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(UDGI): A Novel Growth Inhibitor of Estrogen Receptor Negative
Breast Cancer Cells

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13. ABSTRACT (Maximum 200 words) <p>In our previous work we examined the molecular basis for the association of estradiol and tamoxifen with endometrial carcinoma. Our results showed the inhibitory effects of uterine fluid on the growth of breast cancer cells. Crude uterine derived growth inhibitor (UDGI) activity was reversible and non-toxic in the mechanisms of action. The present work was conducted to characterize, identify, and purify the protein(s) primarily responsible for the described UDGI activity and to characterize its biological activity <i>in vitro</i>. The 21-22 kDa UDGI protein was purified to near homogeneity and we are in the process of analyzing the UDGI amino-terminal sequence. To further define UDGI, antibodies against it and the cloning of cDNA encoding the UDGI protein are required to determine its primary structure and to assess likely biological actions. In addition, the characterization of UDGI expression patterns and regulatory pathways are needed to define molecular regulatory mechanisms and to assess its possible functions in breast epithelial cell growth and differentiation.</p>				
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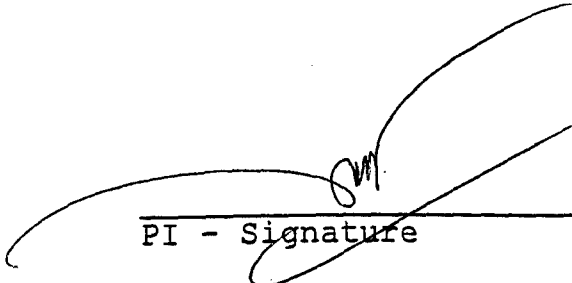
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PI - Signature

June 10, 1998
Date

**Characteristics of Uterine Derived Growth Inhibitor (UDGI):
A Novel Growth Inhibitor of Estrogen Receptor Negative Breast Cancer Cells**

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INTRODUCTION

I. BREAST CANCER

Breast cancer is the most common form of cancer among non-smoking women in the Western society. Approximately one in nine of all women in North America living to age 80 will develop breast cancer (1). The incidence of breast cancer appears to be increasing with an annual worldwide rate of over one million predicted by the turn of this century (2). This high occurrence of metastatic breast cancer is a major challenge, particularly as the probability of survival beyond 5 years is low for patients with metastatic disease.

Breast cancer is one of a small number of malignancies in which both genesis and growth have been linked to hormonal factors. About one-third of all metastatic human breast cancers is responsive to existing endocrine therapies (3). Antiestrogens inhibit the proliferation of *in vitro* human breast cancer cells expressing the estrogen receptor (ER), and have proven to be clinically useful for ER positive tumors (4-7). However, the efficacy of current hormonal therapies is restricted by the progressing resistance of an overwhelming majority of tumors to the therapies. It is often noted that during the course of hormonal therapy, ER positive breast cancer cells lose their dependence on estrogens, their ability to express ER, and their responsiveness to endocrine therapies (1).

Growth factors comprise a key component of stromal-epithelial interactions. Growth factors that have been implicated in mammary gland biology include: the FGF family, TGF- β family, TGF- α , the insulin-like growth factor family, EGF, heparin-binding EGF, TNF- α , and heparin growth factor-scatter factor (reviewed in 8). Each of these growth factors are bound to the extracellular matrix, cell membrane, or other binding proteins in an inactive or sequestered form and their activity is regulated by secreted proteases and protease inhibitors. The actions of proteases and protease inhibitors are fundamental to tissue homeostasis through the regulation of both growth factor bioavailability and cell interaction with the extracellular matrix. Alterations in protease and protease inhibitor actions affect tissue development patterns, angiogenesis, cell motility, and tumour invasion (9-12).

II. UTERINE SECRETION FUNCTIONS

It is clear that the female genital tract, including the uterus, exhibits considerable control over the ability of a conceptus to develop (13,14). The uterus expresses and secretes a number of growth factors (15-20) and other regulatory polypeptides (19,21) in response to ovarian steroid hormones. These polypeptides are thought to play a part in directing or limiting the growth and development of the uterus.

Although several growth inhibitors have been identified over the last few years, there is no reason for us to believe that we have identified them all. Our previous work examining the molecular basis for the association of estradiol and tamoxifen with endometrial carcinoma (19,22) showed the inhibitory effects of uterine fluid (UF) on breast cancer cell growth. We hypothesized that the uterus synthesizes and secretes a growth inhibitor to limit the proliferative response to estradiol stimulation. This led to our interest in characterizing the growth inhibitory activity of UF.

