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13. ABSTRACT (Maximum 200) We are examining the possibility that the membrane transporter, Pgp, could continually or episodically (under the influence of various steroid hormones or environmental factors) transport hydrophobic toxic molecules, including pesticides, into the lumen of breast epithelia over long periods of time (years). Toxins transported into the lumen would be concentrated well above blood concentrations and would continue to diffuse from the lumen back to the cell, affording them time to damage or modify DNA. Published immunocytochemical studies reveal that Pgp is present in human breast ductules, but we have not seen any functional evidence for its presence. We have preliminary evidence for another transporter, the multispecific organic anion transporter (MOAT), which transports fluorescent methotrexate into ductule lumens. A second phase of this research, utilizing primary tissue cultures and cell lines of human breast epithelial tissue. We will use these model systems to determine transporter substrates and potential hormonal regulation of these transporters.			
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FOREWORD

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Karl Karnaky, Jr.

9/30/97

PI - Signature

Date

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INTRODUCTION:

The Bioconcentration Concept:

We originally proposed to examine the possibility that a well-described membrane transporter, the multidrug transporter or P-glycoprotein (Pgp), or a molecule like it, could continually or episodically (under the influence of various steroid hormones) transport toxic molecules into the lumen of breast epithelia over long periods of time (years). This transport process could be very significant because the lumen of breast epithelia in nulliparous woman would essentially be a stagnant luminal pool: the connection to drain any of its secretory products is normally closed by a keratotic plug. Toxins transported into the lumen would be concentrated well above blood concentrations and would continue to diffuse from the lumen back to the nucleus, where they could damage nuclear DNA.

Major New Developments Since Submission of the Original Grant:

1. We have found an important study (Pavelic et al., 1993) reporting the localization of Pgp in breast epithelium with four different Pgp antibodies. We had not discovered this reference when we originally wrote the grant, but it clearly shows that the Pgp is present in at least that sample of women.

2. We have preliminary evidence for the presence of another transporter, the multispecific organic anion transporter, MOAT. The MOAT is a member of a family of transporters which are located in duodenum, jejunum, kidney, but mainly in the liver (Ito et al. 1997). In the liver this transporter appears to be responsible for the excretion of bile acid sulfates and glucuronides (Suchy et al., 1997). In killifish kidney proximal tubules, confocal microscopy demonstrated secretion of fluorescent methotrexate and Texas Red into the lumen; this transport was inhibited by leukotriene C₄ (Masereeuw et al., 1996).

BODY:

Exploratory Experiments

Before using precious human breast tissue we wanted to explore basic techniques in mouse mammary gland tissue. We were able to obtain mainly discarded mice from other labs.

Establishing a ductule isolation procedure:

Procedure:

We started by trying different mincing and sectioning techniques on fish liver. We tried razor blades and also making thin sections with a Vibrotome (tissue embedded in agar).

Results:

These methods did not work.

Next we focused on collagenase digestion methods, which gave good results.

Isolation of glands using collagenase:

Abdominal mammary glands were dissected from mouse strain FVB/N and placed in Hank's Buffered Saline Solution with 1% collagenase type I [283 units/mg of solid; we used 10 mg/ml]. The mixture was transferred to 95% O₂/ 5% CO₂ gassed, plastic tissue culture bubbles and shaken vigorously in 37 °C room for about 90 min. After dilution and centrifugation at approximately 600 rpm, the fat layer was discarded and organoid pieces were viewed under a dissecting scope. Organoid pieces were pipetted onto glass slides for experimental use.

We discovered two problems: 1) different batches of the enzyme have different efficacies, so each new batch of enzyme must be tried to determine the ideal time needed for that batch; 2) too much time in the enzyme would result in totally dissociated cells, and we could not identify ductules at all. We also tried some tissue from discarded rats, and found that our mouse procedure did not work at all with this type of animal.

Results:

This procedure gave short to long lengths of ductules. Occasionally we produce branching ductules with terminal buds attached. This procedure produces ductules for exposure to fluorescent substrates of Pgp and MOAT.

Incubation of tissue in fluorescent substrates of Pgp and MOAT:

Isolated ductules were incubated in 2 μM daunomycin (to detect Pgp transport) or 1-2 μM fluorescent methotrexate (FL-MTX) (to detect a second type of transporter, MOAT, the multispecific organic anion transporter) for 60-90 min at 37 °C. Tissues were mounted on slides and examined at room temperature in a conventional inverted fluorescent microscope (Zeiss IM 405). Later we maintained the slide-mounted tissue at 37 °C by utilizing a fluorescent microscope in a warm room at that temperature. Typically we captured bright field and fluorescent micrographs of the tissue. Brightly fluorescent lumens represent positive evidence of transport. For appropriate inhibition studies, we used a 10 min pre-exposure to verapamil (to block Pgp function) or methotrexate (which is non-fluorescent) or 1 mM KCN to block MOAT transport.

Results:

We found no evidence of Pgp function in any isolated ductules, since we did not see any daunomycin in the ductule lumen. We tried to stimulate Pgp expression with a 24 h exposure to 10^{-6} M β -estradiol before exposure to daunomycin, but this did not change our results.

