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13. ABSTRACT *(Maximum 200 words)*

We reported the purification of UDGI, a uterine derived growth inhibitor. UDGI protein shares 98% identity to ps20 growth inhibitor isolated from urogenital sinus mesenchymal cells. Although purified UDGI potently inhibited breast cancer cell growth, recombinant UDGI exhibited mild inhibition. UDGI antibody recognized native UDGI from uterine fluid and conditioned media of UDGI-transfectant cells with molecular mass of 24, 27 and 29 kDa. UDGI cDNA was isolated from a rat uterus cDNA library. UDGI transcripts were detected in rat mammary tissue and in various female rat tissues with highest expression in the lung and heart. *In vitro*, UDGI secretion was reduced by IGF-I, IGF-II, insulin and TGF-α. *In vivo*, UDGI expression was inhibited by estradiol and induced by pure antiestrogen ICI 182780 (ICI) and tamoxifen. UDGI mRNA was barely detected in mammary tissue of old female rats and in DMBA mammary tumours. Suppression of tumour growth by ICI and tamoxifen was associated with upregulation of UDGI gene expression. Acceleration of apoptosis was observed in stable UDGI-transfectant MCF-7 cell lines following serum starvation. The results suggest that UDGI may function as a mediator of local growth and apoptosis mechanisms.

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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 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 cases 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 tumours (4-7). However, the efficacy of current hormonal therapies is restricted by the progressing resistance of an overwhelming majority of tumours. 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-ß 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.

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 [^{3}H]-thymidine from ICN.

Uterine fluid collection. Silastic tubes containing 17-ß estradiol were implanted into the back of 50 day-old ovariectomized rats. Two weeks after estradiol implantation, the animals were sacrificed with carbon dioxide. The UF was removed from both uteri and centrifuged at 14000 g at 4^{0}C for 30 min to remove cellular debris. The supernatant was stored at -75^{0}C until analysis.

Cell Proliferation Assays. Cell proliferation was determined by evaluating [^{3}H]-thymidine incorporation. For the [^{3}H]-thymidine incorporation assay, the cells were seeded at 1.0 x 10^4 cell/well in 96-well plates and allowed to attach for 24 h. The cells received a 50μl 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 [^{3}H]-thymidine (2 μCi/ml) during the final 2 h of incubation. The assay was terminated by fixing the cell monolayers *in situ* with 10% TCA (250 μl/well) for 2 h at 4^{0}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 hydrolysed with 1 N NaOH (200 μl/well, 5 min, 50 ^{0}C), and 150 μl aliquots were added to scintillation vials containing 150 μl of 1 N HCl to neutralize the pH. The radioactivity was determine by scintillation counting and the results are presented as the reciprocal of incorporated counts/min to reflect the inhibition of [^{3}H]-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 [^{3}H]-thymidine incorporation.

Gel filtration chromatography. To assign a protein species to a peak of biological activity, 500 μl of UF were dialysed 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 [^{3}H]-thymidine incorporation assay.

For additional studies and purification of UDGI 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. Dialysed 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 [^{3}H]-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 resolubilzed 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 [^{3}H]-thymidine incorporation assay.

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

Protein sequencing: Approximately 1-2 μg of purified UDGI proteins were electrophoresed through SDS-PAGE gels in the buffer containing 150 mM thyoglycolic acid at 45 volts to reduce amino-terminal blockage. The gels were calibrated with transfer buffer (35 mM Tris-base, 192 mM glycine) for 30 min and assembled with Pro-Blot polyvinylidene difluoride (PVDF) membranes in a Bio-Rad Trans-Blot cell apparatus. Proteins were electrobotted using 27 V for 16 h at room temperature. Proteins were visualized by Coomassie staining, the UDGI protein bands were excised, and the sequence was determined using Applied Biosystems 477A and 773A protein sequencers.

DNA sequencing: Multiple pass DNA sequencing was performed on an Applied Biosystem model 377 Sequencer version 2.1.1 (Seldon Biotechnology, McGill University) using Ampli-Tag polymerase and UDGI specific primers and double-strand DNA. Sequences were assembled using MacVector version 1 and AssemblyLign version 1.0 (Kodax).

Sequence Analysis: Nucleotide sequence searches were performed on available data bases using the BLASTN and TBLASTN (blast enhanced alignment utility) algorithms.

Preparation of UDGI antisera: For initial immunization, purified UDGI was solubilized in sterile distilled water (500 μg/ml), mixed with Freund's complete adjuvant (1:1 v/v) and injected in 1 ml (250 μg) aliquot at multiple subcutaneous sites of two female New Zealand rabbits. At 3 weeks following immunization, each rabbit received a booster of 100 μg of peptide in Freund's incomplete adjuvant injected subcutaneously. The boosting was repeated one every 5 weeks. Serum was collected every 2 weeks after the third boost and was tested for UDGI specific

antibody. High titer anti-serum was obtained after the fifth boost and was used to detect UDGI.

MCF-7 cell stable transfectant cell lines. The entire coding region of UDGI cDNA was cloned into mammalian expression vector pcDNA3.1 (Invitrogen) to create the UDGI-pcDNA3.1 expressing vector. The UDGI-pcDNA3.1 sequence was confirmed by sequencing. MCF-7 cells were seeded at 2x 10^5 in 100 mm culture dishes in 90% α-MEM (Life Technologies, Inc.) containing 10% FCS with Garamycine 24h prior to transfection. Cells were transfected with 5 μg of UDGI-pDNA3.1 DNA or pDNA3.1 control plasmid DNA and 28 μl of Lipofectamine reagent (Life Technologies) following manufacturer recommendations. 48h following transfection, cells were split 1:10 and replaced with growth medium containing 800 μg/ml G418 (Calbiochem, La Jolla, CA). After 4 weeks, clones were isolated, expanded and assayed for UDGI expression by western blot analysis in conditioned medium.

Expression and purification of UDGI-His. The coding region of UDGI without the signal peptide, was cloned into pQE-30 bacterial expression vector (Qiagen), which allows the incorporation of a His-Tag at the carboxyl terminus. The UDGI-QE containing clone was confirmed by sequencing and transformed into the M15 bacterial expression cell line (Qiagen). 500 ml cultures were grown and induced by 1 mM isopropyl-1-thio-beta-D-galactopyranoside for 2h, and soluble cell extract generated by resuspending in buffer containing 8 M urea, 0.1 M NaH_2PO_4 and 0.01 M Tris.Cl, pH 8.0 for 30 min followed by centrifugation at 13000 rpm for 30 min. Soluble recombinant UDGI His-tagged protein was purified on a nickel-charged resin (Qiagen). The column was washed 3 times with buffer containing 8 M urea, 0.1 M NaH_2PO_4 and 0.01 M Tris.Cl, pH 6.3 and eluted with buffer containing 8 M urea, 0.1 M NaH_2PO_4 and 0.01 M Tris.Cl, pH 4.5 as recommended by the manufacturer. Collected fractions were analysed by SDS-PAGE. Fractions containing proteins were pooled and dialysed against 1 M of acetic acid (pH 2.5) overnight at 4°C. The dialysed samples were quickly frozen, lyophilized, and stored at -20 °C.

Western Analysis. UDGI-transfectant MCF-7 cells and primary mammary cells were grown in growth medium. Both were switched to serum-free medium containing appropriate treatment. Conditioned medium was collected every 24 or 48 hours, clarified by centrifugation, and stored at -20 °C until analysis. Western analysis was performed as described (24). Blots were incubated with rabbit anti-UDGI antibody (1:2500) and horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody (1:7500). Blots were visualized with a chemiluminescent detection system (ECL, Amersham) and exposed to film for 10 sec to 45 sec.

UDGI expression in mammary tissues of 2 month-old rats, 14 month-old rats, in DMBA mammary tumour and following estradiol and anti-estrogen treatments. Animal experiments were approved by McGill University Animal Care Committee. Intact female Sprague-Dawley female, either 60 days old or 14 months old at the beginning of the experiments, were obtained from Charles River, Quebec.