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EXPERIMENTAL PROCEDURES

The following products were used to carry out the experiments: Bio-Gel P-100 (100-200 mesh) and P-30 (100-200 mesh) chromatography gels, glycine, and acrylamide from Bio-Rad; diethylaminoethyl cellulose (DE-52) ion exchange resin and glass fiber filters (934-AH, 2.4 cm) from Whatman; dialysis tubing no. 3 (M_r cutoff = 3,500) from Spectrapore (Los Angeles, CA); TCA, acetonitrile and formic acid from Pharmacia; tissue culture media and fetal calf serum from Gibco; 96-well plates from Nunc; and [^3H]-thymidine from ICN.

Uterine fluid collection. 50 day-old ovariectomized rats are implanted with silastic tubes containing 17- β estradiol. Two weeks after estradiol implantation, the animals are sacrificed with carbon dioxide. The UF is removed from both uteri and centrifuged at 14000 g at 4°C for 30 min to remove cellular debris. The supernatant is stored at -75°C until analysis.

Cell Proliferation Assays. Cell proliferation was determined by evaluating [^3H]-thymidine incorporation. For the [^3H]-thymidine incorporation assay, the cells were seeded at 1.0×10^4 cell/well in 96-well plates and allowed to attach for 24 h. The cells received a 50 μl aliquot of the sample to be tested (previously vacuum-dried and resolubilized in 60 μl growth medium in sterile conditions) and were incubated for an additional 24 h. The cultures were pulsed with [^3H]-thymidine (2 $\mu\text{Ci/ml}$) during the final 2 h of incubation. The assay was terminated by fixing the cell monolayers *in situ* with 10% TCA (250 $\mu\text{l/well}$) for 2 h at 4°C, washing with 100% methanol (5 min, RT) followed by 2 washes with distilled water. The plates were then allowed to air-dry for 10 min. The monolayers were hydrolyzed with 1 N NaOH (200 $\mu\text{l/well}$, 5 min, 50 °C), and 150 μl aliquots were added to scintillation vials containing 150 μl of 1 N HCl to neutralize the pH. The radioactivity was determined by scintillation counting and the results are presented as the reciprocal of incorporated counts/min to reflect the inhibition of [^3H]-thymidine incorporation as a peak of activity.

Ion exchange chromatography. UF was filtered through a 0.45 μm filter and applied directly to a DE-52 anion exchange column (1.5 x 9.5 cm, hydrostatic pressure = 30 cm) equilibrated in 20 mM of ammonium carbonate buffer (pH 8.85). The column was washed with 3 bed volumes of the same buffer, eluted with a linear gradient of ammonium carbonate (20-300 mM, pH 8.9, 150 ml) and 5 ml/fraction were collected. The absorbance at 280 nm (A_{280}) and conductivity were determined for each fraction. 30 μl aliquots of each fraction were directly vacuum-dried in sterile microfuge tubes and resolubilized in 100 μl of sterile growth medium. 60 μl aliquots/well were assayed directly for Hs578T cell growth activity by evaluating [^3H]-thymidine incorporation.

Gel filtration chromatography. To assign a protein species to a peak of biological activity, 500 μl of UF were dialyzed against 1 M acetic acid (pH 2.25) and lyophilized. The lyophilized proteins were redissolved in 1 M acetic acid (1ml) and chromatographed through a Bio-Gel 200 gel filtration column (2.5 x 70 cm), equilibrated in 1 M acetic acid by gravity flow (45 cm), and 3 ml fractions were collected. 100 μl aliquots were vacuum-dried and resolubilized in 65 μl of growth medium. 60 μl were added to Hs578T cells for [^3H]-thymidine incorporation assay.

For additional studies and purification of UDG1 on a large scale, UF was processed through the ion exchange chromatography step. Pooled fractions from biologically active peaks were prepared by dialysis (Spectrapore no. 3 tubing, 3,500 M_r cutoff) against 1 M acetic acid (pH 2.25) overnight at 4°C. Dialyzed samples were frozen, lyophilized and stored at -20°C. The lyophilized samples were solubilized in 1 M acetic acid (1 ml) and applied to a Bio-Gel P-100 column (1.4 x 70 cm), equilibrated in 1 M acetic acid (pH 2.25). The proteins were eluted with a hydrostatic pressure of 55 cm, and 1.4 ml fractions were collected. 100 µl aliquots from each fraction were vacuum-dried in sterile microcentrifuge tubes and resolubilized in 65 µl of growth medium. 60 µl were added to Hs578T cells for [³H]-thymidine incorporation assay.

Reverse phase high performance liquid chromatography. All samples for HPLC analysis were first processed through ion exchange chromatography and gel filtration chromatography. Samples (pooled fractions from bioactivity peak) were either vacuum-dried or lyophilized and resolubilized in 50% formic acid (0.5 ml). Samples were applied (3 consecutive 150 µl applications) to a Waters C-18 reverse phase column fitted to an HPLC system composed of a Waters 712 WISP automatic injector, a Beckman 412 controller, 110 A pumps, and 165 variable wavelength detector. The proteins were eluted with a 1.0 ml/min/fraction flow rate with 0.1% trifluoroacetic acid in a linear acetonitrile gradient. 100 µl aliquots from each fraction were vacuum-dried in sterile microcentrifuge tubes and resolubilized in 65 µl of growth medium. 60 µl were added to Hs578T cells for [³H]-thymidine incorporation assay.

