± 3
Mammary carcinoma	BT-20	53 ± 17
Mammary carcinoma	MDA-MB-231	25 ± 4
Mammary carcinoma	ZR-75-1	37 ± 4
Mammary carcinoma	Evejos	21 ± 4
Mammary epithelium	HBL-100	19 ± 15
Lung adeno-carcinoma	A 427	3 ± 1
Colon carcinoma	SW 948	−8 ± 5
Fibrosarcoma	HT 1080	8 ± 16
Promonocytic leukemia	HL-60	9 ± 3
Bladder carcinoma	Hill	4 ± 7
Lymphoblastic leukemia	Raji	−2 ± 11
Myelomonocytic leukemia	Wehi-3	1 ± 6
Squamous carcinoma	A 253	0 ± 6
Squamous carcinoma	UM-SCC-17a	−12 ± 11
Squamous carcinoma	UM-SCC-38	−6 ± 8
Cervical carcinoma	HELA	−17 ± 20

To examine mammastatin production by normal and transformed human cell lines, we performed immunoperoxidase staining of detergent-permeabilized normal and transformed cells using the 3C6 monoclonal antibody (Fig. 3). As a control, irrelevant IgM antibodies were used and showed no staining (*20*). Normal human mammary cells contain immunoreactive mammastatin, which is present in greater than 90% of the cells (Fig. 3A). In contrast, only 10 to 15% of MCF-7 cells showed even weak staining. Similarly, immunoperoxidase staining of the other transformed cell lines (Table 2) showed inconsistent low levels of staining (*21*).

These studies show that normal human mammary cells produce proteins of approximately 47 and 65 kD that inhibit the growth of transformed mammary cell lines in culture. These include both estrogen receptor–positive, as well as estrogen receptor–negative, cell lines. This may indicate that mammastatin does not require estrogen receptor for its action. However, it remains to be determined whether estrogen or other steroid hormones regulate the production of mammastatin by human mammary cells.

Decreased immunoperoxidase staining of transformed mammary cell lines compared to normal mammary cells may indicate decreased production of inhibitor by these cells. Indeed, we have been unable to detect immunoreactive or biologically active mammastatin in conditioned medium of transformed mammary cell lines (*22*). Decreased production of mammastatin by transformed mammary cells might contribute to the loss of normal growth control in these cells. If this were the case, then new therapeutic strategies might be developed that use mammastatin to inhibit the growth of human breast cancer.

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11. MCF-7 cells were plated at 10^4 cells per milliliter and at day 1, 10% NHMC-conditioned media or purified mammastatin (10 or 20 ng/ml) was added with or without monoclonal antibody 3C6 (5 μg/ml). Cell number was determined at day 7.
12. Affinity-purified mammastatin was dialyzed into 1/10 PBS and then concentrated by lyophilization (10×). Concentrated sample was loaded onto a 75-cm S-200 sephacryl column and eluted with PBS. Fractions were analyzed for MCF-7 growth inhibitory activity as in Fig. 2.
13. Purified mammastatin (100 ng/ml) was heated to 37°, 55°, 70°, or 100°C for 60 min. Inhibitory activity of these samples was then determined as in Fig. 2.
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20. Immunoperoxidase staining of cell cultures was performed with 3C6 and an irrelevant IgM at equal concentrations. Samples that stained with 3C6 were negative with irrelevant antibody.
21. Immunoperoxidase staining was performed on the cell lines listed in Table 2 as described in Fig. 3. BT-20, MDA-MB-231, ZR-75-1, evejos, and HBL-100 showed less than 10% weakly staining cells. Non-mammary cell lines were negative.
22. Conditioned medium was obtained from MCF-7, BT-20, and MDA-MB-231 cell lines as described for NHMC and tested for MCF-7 growth inhibitory activity. These media were also analyzed by immunoblot. Neither inhibitory activity or immunoreactive mammastatin was detected.

23. We would like to thank H. Soule of the Michigan Cancer Foundation for his gift of normal human mammary cells and NHMC-conditioned medium, G. Lowrie and C. Liu for their technical assistance, and R. Leonard for his assistance with immunoperoxidase staining of human cell lines.

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Switching of a Neuron from One Network to Another by Sensory-Induced Changes in Membrane Properties

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A neuron that is an integral member of the pyloric neural network of the lobster stomatogastric nervous system leaves this network and instead fires exclusively with another stomatogastric nervous system network, the cardiac sac network, whenever the cardiac sac network is active. This switch is associated with the neuron losing, in a long-lasting fashion, regenerative oscillatory membrane properties that underlie its participation in the pyloric network. Functional membership of neurons in central networks is thus not fixed, and long-lasting neuromodulatory influences, controlled at least in part by sensory inputs, can switch neurons from one network to another.

NERVOUS SYSTEMS GENERATE behaviors and modify those behaviors in response to sensory stimuli. Many behaviors involve the coordination of several different groups of neurons, known as neural networks, with each network generating specific aspects of the total behavior. An understanding of the mechanisms underlying this coordination is therefore of fundamental importance in order to explain nervous system function. Work in vertebrate and invertebrate preparations suggests that considerable flexibility exists within individual networks and that a single anatomically defined network can assume several different functional configurations, each of which produces a different output (*1, 2*). Although these results suggest that neurons can belong to different configurations of a single network, they do not address whether a neuron can belong to two or more different networks otherwise composed of different neurons. We describe here a neuron that switches between two different networks depending on whether one or both networks are active and show that this switch is associated with changes in the intrinsic membrane properties of the cell.

We used in vitro preparations (*3*) of the stomatogastric nervous system (STS) of the lobster, *Palinurus vulgaris*. The crustacean STS contains four neural networks that generate the motor patterns of the four different regions of the foregut (*4*). The pyloric network generates the motor patterns of the

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