To study effect of estradiol on UDGI gene expression. 0.5 cm, 1.0 cm and 1.5 cm silastic tubes (0.04 in. ID, Dow corning, Michigan) containing 17-ß oestradiol were implanted into the back of the neck of different groups of rats. Control rats were experienced the same surgical implantation with empty silastic tubes. Based on previous published work (25), the release rate of 17 ß-oestradiol from silastic implants was documented to be 2.4 μg/cm/day.

To examine the effects of tamoxifen on UDGI gene expression, rats were implanted with either 2 cm or 4 cm silastic tubes (0.12 in. ID, Dow corning, Michigan) containing tamoxifen (Sigma) were implanted into the back of the neck of different groups of rats. The release rate was 25 μg/cm per day.

To investigate the effects of ICI 182780 on UDGI gene expression, 2 month-old rats were injected with 1 mg, 1.5 mg, and 2 mg/kg BW ICI 182780 (Zeneca Pharmaceuticals) in castor oil per week for 3 weeks. Control rats received only castor oil. To induce UDGI expression in mammary gland of old rats, 14 month-old rats were injected with either castor oil or 2 mg/kg BW ICI 182780.

To study the UDGI expression in mammary tumours and the ability of antiestrogens to induce UDGI expression in DMBA breast tumours. The DMBA tumour model was employed (26). Rats bearing DMBA tumours were treated with either vehicle or 1 mg ICI/kg BW or 5 mg tamoxifen/kg BW. Antineoplastic activity was quantitated as mean tumour burden (mm^3) in treated animals after 2 weeks.

At the end of the experiment, animals were sacrificed using carbon dioxide at the end of the experiment. The mammary tissues or tumours were excised, trimmed and frozen in liquid nitrogen and stored at -70 ^{0}C for RNA extraction. Part of the mammary tissue was fixed in 10% buffered formalin for histochemical studies.

Detection of apoptosis: Five μM sections of mammary tissue were used to detect apoptotic cells using TUNEL assay. Fragmented DNA was labelled using the ApoAlert DNA fragmentation assay (Clontech Laboratories, Palo Alto, CA) as described by the manufacturer.

Northern analysis of UDGI gene expression in mature female rat tissues. Total RNA was isolated from tissues and DMBA tumours using RNAZol-B (Teltest, TX). Northern Blots were performed as described (27). Blots were hybridized either with UDGI or ß-actin (ATCC) or GAPDH cDNA inserts.

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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 [^{3}H]-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).

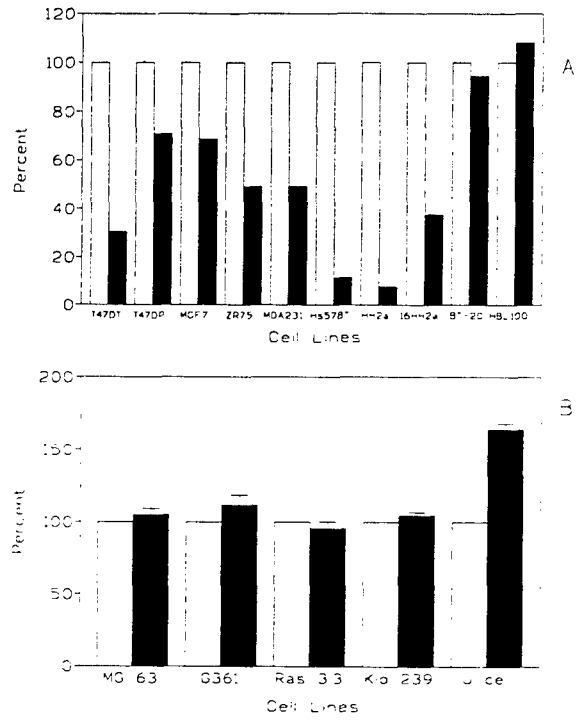

Figure 1. Effect of uterine fluid (UF) on mammary cell (A) and non-mammary cell (B) DNA synthesis. Confluent cultures of target cells for inhibition were trypsinized and plated at 2.5 x 10^4 cells in 24-plate multi-well dishes in 2.5% FCS. After 48h, the cell monolayer was rinsed twice with phenol-red 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, [^{3}H]-thymidine incorporation into DNA was determined as described (28). 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 observed. Furthermore, UF had neither stimulatory nor inhibitory effects on human melanoma (M 361), human osteosarcoma (MG 63), mouse, or 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. [^{3}H]-thymidine incorporation into DNA was determined as described above. As shown in figure 2, UF inhibited DNA synthesis in three cell lines in a dose-dependent manner.

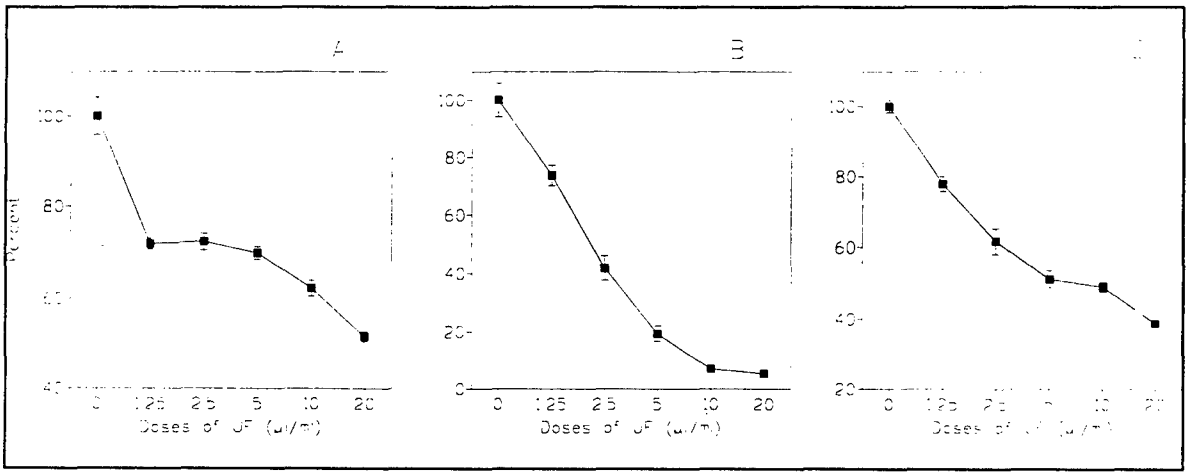

Figure 2. Effect of uterine fluid (UF) on MCF-7 (A) and Hs578T (B) and MDA 231 (C) cell DNA synthesis. Cells were grown as described in figure 1. Cells were treated with the indicated doses of UF (μl/ml) in serum free media for 24h. At the end of incubation, [^{3}H]-thymidine incorporation was determined as described (28). 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 [^{3}H]-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 consistent 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 (s) 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 analysed by SDS-PAGE. UDGI activity was collected from rat UF following estradiol implantation, as described in "Experimental Procedures".

Ion exchange chromatography was initially performed to analyse the biological activity of UDGI. The samples were dialysed 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.