SDS-polyacrylamide gel electrophoresis (PAGE). Samples were vacuum-dried, resolubilized in Laemmli sample buffer and analyzed by SDS-PAGE following the procedures of Laemmli (23).

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RESULTS

We undertook the first study of the antiproliferative effect of UF *in vitro*, using rat primary endometrial cells, non-mammary cell lines and variety of human and bovine, transformed and non-transformed mammary epithelial cell lines. Figure 1 shows the effects of UF on [³H]-thymidine incorporation into various cell lines and primary uterine cells. The addition of 1% of UF to the culture for 24h resulted in a marked inhibition of proliferation of the breast cancer cell lines. The inhibition was very pronounced for the ER negative cell lines (HH2a, Hs578T, MDA 231, and 16HH2a). The ER positive T47D cell line was distinctly inhibited by UF as well (Fig. 1A). The human HBL-100 and BT-20 cell lines were not inhibited by UF (Fig. 1A).

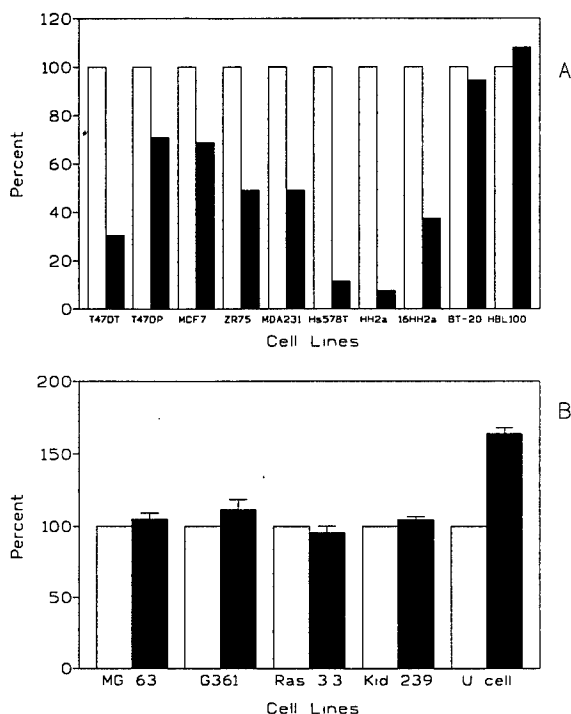


Figure 1. Effect of uterine fluid (UF) on mammary cell (A) and non-mammary cell (B) DNA synthesis. Confluent cultures of target cells for inhibition were trypsinized and plated at 2.5×10^4 cells in 24-plate multi-well dishes in 2.5% FCS. After 48h, the cell monolayer was rinsed twice with phenol-red serum free (PSF) media and incubated for 24h in PSF media containing 2.5% double charcoal stripped serum. After 24h, the cells were washed twice with PSF media and then incubated in triplicate without (white bars) or with (black bars) 10 µl/ml of UF added to the PSF media for 24h. At the end of incubation, [³H]-thymidine incorporation into DNA was determined as described (24). The experiments were repeated 3 times with similar results and the means of triplicate determinations were plotted as percent of control.

This inhibition was very specific for breast epithelial cells, since the UF enhanced DNA synthesis in rat primary uterine cells (U cell, Fig 1B). The inhibition of the rat UF was not species-specific, since inhibition of bovine and human breast epithelial cell lines was seen. Furthermore, UF had neither stimulatory nor inhibitory effects on human melanoma (M 361), human osteosarcoma (MG 63), mouse, rat intestinal epithelial (Ras 3.3) and human kidney epithelial (Kid 239) cell lines (Fig. 1B).

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To study the dose-response of human breast cancer cells to UF, MCF-7, Hs578T and MDA 231 cells were cultured as described above. Various concentrations of UF were added to the cultures and the incubations continued for another 24h. [^3H]-thymidine incorporation into DNA was determined as described above. As shown in figure 2, UF inhibited DNA synthesis of three cell lines in a dose-dependent manner.