We found preliminary evidence for secretion of the MOAT substrate, fluorescent methotrexate, into the lumen of mouse mammary gland ductules (APPENDIX PAGE 1, Figure 1: LEGEND FOR FRESHLY-ISOLATED DUCTULES OF MOUSE MAMMARY GLAND (conventional fluorescence microscope used, not a confocal microscope) : A) exposed to fluorescent methotrexate (FL-MTX) for 10 min, phase contrast microscopy; B) same ductule as A, examined with fluorescent microscopy; note FL-MTX concentrated in lumen of ductule; C) exposed to 100 μ M MTX [which is NOT fluorescent], then 10 min. with FL-MTX and MTX: shows NO luminal concentration, since the FL-MTX was effectively inhibited; rather, cells have scattered label; D) control, no exposure to FL-MTX or MTX; there is little autofluorescence in this tissue). A second example is shown on APPENDIX PAGE 2 (TOP: bright field micrograph of isolated ductule, exposed to fluorescent methotrexate; BOTTOM: fluorescent micrograph of same tubule, revealing fluorescent methotrexate inside the lumen of most of the ductule length, including the branches).

Two problems were encountered in these studies: 1) there was great variability in the results, with only a few ductules showing this preliminary sign of transport. At this time we do not know whether this reflects naturally occurring differences in expression between animals, differing hormonal states, or regional differences in expression within one animal.

We also tried to localize Pgp with immunocytochemistry.

Immunocytochemistry:

Tissue was embedded in OCT compound and frozen in liquid N_2 . 5 μ m sections were cut with a cryostat, mounted on glass slides, fixed for 10 min in 3.7% formaldehyde in (phosphate-buffered saline) PBS, and washed. Next the sections were blocked for at least 5 min with NGG-Sap-PBS (2-4 mg/ml normal goat globulin; 0.1% saponin; PBS w/o Ca^{2+} or Mg^{2+}) and then washed. The sections were exposed to primary antibody for 25-30 min; the blocking agent was left on control tissue. Tissues were then washed. Next the tissue on both slides was exposed to 2^o antibody for 25-30 min and then washed. 3,3'-diaminobenzidine hydrochloride (DAB) was added to both slides for 5 min (3-5 mg DAB in 10 ml of PBS plus 3 μ l 30% H_2O_2).

Results:

Cross-reactivity in the mouse tissue led to very high backgrounds. We could not see ductule label above this background.

HUMAN STUDIES

The preliminary experiments listed above helped us establish methods to use for human breast reduction tissue. We began experiments on human tissue 3 months ago. Availability is variable from week to week, and only averages about one operation per week.

We quickly learned that it is vital to trim stroma from areas of fat before exposing the tissue to collagenase. Using the conventional fluorescent microscope we have not seen any luminal labelling with Pgp or MOAT substrates. We have looked at one set of tissue exposed to MOAT substrates using the confocal microscope, and have not seen any label in the lumen.

We just have started primary cultures of breast tissue. The tissue is dissected into small pieces with a dissecting microscope mounted in a hood. These pieces are placed on the surface of a tissue culture flask and covered with 2 ml of medium (Dulbecco's Modified Eagle's Medium) with antibiotics. Several days later we will replace this complete medium with serum-free medium (Ham's F-12: DMEM) plus antibiotics, insulin, transferrin, triiodothyronine, hydrocortisol, and epidermal growth factor.

We have also just started culturing ductules isolated with the collagenase procedure detailed above. We have no results to report as yet. We have also just started immunocytochemistry, and in the first few samples have not seen evidence for Pgp staining in breast ductules.

CONCLUSIONS:

In summary, we have established many of the methods we will need to fully explore the phenomenon of bioconcentration in freshly isolated, normal breast ductular epithelium. Since the Pgp has been localized in breast epithelium (Pavelic et al., 1993), it was surprising that we have not seen physiological evidence of transport by this transporter. There are numerous possible explanations for our observations. We have only looked at a few samples and only one in eight samples might show this phenomenon (corresponding to the number of women who have breast cancer); Pgp activity might be under hormonal control, and the patients undergoing breast reduction operations are not in the ideal hormonal state; Pgp might function in only certain regions of the ductules, and we have not sampled those regions; finally, the tissue has been removed from the patient over 24 hours before we can actually test for transporter activity, and the transporter might have been downregulated.

Because of the immunocytochemical evidence for Pgp in normal breast tissue, we will continue to look for its presence. Our major objective will be to attempt to stimulate function with estrogen and progesterone.

On the other hand, it is exciting that we have preliminary

evidence for MOAT, another transporter of toxic molecules. Transport by MOAT may result in concentration of toxic molecules in the ductule lumen, in a manner similar to that of Pgp.

Relation of our first year of work to STATEMENT OF WORK of this grant:

Due to the unpredictable nature of surgical tissue availability and the uncertainty with how long it would take to develop basic methods, we listed the major tasks focused on **surgical tissue** to be completed over 24 months. I believe we are on an excellent time table right now. We have begun Tasks 1-5. Tasks 6 and 7 will be done in the next 6 months. In our original timetable we were going to focus on **cell lines** of breast epithelial cells during the first 12 months. However, we have concentrated on surgical tissue during the first year. We will tackle the cell lines during the second year.

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Figure 1