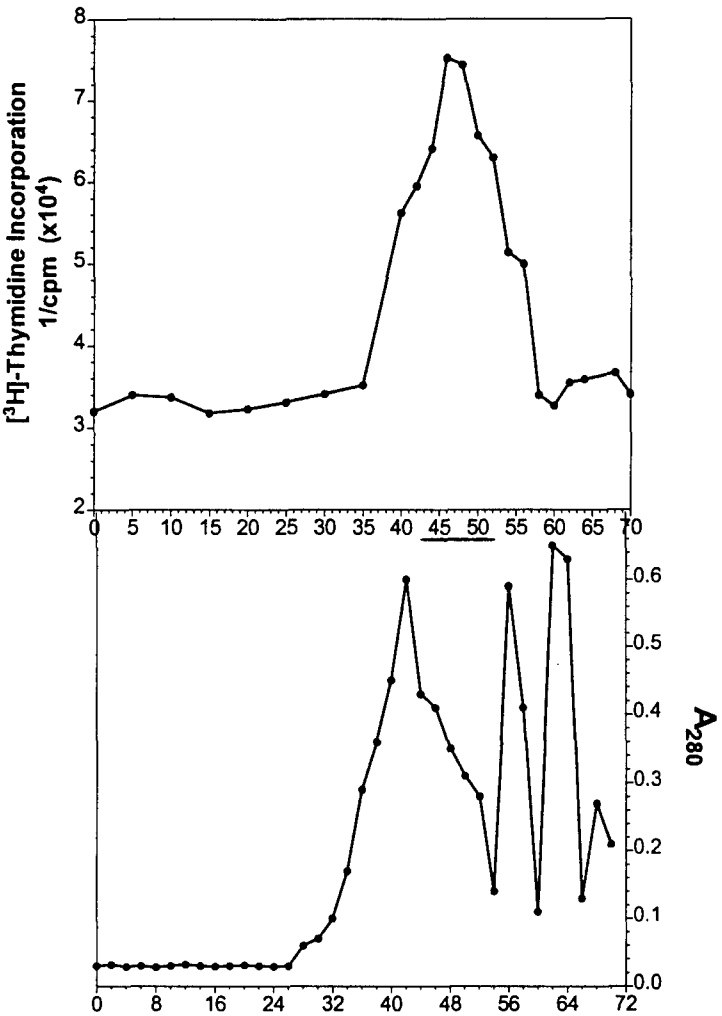

Fig. 3. Ion exchange chromatography. Proteins from rat UF were chromatographed through a DE-52 anion exchange column. 100 μl aliquots from each fraction were vacuum-dried and assayed for inhibition of [^{3}H]-thymidine incorporation in Hs578T cells as described in "Experimental Procedures". Activity was plotted as the reciprocal of incorporated counts/min (1/cpm) to illustrate the inhibition of [^{3}H]-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. The 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 dialysed against 1 M of acetic acid (pH 2.5) overnight at 4^0C. The dialysed samples were quickly frozen, lyophilized, and stored at -20 ^{0}C. UDGI activity prepared in this manner was further analysed 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 analysed by SDS-PAGE (Fig. 5, lower panel, arrow, fractions 80 to 86).

Owing to the ability of HPLC to separate proteins of similar size based on hydrophobic properties, the last purification step utilized reverse phase HPLC. Pooled fractions from gel filtration chromatography were vacuum-dried, resolubilized in 50% formic acid, and analysed using reverse phase HPLC, as described in "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 in turn, was directly correlated with the elution pattern of the 21-22 kDa protein species (Fig. 6, lower panel). This protein was 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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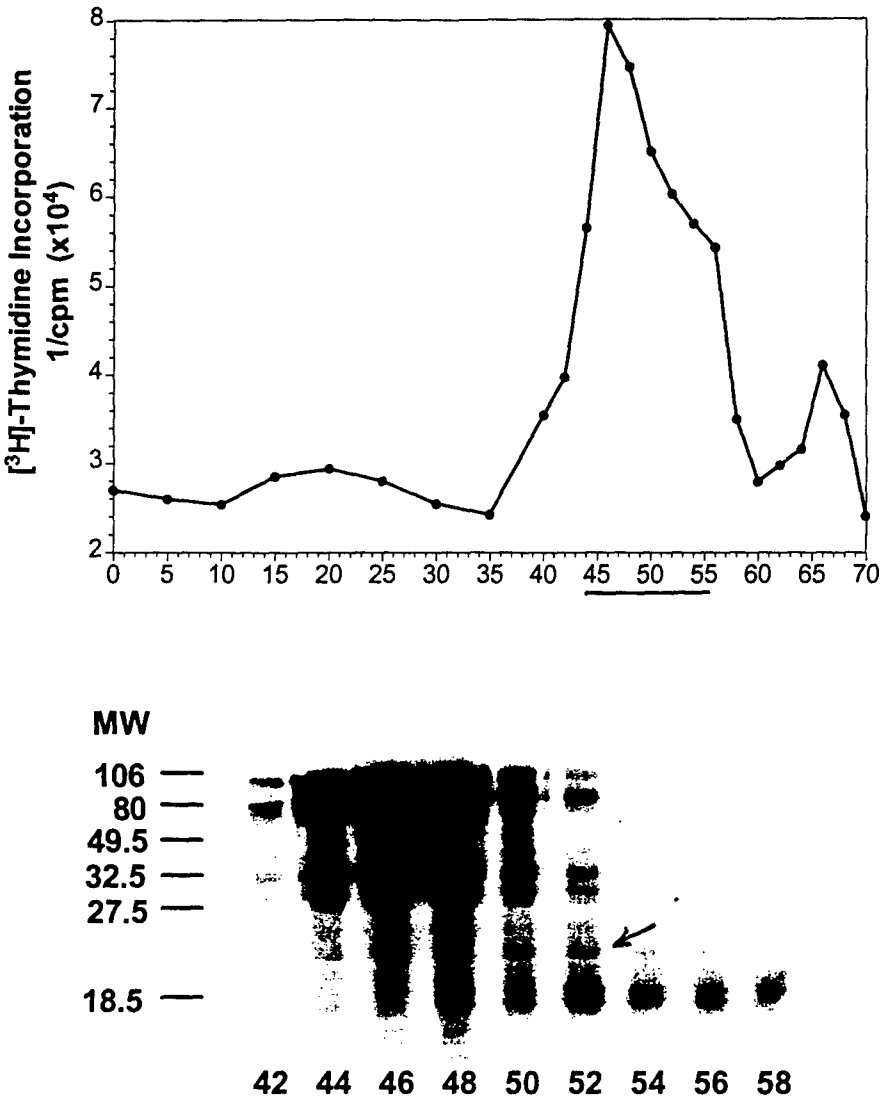

Fig. 4. Analytical gel filtration chromatography. Proteins from the UF were chromatographed through a DE-52 anion exchange column, as described in Fig. 3, and analysed by gel filtration chromatography. The fractions were assayed for activity with Hs578T cells, as described in Fig. 3 and in "Experimental 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 46 to 52) correlated directly with the position of the eluted bioactivity peak. Molecular size markers are shown.

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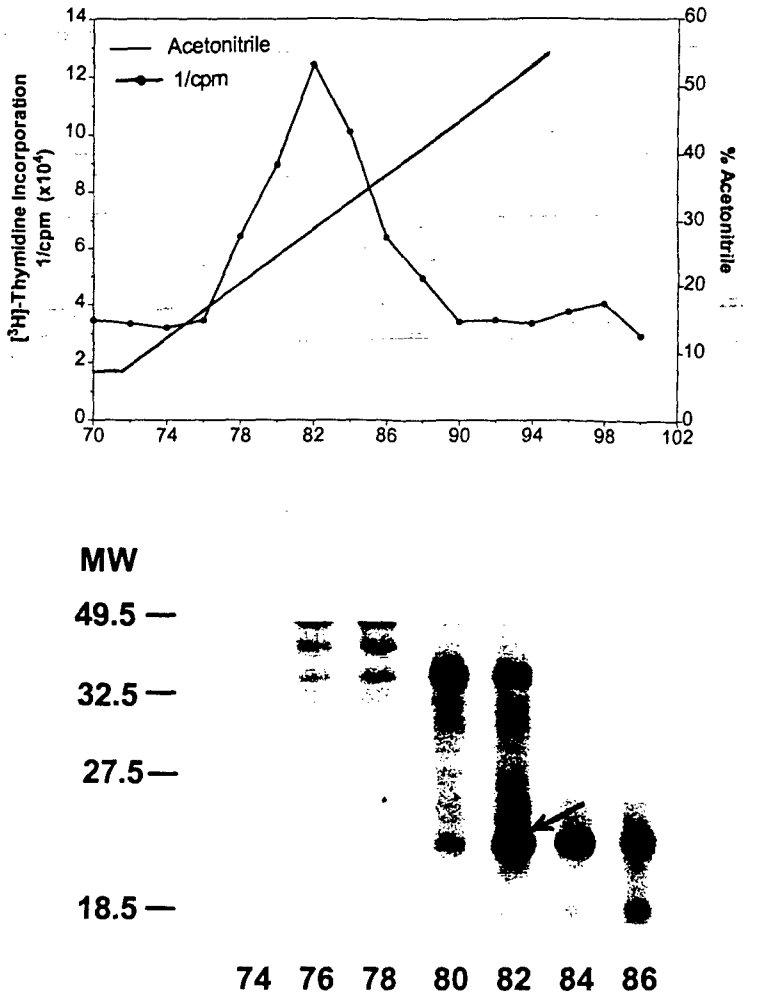