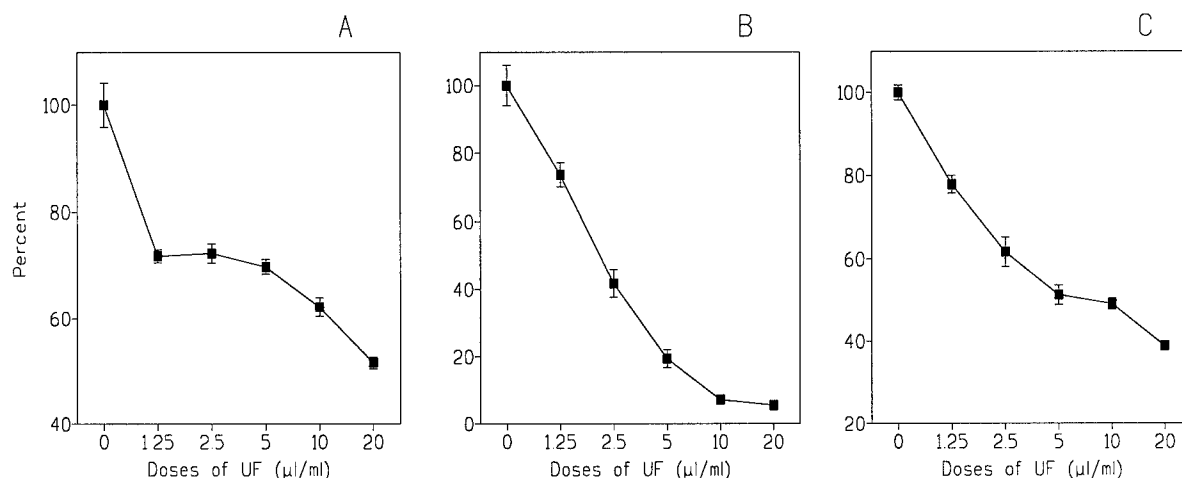


Figure 2. Effect of uterine fluid (UF) on MCF-7 (A) and Hs578T (B) and MDA 231 (C) cell DNA synthesis. Cells were grown as described in figure 1. Cells were treated with the indicated doses of UF ($\mu\text{l/ml}$) in serum free media for 24h. At the end of incubation, [^3H]-thymidine incorporation was determined as described (24). The experiments were repeated 3 times with similar results and the means of triplicate determinations were plotted as percent of control.

The addition of 2% UF into the culture resulted in a 50%, 95% and 61% inhibition of [^3H]-thymidine incorporation into MCF-7 (Fig. 2A), Hs578T (Fig. 2B) and MDA 231 (Fig. 2C) cell lines, respectively. The results from this study suggest that rat UF contains a very potent growth inhibitor for breast epithelial cells. Since the inhibitory activity was observed in both ER-positive and ER-negative cell lines, the inhibitor does not require the ER for its action.

The data are compatible with the hypothesis that UF contains a mammary growth inhibitor named uterine derived growth inhibitor (UDGI). The present work was conducted to characterize, identify, and purify the protein(s) primarily responsible for the described UDGI activity and to characterize its biological activity *in vitro*.

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Initial studies were conducted to determine whether a putative protein could be assigned to the peak or peaks of UDGI biological activity in order to assess the feasibility of purification and to focus on a particular protein for preparative purification. Accordingly, gel filtration chromatography and reverse phase HPLC were used to analyze UDGI activity, and the eluted proteins were analyzed by SDS-PAGE. UDGI activity was harvested from rat UF following estradiol implantation, as described in "Experimental Procedures".

Ion exchange chromatography was initially done to analyze the biological activity. The samples were dialyzed against 20 mM of ammonium carbonate (pH 8.85), applied to and eluted from a DE-52 anion-exchange column according to the methods described in "Experimental Procedures". Fig. 3 shows the biological activity and A_{280} protein elution profiles. The elution of the major growth inhibitory peak was observed from estradiol-induced rat UF.

