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13. ABSTRACT (Maximum 200) The purpose of this project was to develop a reliable, non invasive technique to gain access to the lining of the milk ducts. In a previous IDEA grant (DAMD 17-94-J-4281) we demonstrated the feasibility of an intraductal approach to breast disease, the ability to obtain ductal cells through washings, and the general anatomy of the nipple duct orifices and ductal systems. After studying the microanatomy of the ductal orifices, we proceeded to develop a technique for identifying the breast duct orifices. This involves dekeratinizing the nipple and applying a fluorescing keratin antibody to the ductal epithelium. Once this approach has been demonstrated we will proceed to utilize it to further confirm the ductal orifice anatomy. The second accomplishment has been to demonstrate that we can reliably retrieve cells from a duct cannulated with a double lumen catheter.				
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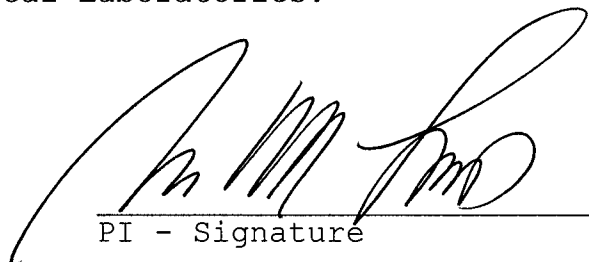
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INTRODUCTION

Subject: The subject of this work is a new approach to the study, detection and treatment of breast cancer.

Purpose: The purpose of this project is to develop a reliable non-invasive technique to gain access to the lining of the milk ducts.

Scope:

Project one: anatomy

1. devising a local approach to highlighting the ducts
 - a. microanatomy of the nipple
 - b. dekeratinization
 - c. florescence
2. confirming the anatomy
 - a. in cadavers
 - 1) casting
 - b. digitally on MRI
 - c. of the nipple duct orifices in the normal woman study
 - d. of the ductal systems in the calcification and mastectomy studies

Project two: cell retrieval

1. use a prototype catheter in a woman about to undergo a mastectomy and demonstrate that we could get cells back
2. determine the optimal way to both retrieve cells and outline duct
3. demonstrate that the whole duct was being washed
4. devise a technique to discriminate premalignant calcifications by washings

Background: One limiting factor for all of breast cancer research and treatment has been our lack of knowledge about the anatomy of the breast and our lack of intermediate markers of breast cancer risk. The main reason we have been unable to identify such markers is that we do not have ready, reproducible non-invasive access to the lining epithelium where breast cancer starts. Breast duct endoscopy and pathological/cytological analysis has the potential to be a means of gaining access to the ductal epithelial cells and diagnosing, treating and studying the precancerous state as well as demonstrating the normal anatomy.

The purpose of our previous IDEA grant (DAMD1794-J-4281) was to demonstrate the feasibility of this approach. We progressed through three stages:

- 1) Demonstrating the feasibility of intraductal endoscopy with a flexible ductoscope;
- 2) Demonstrating the ability to retrieve ductal epithelial cells;
- 3) Describing the normal anatomy of the breast ducts and the nipple orifices.

Initially we attempted breast duct endoscopy in nine patients and were able to cannulate seven. We had difficulty obtaining washings through aspiration from the duct but were able to retrieve fluid from the surface of the nipple through a capillary tube. In

five of the nine cases we obtained epithelial cells in the washings. In one the cells were consistent with proliferative disease. In three there was atypical epithelium and in one there were frank ductal carcinoma cells. There were no complications or untoward events in these first nine patients. In addition to the technical observations we also demonstrated a number of important observations with respect to both the normal ductal system as well as ductal carcinoma *in situ* (DCIS). In the studies where contrast dye was injected, the ductal system appeared to be a non-anastomosing three dimensional network composed of ducts and acini which are difficult to correctly assign to their ductal system of origin in routine two dimensional histological sections. In studies where several different dyes were injected in different ductal systems, DCIS appeared confined to a single ductal system despite the illusion of being present within a number of different ducts on routine sections.

In phase two of the IDEA grant we found that we could identify one half to one third of the ductal orifices (assuming 5-9) and obtain ductal cells from one third of the ducts washed. In three cases we were able to analyze cells for DNA, ploidy, G actin and EGF. We were unsuccessful obtaining cells in washings in 19 patients (51%). Because of either technical difficulties (not being able to dilate the duct sufficiently, not being able to instill saline, not being able to withdraw saline) or acellular return.

In our anatomical studies we found that the median number of lactating duct orifices identified per breast was 5 with a range of 1-12. This corresponds with earlier estimates by Sartorius of 6-12. The pattern of duct orifices and ductal systems is best displayed as a three dimensional cone with ducts extending not radially but toward the chest wall in two concentric circles. The inner group of four ductal systems is fairly constant as is one extending into the upper outer quadrant. The variability comes with the peripheral systems in the upper lower and medial breast.