Fig. 5. Analytical reverse phase HPLC. Upper panel, proteins were chromatographed with a C-18 reverse phase column, as described in Fig. 3 and in "Experimental Procedures". 100 μl aliquots from each fraction were vacuum-dried and assayed for inhibition of [^{3}H]-thymidine incorporation in Hs578T cells, 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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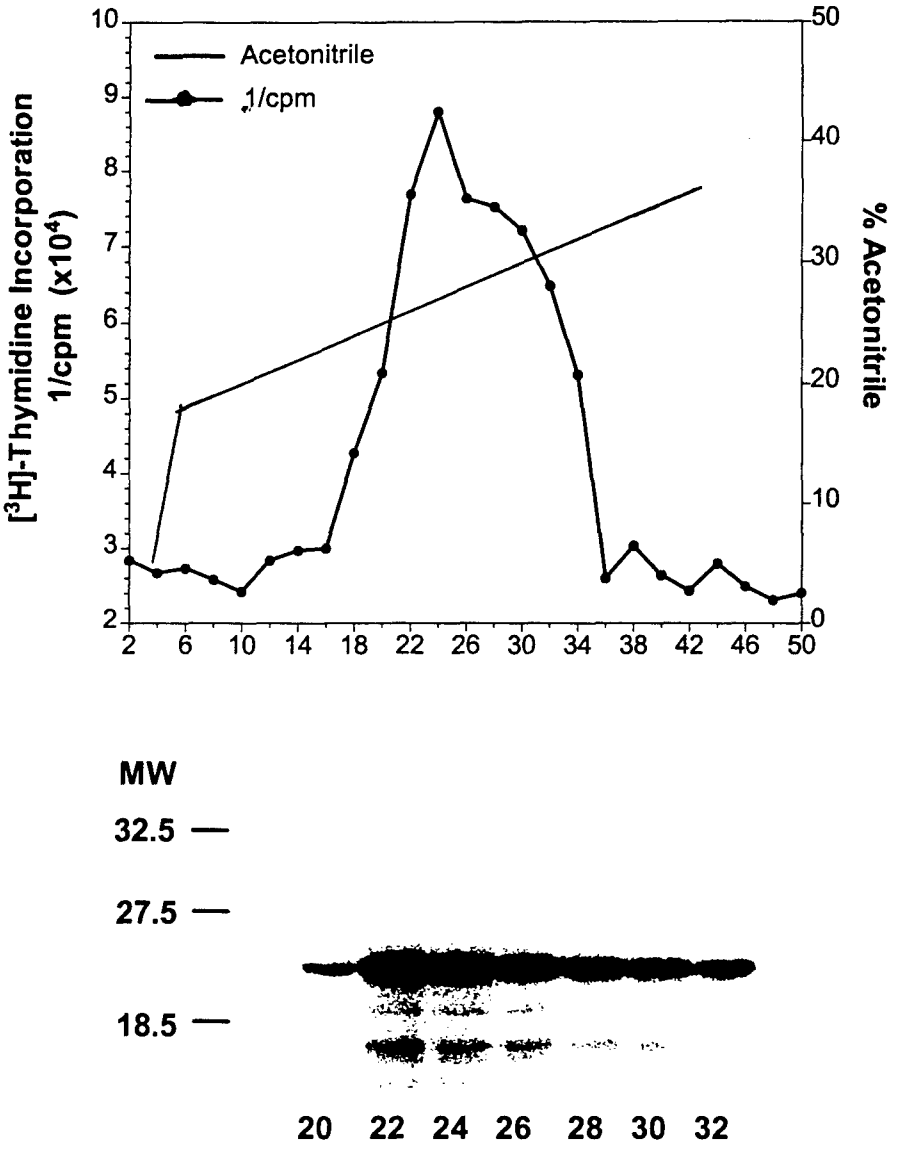

Fig. 6. Preparative HPLC chromatography. Upper panel, fractions from gel filtration chromatography were pooled and used for reverse phase HPLC as described in "Experimental Procedures". Shown is the elution profile of the biological activity with Hs578T cells and an acetonitrile elution gradient. The 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 shown.

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For microsequence analysis, purified UDGI protein was electrophoresed through SDS-PAGE gels, blotted to PVDF membranes, and analysed for amino-terminal sequence. A single sequence was detected with unambiguous assignments made for the position 1-23. Analysis with SwissProt, SPUpdate, GenPept, and GPUpdata data bases indicated that this region was 96% homoloous with a previously characterized protein termed urogenital sinus derived growth inhibitor sp20 (29). The difference in homology between the sp20 protein and the protein identified in this study owes to one amino-acid at position 16 (as illustrated below)..

UDGI: NH-2-Thr-Trp-Glu-Ala-Met-Leu-Pro-Val-Arg-Leu-Ala-Glu-Lys-Ser-Gln-Val-Glu-Glu-Val-Ala-Ala-Thr-Gly

ps20: NH-2-Thr-Trp-Glu-Ala-Met-Leu-Pro-Val-Arg-Leu-Ala-Glu-Lys-Ser-Gln-Arg-Glu-Glu-Val-Ala-Ala-Thr-Gly

To confirm whether UDGI and ps20 were derived from the same cDNA, we degenerate a UDGI primer corresponding to amino acids 10 to 17 (which is 3 nucleotides different from the published sequence for ps20) and used a PCR strategy in combination with vector primer to amplify a fragment of UDGI cDNA from the rat pcDNA3.1 uterus cDNA library. A 800 bp fragment was produced by PCR. This fragment was further cloned into the pcDNA3.1 vector and sequenced. The deduced amino acid sequence of this clone matched directly with the first 23 amino acid sequence of UDGI and shared 98% identity to the amino acid sequence of ps20 protein (29). The results suggest that UDGI is likely the ps20 protein.

To examine UDGI gene expression *in vivo*, Northern blot analysis was performed on total RNA isolated from rat mammary tissue at different stage of pregnancy. Despite previous reports of a single transcript for ps20 gene (29), two transcripts of aproximately 1.4 and 1.1 kb were clearly detected by the UDGI cDNA probe (Fig. 7). This observation raised the questions of whether the 1.4 and 1.1 kb transcripts were transcribed from a single gene or a different gene, and whether the difference in transcript size observed was due to differential splicing or difference in the 5' or 3' untranslated sequences.

Figure 7. Expression of UDGI in the mammary gland during different stages of pregnancy, during lactation and involution. Mammary tissues were non-pregnant (lane **1**), 3 days (lane **2**), 6 days (lane **3**), 9 days (lane **4**), 12 days (lane **5**), 15 days (lane **6**) and 18 days (lane **7**) of pregnancy; 2 days (lane **8**) and 5 days (lane **9**) of lactation; and 2 days after pups were removed (lane **10**). Total RNA derived from mammary gland was subjected to Northern blot. Blots were hybridized with GAPDH (**A**) and rat UDGI (**B**) cDNAs. Densitometric scanning of the UDGI bands are shown in (**C**). Note that two transcripts for UDGI were detected.

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When Northern analysis was performed on total RNA isolated from various rat tissues, two UDGI transcripts were also detected by UDGI cDNA (Fig. 8). The ratio of these transcripts varied from tissue to tissue. Of multiple tissues survey, the highest levels of UDGI mRNA was observed in the lung (Fig. 8). The 1.4 kb transcript was the predominant species of UDGI transcript in bladder and red muscle.