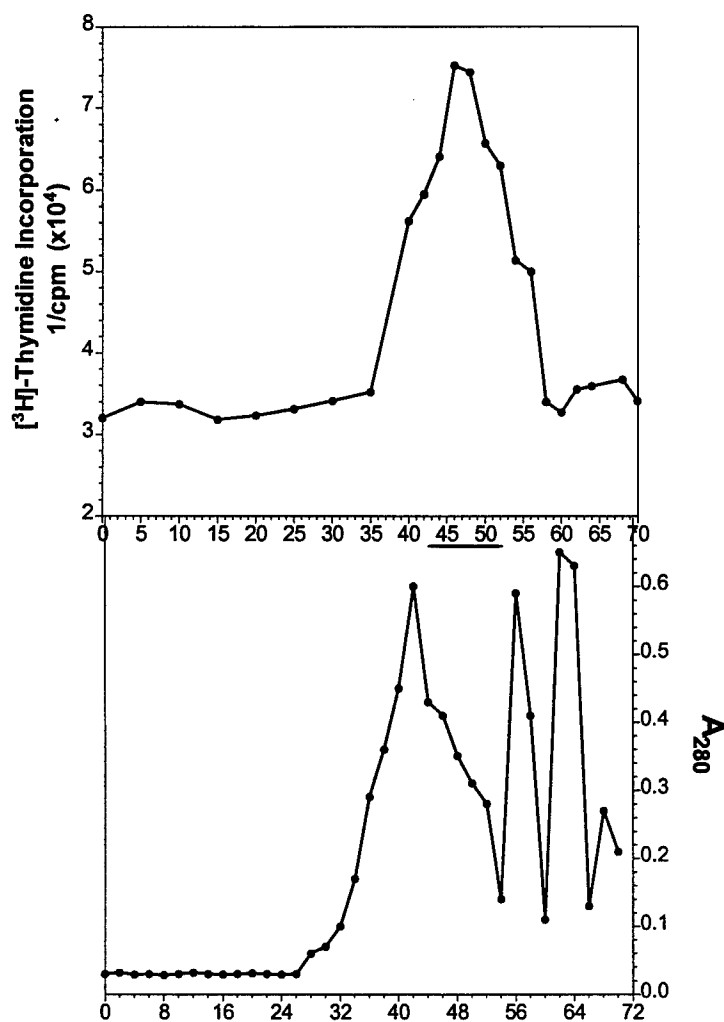


Fig. 3. Ion exchange chromatography. Proteins from rat UF were chromatographed through a DE-52 anion exchange column. 100 μl aliquots from each fraction were vacuum-dried and assayed for inhibition of $[^3\text{H}]\text{-thymidine}$ incorporation in Hs578T cells as described in "Experimental Procedures". Activity was plotted as the reciprocal of incorporated counts/min ($1/\text{cpm}$) to illustrate the inhibition of $[^3\text{H}]\text{-thymidine}$ incorporation as a peak of activity. Activity eluted as a single peak and the fractions were pooled and processed for analytical gel filtration chromatography and HPLC, as described in other figures. Bottom panel shows the corresponding A_{280} pattern of the total protein.

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Fractions from the ion exchange chromatography representing the major peak of the eluted biological activity (Fig. 3, 42 to 52) were collected and pooled. The pooled sample was dialyzed against 1 M of acetic acid (pH 2.5) overnight at 4°C. The dialyzed samples were quickly frozen, lyophilized, and stored at -20 °C. UDGI activity prepared in this manner was further analyzed by gel filtration chromatography for assignment of size using a variety of buffers including acetic acid, ammonium carbonate, and ammonium acetate. Of these buffers, gel filtration chromatography in 1 M of acetic acid optimally reduced any interaction with the column matrix and allowed for a reproducible recovery of an activity peak as shown in Fig. 4. The biological activity was detected as a single peak, eluting consistently in the calculated 21-22 kDa size range. SDS-PAGE analysis of the eluted fractions (Fig. 4, lower panel) showed the elution pattern of a 21 to 22 kDa protein to be directly correlated to the elution peak of biological activity. To further establish the correlation of this protein species with peak activity, additional samples were pooled, chromatographed through C18 reverse phase HPLC columns, and eluted with a linear gradient of acetonitrile as shown in Fig. 5. In direct agreement with gel filtration, the major peak of UDGI from HPLC was associated with a protein of approximately 21-22 kDa, as analyzed by SDS-PAGE (Fig. 5, lower panel, arrow, fractions 80 to 86).

The last purification step utilized reverse phase HPLC owing to the ability of HPLC in separating proteins of similar size based on hydrophobic properties. Pooled fractions from gel filtration chromatography were vacuum-dried, resolubilized in 50% formic acid, and analyzed with reverse phase HPLC, as described under "Experimental Procedures". The column was eluted with a shallow gradient of acetonitrile to produce optimal separation of the major peak versus minor peak proteins. Fig. 6 shows the biological activity elution profiles and the corresponding SDS-PAGE analysis with peak activity. The biological activity eluted as a well defined peak, which was directly correlated with the elution pattern of the 21-22 kDa protein species (Fig. 6, lower panel), purified to near homogeneity, as determined by SDS-PAGE analysis and silver staining. The purification procedure as described yielded approximately 500-700 ng of the 21-22 kDa protein from 2 ml of UF.