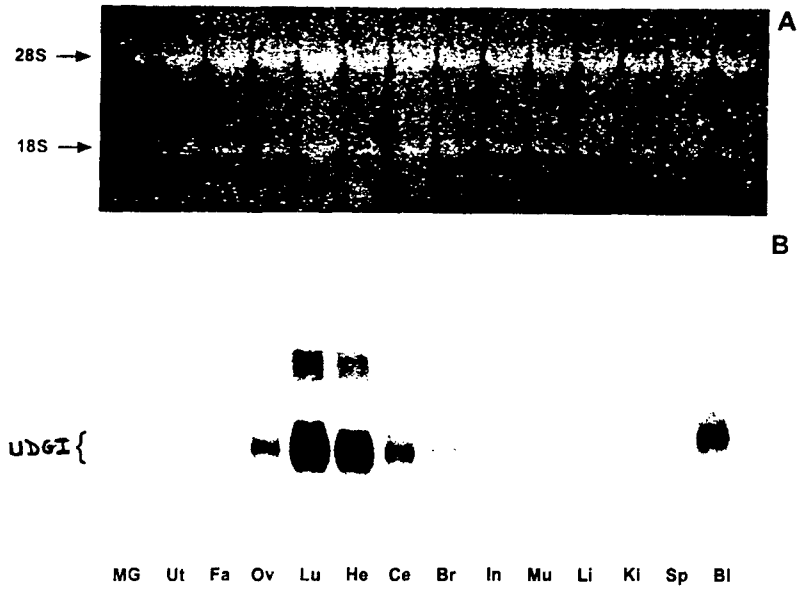

Figure 8. Northern blot analysis of UDGI gene expression in female adult rat tissues. Total RNA derived from various tissues of 3 month old female rat was subjected to Northern blot. Blots were hybridized with and GAPDH (**A**) and rat UDGI (**B**) cDNAs.

To extend our study regarding the function of UDGI expression (ps20) in growth regulation of normal mammary cells and breast cancer cells, we developed a rabbit polyclonal antibody against purified UDGI protein. When uterine fluid (the fluid from which UDGI was identified) was analysed by Western analysis using UDGI antibody, three specific bands of apparent molecular mass 24, 27 and 29 kDa were specifically recognized by UDGI antibody (Fig. 9). The 27 kDa species was the major species. Preabsorbed control gave no signal. Similar results were observed when conditioned media from primary mammary cells was analysed by Western analysis. However, the 24 kDa form became the major species when cell extracts were used. The results suggest that UDGI may undergo post-translational modification.

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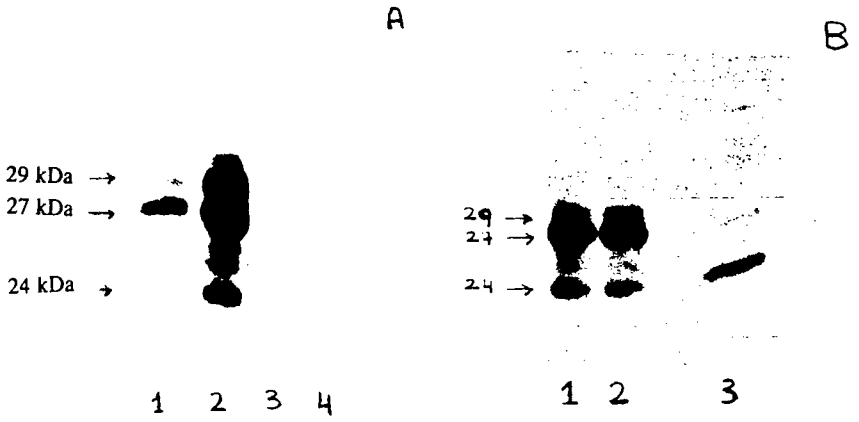

Figure 9. Western blot analysis of purified UDGI, rat primary cell conditioned media and rat primary cell extract. Proteins derived from uterine fluid (lane 1A), purified UDGI (lanes 2A-4A), rat primary conditioned media (Lanes 1B and 2B) and rat primary cell extract (Lane 3B) were subjected to Western analysis. Blots were incubated with either anti-UDGI antibody (Lanes 1A, 2A, 1B-3B), or preabsorbed UDGI antibody (lane 3A), or preimmune serum (lane 4A). Molecular weight of immuno-reactive bands are shown.

To determine if post-translational modification plays a role in UDGI activity, the UDGI open reading frame (without the signal peptide) was subcloned into a bacterial expression vector and expressed as a carboxyl-terminal histidine-tagged protein in bacteria (Fig. 10). Western analysis with UDGI antibody demonstrated specific immunoreactive with recombinant UDGI protein (data not shown).

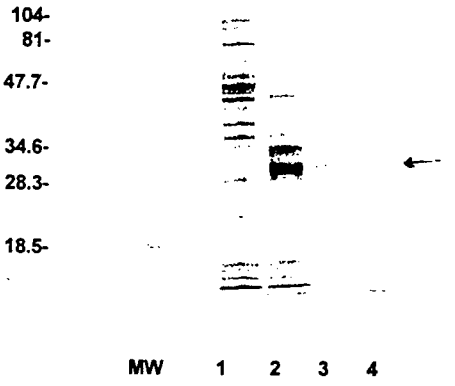

Figure 10. Expression of UDGI-His-tagged protein. Crude extracts of bacterial cells prior to induction (lane 1), following induction (lane 2), and after purification (lanes 3 and 4) were resolved by SDS-PAGE and visualized by Coomassie Blue stain.

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Because Hs578T and MCF-7 cells are target cell types shown to be sensitive to purified UDGI, MCF-7 and Hs578T cells were assayed for UDGI-induced inhibition of [^{3}H]-thymidine incorporation. As shown in Figure 11, recombinant UDGI induced only a minor inhibition of [^{3}H]- thymidine incorporation in both MCF-7 and Hs578T cells. The data suggest that post-translational modification of UDGI may be required for full activity.

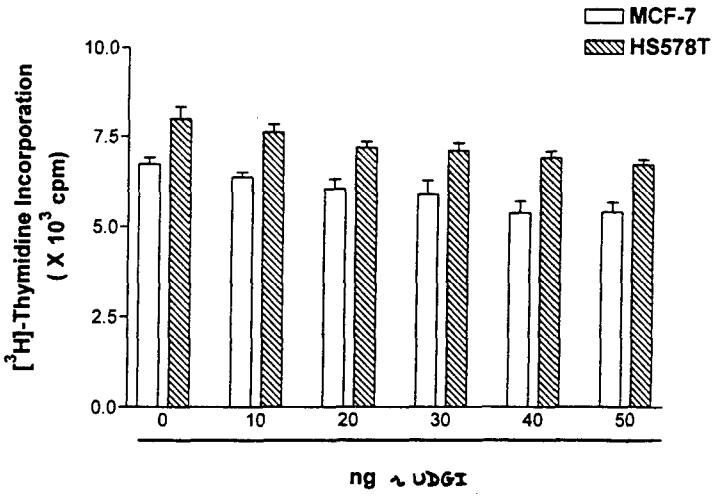

Figure 11. Effect of recombinant UDGI on MCF-7 and Hs578T cell DNA synthesis. Cells were grown as described in Figure 1. Cells were treated with the indicated doses of recombinant rat UDGI (ng/ml) in serum free media for 24h. At the end of incubation, [^{3}H]-thymidine incorporation was determined as described (28). The experiments were repeated 3 times with similar results and the means of triplicate determinations were plotted as percent of control.

Since growth of mammary epithelial cells in the mammary gland is influenced, to some extent by female steroid hormones, and purified UDGI inhibits breast cancer cells *in vitro*, Northern and Western blot analyses were used to determine the expression of UDGI gene in the mammary gland of 2 and 14 month old female rats. In addition, the expression of UDGI gene in normal mammary tissue and tumours, prior to and after antiestrogen treatment, was also investigated. The UDGI gene expression was then correlated with proliferation, apoptosis or tumour volume. Given the extensive use and efficacy of antiestrogens in breast cancer treatment, and ongoing research regarding their usefulness in breast cancer prevention and treatment, new data regarding the action of ICI and tamoxifen on UDGI expression deserve investigation.

In 2 month old rats, estradiol caused mammary epithelial cell proliferation (Fig. 12) concomitant with suppression of UDGI expression (Fig. 13 and 14). Pure anti-estrogen ICI 182780, which blocked breast epithelial cell proliferation and caused apoptosis (Fig. 12), greatly stimulated UDGI expression (Fig. 15 and 14). Mild stimulation of UDGI expression by tamoxifen was also observed (Fig. 16 and 14). The observations indicate that, *in vivo,* UDGI expression is associated with growth inhibition and apoptosis.

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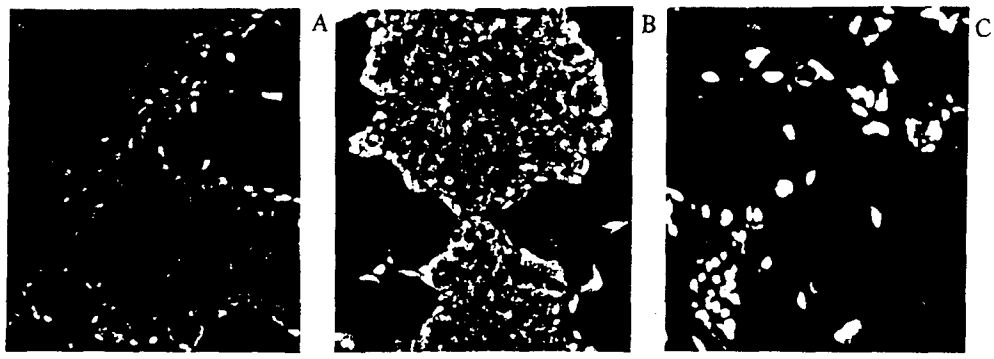

Figure 12. Effects of ICI and estradiol on mammary epithelial cell apoptosis. Rats were treated with either vehicle or estradiol or ICI 182780. Five μM sections of mammary gland were used. Fragmented DNA were labelled using the ApoAlert DNA fragmentation assay (Clontech Laboratories, Palo Alto, CA). Control mammary sections (**A**), estradiol-treated mammary sections (**B**) and ICI-treated mammary sections (**C**).

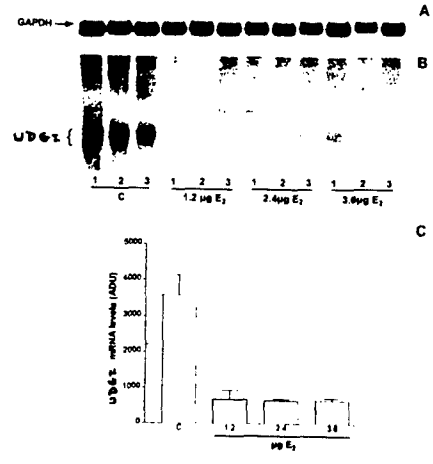

Figure 13. Effects of estradiol on mammary gland UDGI expression. Rats were treated with indicated concentrations of 17-ß estradiol. Total RNA derived from mammary glands was subjected to Northern blot. Blots were hybridized with and GAPDH (**A**) rat UDGI (**B**) cDNAs. Densitometric scanning of the UDGI band is shown in (**C**). Note that UDGI expression was severely inhibited by estradiol treatment.

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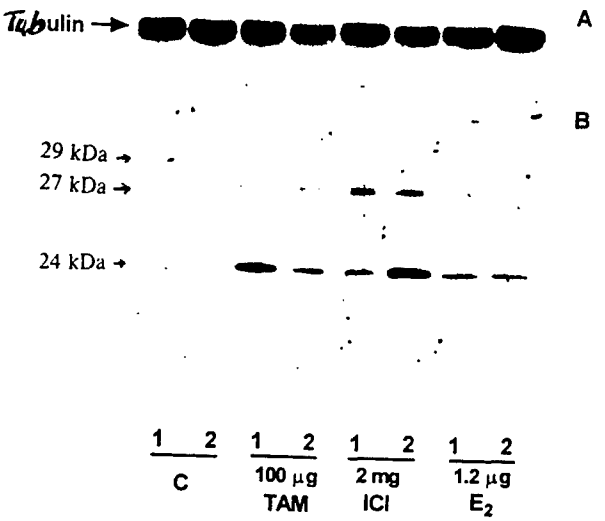

Figure 14. Effects of tamoxifen, ICI 182780 and estradiol on mammary gland UDGI expression. Rats were treated with indicated concentrations of tamoxifen (**TAM**), ICI 182780 (**ICI**), and 17-ß estradiol ($\mathbf{E_2}$) for 3 weeks. Total proteins derived from mammary glands was subjected to Western blot analysis. Blots were blotted with tubulin (**A**) and rat UDGI (**B**) antibodies. Note that tamoxifen and ICI significantly induced UDGI expression.

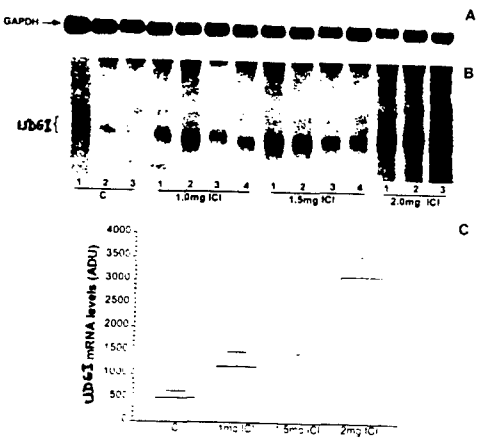

Figure 15. Effects of ICI 182780 on mammary gland UDGI gene expression. Rats were treated with indicated concentrations of ICI. Total RNA derived from mammary glands was subjected to Northern blot. Blots were hybridized with GAPDH (**A**) and rat UDGI (**B**) cDNAs. Densitometric scanning of the UDGI bands are shown in (**C**).

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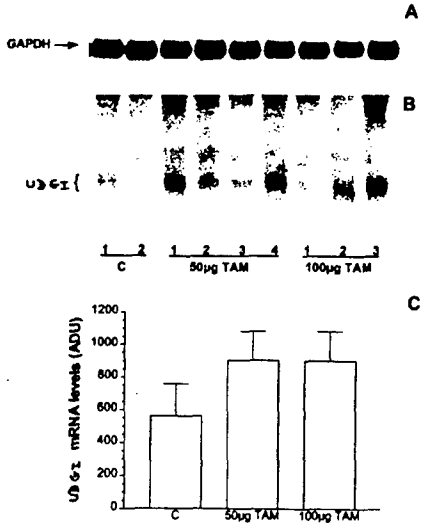

Figure 16. Effects of tamoxifen on mammary gland UDGI expression. Rats were treated with indicated concentrations of tamoxifen. Total RNA derived from mammary glands was subjected to Northern blot. Blots were hybridized with and GAPDH (**A**) rat UDGI (**B**) cDNAs. Densitometric scanning of the UDGI band is shown in (C).

Surprisingly, UDGI mRNA was barely detectable in mammary glands of 14 month old rats (Fig. 17). The loss of UDGI gene expression was completely restored by treament with antiestrogen ICI 182780. Since UDGI is a potent growth inhibitor for breast epithelial cells *in vitro*, the decrease in UDGI expression in mammary gland of old animals may allow the tumour cells to grow. This hypothesis is supported by the fact that high breast cancer incidence is seen in old animals and post-menopausal women.

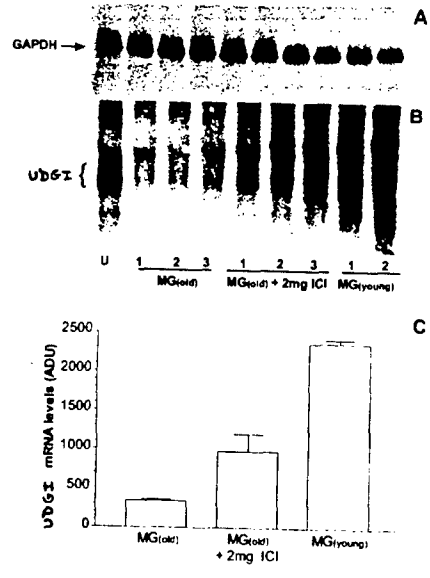

Figure 17. Stimulation of UDGI expression in mammary tissue of old rats. Fourteen-month old rats were treated with indicated concentrations of ICI 182780. Total RNA derived from mammary glands was subjected to Northern blot. Blots were hybridized with and GAPDH (**A**) rat UDGI (**B**) cDNAs. Densitometric scanning of the UDGI band is shown in (**C**). Samples are positive control uterus RNA (**U**), mammary RNA of 2 month old rat (**MG (young)**), and mammary RNA of 14 month old rats (**MG (old)**). Note that the UDGI transcripts were barely detectable in mammary glands of old rats. A significant increase in UDGI gene expression was seen following ICI administration.