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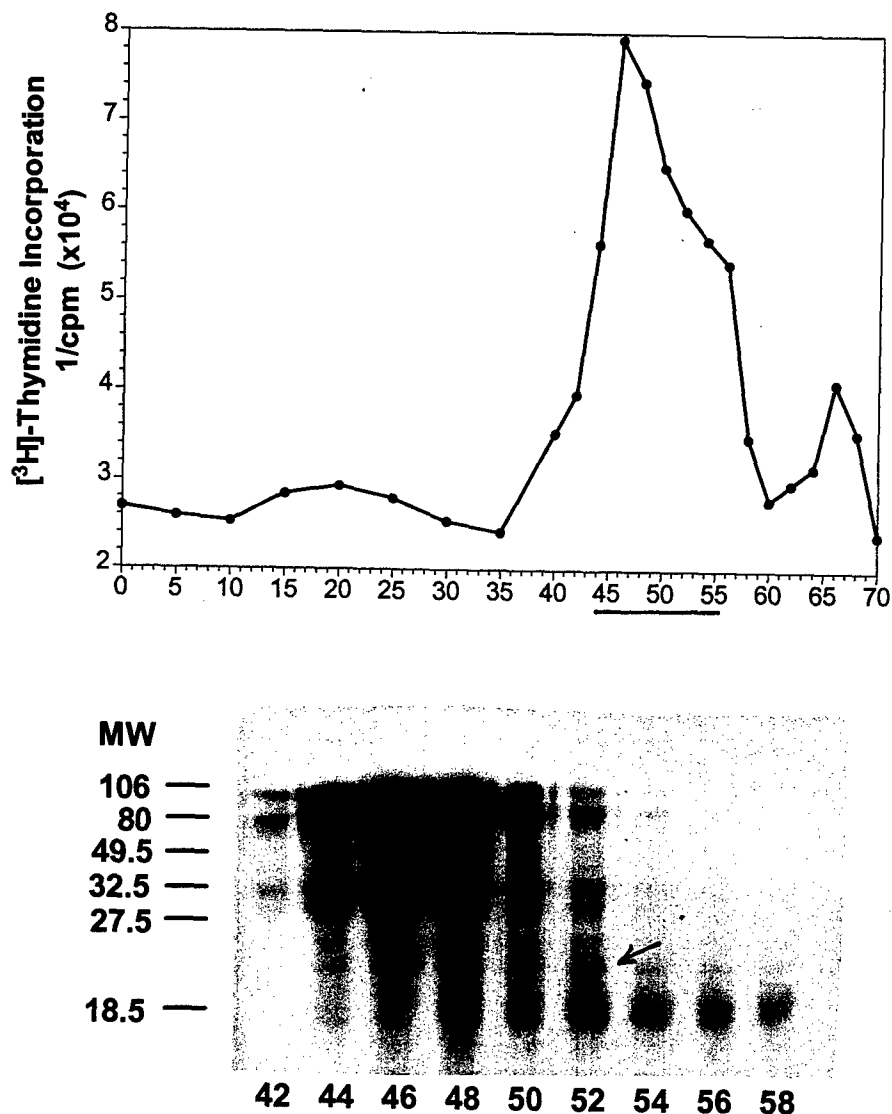


Fig. 4. Analytical gel filtration chromatography. Proteins from the UF were chromatographed through a DE-52 anion exchange column, as described in Fig. 3, and analyzed by gel filtration chromatography. The fractions were assayed for activity with Hs578T cells, as described in Fig. 3 and in "Experimental Procedures". Upper panel, elution profile of the biological activity from the P-100 gel filtration column. The biological activity eluted as a single peak (maximum activity at fractions 46 to 52) is associated with the 21-22 kDa size region. Lower panel, SDS analysis of eluted fractions. Fractions (42 to 58) from gel filtration were vacuum-dried, electrophoresed through a 16% acrylamide gel, and stained using the silver method. The elution pattern of a 21-22 kDa species (lower panel, arrow, fractions 44 to 54) correlated directly with the position of the eluted bioactivity peak. Molecular size markers are shown.

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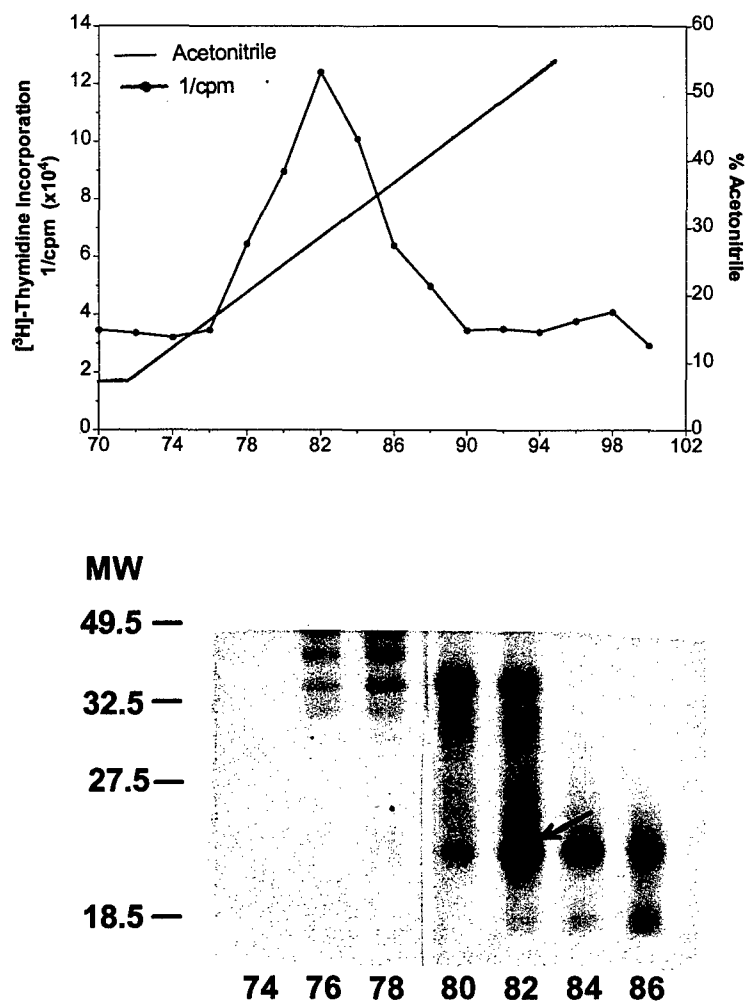


Fig. 5. Analytical reverse phase HPLC. Upper panel, proteins were chromatographed with a C-18 reverse phase column, as described in Fig. 3 and in "Experimental Procedures". 100 μ l aliquots from each fraction were vacuum-dried and assayed for inhibition of [³H]-thymidine incorporation in Hs578T cells, as described in "Experimental Procedures". The biological activity eluted as a single peak (maximum activity at fractions 80 to 86) is associated with the 21-22 kDa size region. Lower panel, SDS analysis of eluted fractions. Fractions (74 to 86) from gel filtration were vacuum-dried, electrophoresed through a 16% acrylamide gel, and stained using the silver method. The elution pattern of a 21-22 kDa species (lower panel, arrow, fractions 80 to 86) correlated directly with the position of the eluted bioactivity peak. Molecular size markers are shown.

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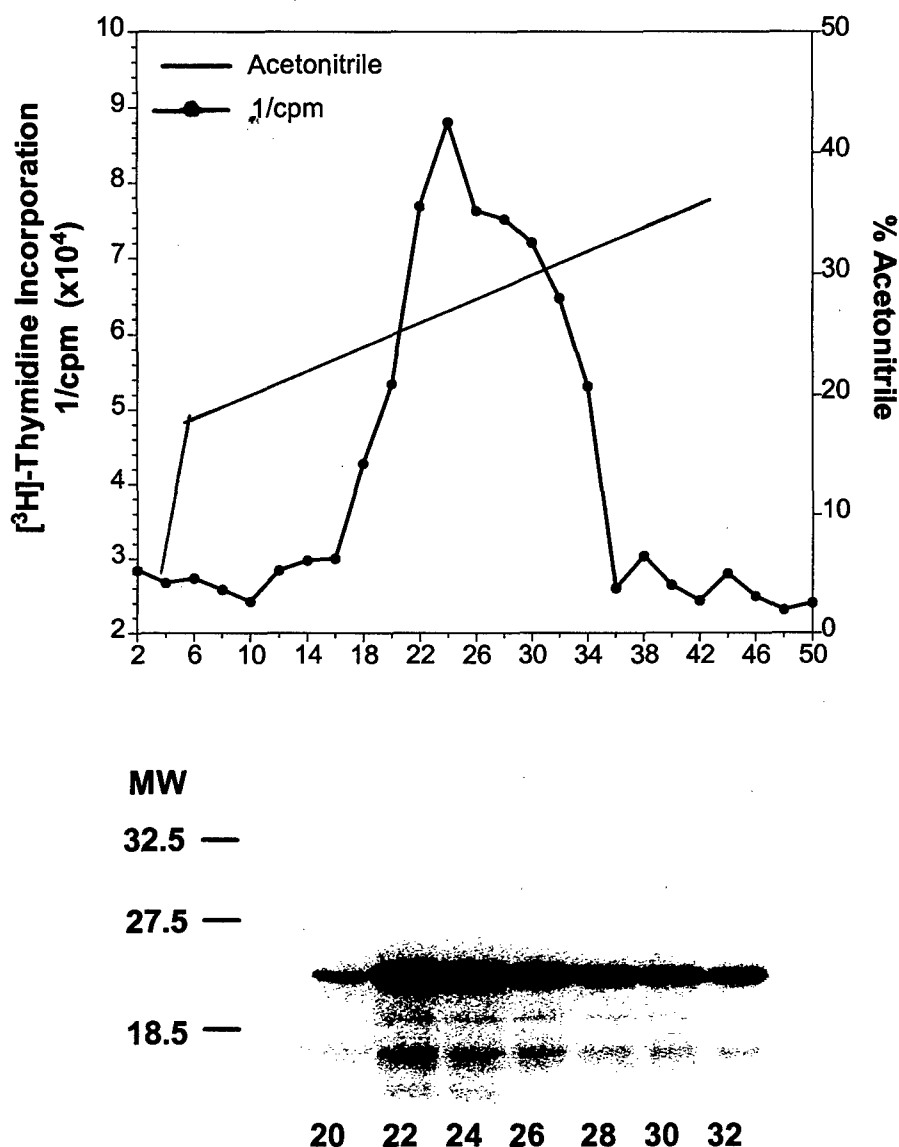