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Assuming that UDGI is a growth inhibitor and apoptotic factor for breast cells, we predicted that UDGI expression in breast tumours would be low. In addition, we wanted to determine whether we could reactivate or induce UDGI expression in mammary tumours by any anti-tumour agents. Agents that can reactivate UDGI expression would be considered for breast cancer treatment or prevention. It is well known that a significant proportion of DMBA-induced rat mammary tumours are antiestrogen-responsive (26,30). Our first attempt was to use the DMBA mammary tumour model (26) to determine if there was a correlation between the antineoplastic activity of ICI 182780 and tamoxifen with the ability of these agents to induce UDGI gene expression. As predicted, UDGI transcripts were barely detectable in all DMBA tumours examined (Fig. 18). UDGI gene expression was greatly stimulated following either tamoxifen or ICI 182780 treatment for 2 weeks (Fig. 18B). The induction was positively correlated with the ability of these agents to suppress tumour growth, as determined by tumour volume (Fig. 18D).

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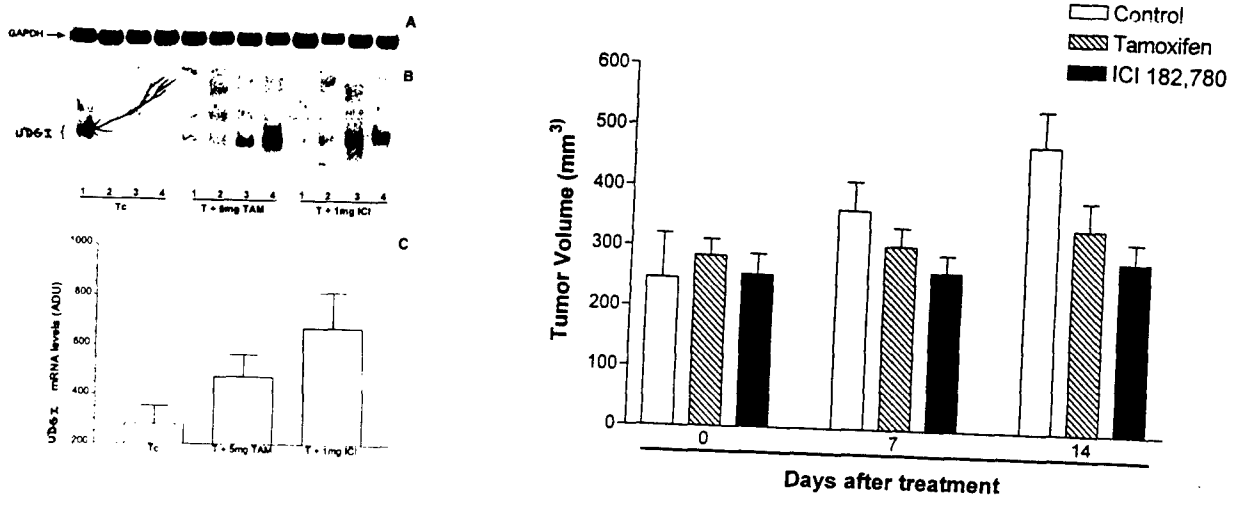

Figure 18. Reactivation of UDGI expression in rat DMBA mammary tumours. DMBA mammary tumours were created as previously described (26). Rats bearing mammary tumours were either left untreated (**Tc**) or treated with indicated dose of tamoxifen (**TAM**) or indicated doses of ICI 182780 (**ICI**) for two weeks. After two weeks, tumours were collected and total RNA was extracted and subjected to Northern blot. Blots were hybridized with and GAPDH (**A**) rat UDGI (**B**) cDNAs. Densitometric scanning of the UDGI band is shown in (**C**).Tumour volume was measured weekly and plotted as shown in (**D**). Note that the UDGI transcripts were barely detectable in DMBA mammary tumours. Significant increase in UDGI transcripts were observed following either tamoxifen or ICI treatment.

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To further demonstrate the role of UDGI protein in regulating mammary cell growth, human breast cancer MCF-7 cells were transfected with a mammalian expression vector containing full length UDGI cDNA (UDGI-pcDNA3.1) or with control pcDNA3.1 vector. Conditioned media (CM) from stable transfectant lines was collected, concentrated and analysed by Western blot analysis with UDGI antibody. As predicted, the 24, 27 and 29 kDa proteins were detected in the CM of MCF-7 cells transfected with UDGI cDNA, but not in CM of mock-transfected MCF-7 cells (Fig. 19). When these cells were grown in medium containing 10% FCS, there was no noticable difference in growth rate between the UDGI transfectant cell lines and the mock transfectants. When the UDGI transfectants were deprived of serum, they exhibited an extensive array of filopodia and rounded up (Fig. 20). By 48h after serum starvation, extensive cell death was observed in UDGI transfectant lines as compared to mock transfectant lines (Fig. 20).

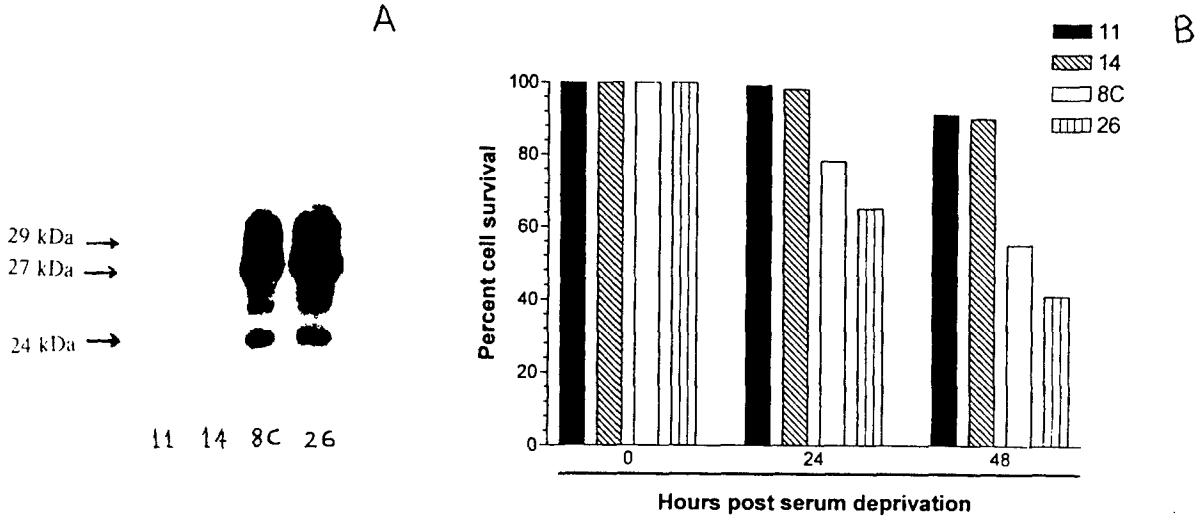

Figure 19. Overexpression of UDGI accelerates cell death. (**A**) Western blot analysis of UDGI secretion into conditioned media of mock- transfected lines (11 and 14) and two clones expressed UDGI (8C and 26) using UDGI antibody. (**B**) Viability assays. Cultures of pcDNA3.1 mock-transfected lines (11 and 14) and two clones expressed UDGI (8C and 26) were deprived of serum. The viability was assessed by trypan blue exclusion at 24 and 48 hours following serum deprivation and was plotted as percent cell survival.

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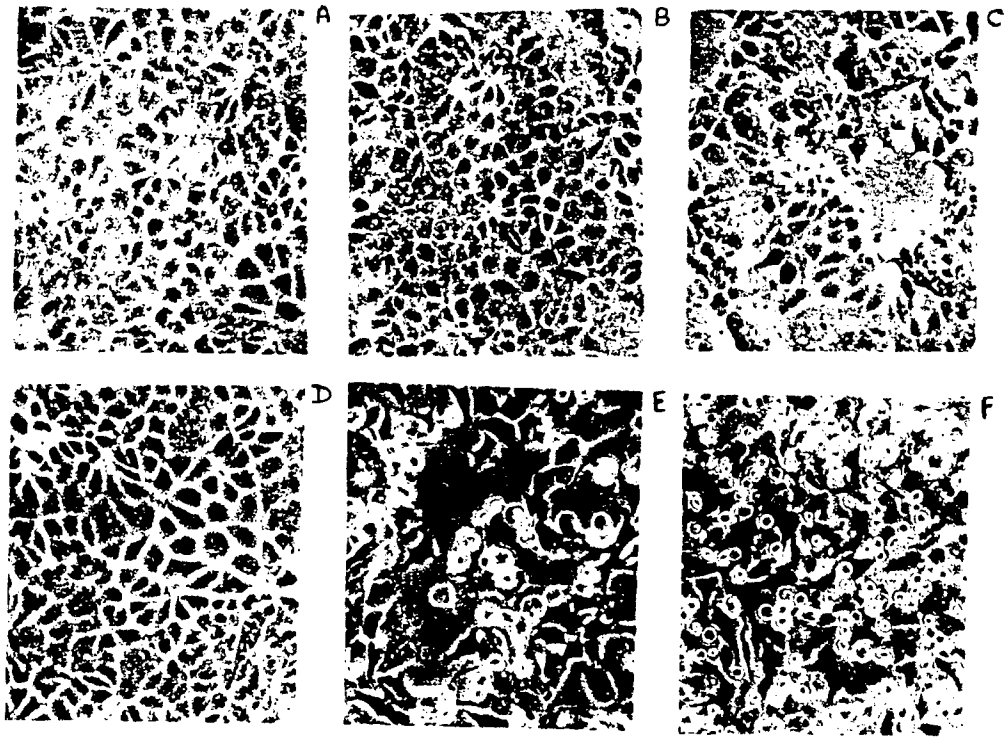

Figure 20. Morphology of UDGI transfectant cells before and after serum starvation. Representative photographs of mock transfected cell line 11 (**A, B, C**) and UDGI-transfected cell lines (**D, E, F**) at 0 (**A and D**), 24h (**B and D**) and 48h (**C and F**) following serum deprivation. X250.

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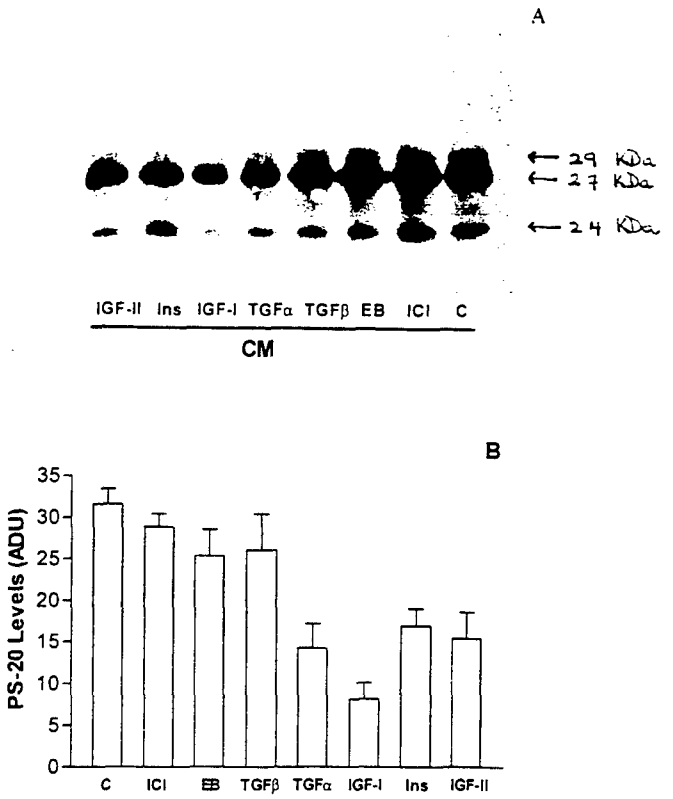

Figure 21. Effects of IGFs, insulin, TGF-ß, TGF-α, ICI 182780 and vitamin D analog EB1089 on UDGI accumulation in conditioned media of primary rat mammary gland cells. Primary mammary cells were isolated as described previously (19). Cells were treated with 20 ng/ml IGF-II, 5 μg/ml insulin (Ins), 20 ng/ml IGF-I, 10 ng/ml TGF-α, 10 ng/ml TGF-ß, 1 x 10^{-8} M EB1089 (EB) and 1 x 10^{-8}M ICI 182780 (ICI) for 48 hours. One ml of conditioned media was concentrated and was subjected to Western blot analysis. Membranes were blotted with rat UDGI antibody (**A**). Densitometric scanning of the UDGI bands is shown in (**B**). Note that TGF-α, IGF-I, IGF-II and insulin significantly reduced UDGI accumulation.

STATEMENT OF WORK

To date we have completed Task 1 to 5 of the Statement of Work outlined in the proposal: Purification of UDGI from rat uterine fluid, polyclonal antibody production, UDGI cDNA isolation, transfection of UDGI expression into breast cancer cells, regulation of UDGI gene in normal breast cells. In doing so we came across some unanticipated problems:

1. To analyse 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. cDNA library screening to determine the difference between 1.1 and 1.4 kb transcripts. The human UDGI is also cloned.

2. *In vivo* tumorigenic assay.

3. Immunolocalization of UDGI in normal and mammary tumours.

4. Examine the the mechanism(s) of UDGI-induced apoptosis *in vitro*.

Tasks remaining:

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

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

CONCLUSION

In this annual report, we report the purification of UDGI, a uterine derived secreted protein which possesses growth inhibitory properties. Sequence analysis revealed that UDGI shares 98% identity to ps20 growth inhibitor protein which was previously isolated from urogenital sinus mesenchymal cells (29). UDGI (ps20) belongs to a family composed primarily of secreted serine protease inhibitors. Although purified UDGI was shown to potently inhibits breast cancer cell growth, recombinant UDGI only exhibited mild inhibitory effects. Polyclonal antibodies against purified UDGI recognized native UDGI from uterine fluid, conditioned media of primary mammary cells and stable UDGI-transfectant MCF-7 cell lines with molecular mass of 24, 27 and 29 kDa, respectively. A full length of UDGI cDNA was isolated from the rat uterus cDNA library. Northern blot analysis using UDGI cDNA detected two UDGI transcripts in rat mammary tissue using UDGI cDNA. UDGI mRNA levels gradually decreased as pregnancy advanced. UDGI mRNA was also detected in various female rat tissues with the highest expression in the lung and heart. *In vitro,* using primary mammary cells, UDGI expression was negatively regulated by IGF-I, IGF-II, insulin and TGF-α. *In vivo*, UDGI expression was greatly suppressed by estradiol while pure antiestrogen ICI 182780 and tamoxifen upregulated UDGI expression. UDGI gene expression was undectable in mammary tissue of 14 month old rats and its expression was greatly stimulated following ICI 182780 administration. The induction of UDGI correlated positively with apoptosis in mammary gland. In mammary DMBA tumours, UDGI gene expression was very low and was significantly induced following either tamoxifen or ICI 182780 treatment. Acceleration of apoptosis was observed in stable UDGI-transfectant MCF-7 cell lines following serum starvation. Taken together, these results indicate that UDGI is a growth inhibitor and apoptotic factor for breast epithelial cells, *in vitro*. *In vivo*, the low levels of UDGI expression in the mammary tissue of old rats and in breast tumours may facilitate tumour growth and progression. Induction of UDGI in normal mammary gland during apoptosis and in DMBA mammary tumours during tumour regression by antiestrogens suggest that UDGI may function as a mediator of local growth and apoptosis mechanisms.

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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)

1 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:

PHYLIS M. RINEHART
Deputy Chief of Staff for
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