Fig. 6. Preparative HPLC chromatography. Upper panel, fractions from gel filtration chromatography were pooled and used for reverse phase HPLC as described in "Experimental Procedures". Shown is the elution profile of the biological activity with Hs578T cells and an acetonitrile elution gradient. The biological activity eluted with a consistently observed peak (fractions 20 to 32). The lower panel shows the SDS-PAGE analysis and silver staining of the proteins. The elution pattern of the 21-22 kDa protein correlated with the initial peak (fractions 20 to 32) and was purified to near homogeneity. Molecular size markers are as indicated.

STATEMENT OF WORK

To date we have completed Task 1 of the Statement of Work outlined in the proposal: Purification of UDGI from rat uterine fluid. In doing so we came across some unanticipated problems:

1. To analyze UDGI bioactive proteins, all chromatography buffers had to be both volatile (no salt residues upon drying) and bacteriostatic (sterile, non-supportive of bacterial growth) so that aliquots from gel filtration or HPLC columns could be vacuum-dried in sterile vials and used directly for biological assays (in addition to targeting breast cancer cells in culture) without additional steps of dialysis and sterilization of the sample. During the course of purification we faced several problems concerning the choice of eluting buffers that would meet our requirements.
2. A second problem arose in the reproducible recovery of an activity peak during purification. This was due to an interaction between the protein and the column matrix. To optimize it we had to select several buffers that would optimally reduce the interaction.

Presently we are in the process of and will have answers shortly on:

1. **Microsequence Analysis.** To determine if UDGI is a novel protein or an existing protein, we are in the process of determining its amino-terminal sequence analysis and comparing its sequence to a published sequence. For amino-terminal sequence analysis, the UDGI protein (approximately 1000 ng) from a large scale preparation has to be electrophoresed by SDS-PAGE, blotted to polyvinylidene difluoride membranes and analyzed for its amino-terminal sequence.
2. **Determination of biologically active concentrations.** Purified UDGI was used for dose-response assays with target cells. Purified UDGI induced a linear and saturable inhibition of [³H]-thymidine incorporation in breast cancer cells and inhibited cell proliferation (i.e. increased population doubling time).

Tasks remaining:

Task 2. Monoclonal antibody production. Months 9-20:

- a. Immunization of mice with UDGI protein
- b. Fusion of spleen cells from hyperimmunized mice with genetically marked myeloma
- c. Screening hybridoma supernatants
- d. Production of UDGI from UF using affinity column

Task 3. UDGI cDNA isolation. Months 20-28:

- a. cNA library construction
- b. cDNA library screening
- c. UDGI cDNA sequencing
- d. UDGI expression vector construction

Task 4. Transfection of UDGI expressing vector into breast cancer cells. Months 28-36:

- a. Transfection and colony isolation
- b. Characterization of UDGI expressing clones
- c. In vivo tumorigenic assay

Task 5. Regulation of UDGI gene expression in normal breast epithelial, breast stromal and breast cancer cells. Months 20-36:

- a. Immunochemical study
- b. UDGI gene expression
- c. Regulation of UDGI gene expression by growth factors and hormones

Task 6. Detection of UDGI binding sites. Months 21-30:

- a. Direct binding assays
- b. Affinity labelling of UDGI receptors

Task 6 will be started after task 2 is completed. Task 5 will be partly started after task 2 is completed and can be carried out further task 3 is completed.

CONCLUSION

Data presented in this report shows the purification of a 21-22 kDa protein (termed UDGI) derived from rat UF. Since the amino-terminal sequence information of UDGI is in the process of being analyzed, the identity of UDGI can not be determined. UDGI may represent a novel or existing protein. At the present moment, the possible role of UDGI and the mechanisms of UDGI action are fully unknown. To define UDGI further, antibodies against UDGI and the cloning of cDNA encoding the UDGI protein are required to determine the primary structure and to assess likely biological actions. In addition, the characterization of UDGI expression patterns and regulatory pathways are required to define molecular regulatory mechanisms and to assess its possible functions in breast epithelial cell growth and differentiation. The UDGI may or may not represent an autocrine, paracrine or endocrine effector growth regulatory protein involved in receptor mediated pathways. Understanding the expression patterns and cellular location of UDGI in breast disease and defining molecular mechanisms of action will be of importance in assessing any potential role of UDGI in breast cancer.

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DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

21 JUN 2001

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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