From this initial work we concluded that:

- Breast duct endoscopy is feasible and cells can be obtained from the ducts for analysis. Although technical difficulties preclude this from being a clinically useful technique at this time, it has yielded important pathological and anatomic observations that are clinically relevant.
- The median number of lactating breast duct orifices per nipple is 5 with a range from 1-12. They form a consistent pattern that is bilateral. The breast ducts form a non-anastomosing three dimensional network composed of ducts and acini which do not correspond to quadrants or radial wedges and are difficult to correctly assign to their duct of origin in routine two dimensional histological sections. Rather they project back towards the chest wall in a pattern of two concentric circles.
- Although DCIS appears to be confined to one ductal system, the anatomic complexity bears directly on our ability to gauge the area that needs to be removed as well as the accuracy of both positive and negative margins. Further confirmation of this anatomical pattern will be important in designing breast surgery for DCIS.

The current contract is an extension of this work.

BODY

Project one:

1. Lighting up the nipple duct orifices

Background: Although our previous analysis of the anatomical patterns and general number of ducts was useful information, it did not help us in an individual case. Knowing that most women have 13 or less ductal orifices doesn't help us when confronted with an individual woman. We still needed a technique which would mark all of the nipple duct orifices in any one woman. We explored several approaches to this problem, including:

- a) consumption of a inert colored material which might color the nipple duct fluid and demonstrate the nipple. Although cows who eat certain materials can color their milk, they are lactating. Non lactating women may not have any fluid and therefore coloration would not be useful.
- b) Applying a colored liquid such as betadine and applying suction to the nipple with the hope that the release of suction would suck the colored liquid into the nipple orifice. This was tried on several cadavers without success, probably because of the keratin plug in the nipple duct orifice noted below
- c) Stimulation of nipple duct fluid with oxytocin, a material used in stimulating breast feeding. One of the investigators tried a nasal spray of oxytocin and noted no increase in breast ductal fluid.

None of these were successful. We decided to approach the nipple directly.

Methods and materials:

- a) The careful analysis of the histological nipple duct orifice to determine its microanatomy

In our effort to fully describe the anatomy of the nipple duct orifice we also studied the microanatomy. One nipple areolar complex was removed from a cadaver and fixed. Over a thousand oriented slices were made and stained. On one slide we were able to identify a ductal system extending directly to the nipple duct orifice. The orifice was found to have a keratin plug. There was no transitional epithelial as demonstrated by a low molecular weight keratin antibody which stains ductal epithelial cells and not squamous cells.

Our ability to selectively stain the ductal glandular epithelium led us to hypothesize that we could selectively stain the ductal epithelium and then treat it with a fluorescent antibody which would be visible. This would achieve our task of identifying the nipple duct orifices. We developed this technique in a step wise fashion employing frozen pig nipples (a readily available hairless animal model).

- b) Removal of keratin plugs

The first step was to see if the keratin plugs filling the duct orifices would obscure the florescence. A series of experiments demonstrated that, indeed the keratin was a block to seeing fluorescence. We therefore set out to develop a dekeratinization

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technique. After trial and error we settled on the following dekeratinization cream recipe:

1.8 g of *velvachol*

0.2 g of *methylcellulose*

3 ml of *glacial acetic acid*

(order of addition of recipe components has proven to be important:

velvachol, methylcellulose, then glacial acetic acid

makes a 60% solution of glacial acetic acid cream)

Velvachol is a water miscible pharmaceutical vehicle. Velvachol is hydrophilic and compatible with both acids and alkaline solutions. It is composed of water, petrolatum, mineral oil, cetyl alcohol, sodium lauryl sulfate, cholesterol, methylparaben, butylparaben, and propylparaben.

Methylcellulose is a pharmaceutical suspending agent. Methylcellulose is a cellulose methyl ether which is stable in both alkaline and acidic suspensions and is soluble in glacial acetic acid.

Glacial acetic acid is commonly used as a wet dressing and is also used as a keratolytic agent.

Dekeratinization Procedure:

The cream is evenly distributed and applied to the surface of the nipple. After a 24 hour period the cream is removed by washing with a saline solution. When all the cream has been removed, using a pair of tweezers the outermost layer of skin is peeled off. The surface is then brushed (gently) to remove any remaining tissue. This procedure induces sloughing of the outermost layer of the epidermis and keratolysis. Histological analysis of dekeratinized nipples has shown the removal of the keratin layer without damage to the epithelial tissue.

c) Fluorescent procedure

Having solved the first problem, we then set out to devise an appropriate technique for fluorescing the ducts. The current technique we are exploring is:

Fluorescent Procedure:

Following dekeratinization the nipple(s) are subjected to a fluorescent probe analysis in order to visualize the ductal openings. The nipple is soaked in a saline solution containing the primary antibody, mouse anti-human epithelial membrane antigen (EMA) at room temperature for 4 hours. Afterwards, the nipple(s) are washed (4x) for 15 minutes with saline at room temperature. The nipple(s) are then soaked in a saline solution containing the secondary antibody, goat anti-mouse IgG (H and L chains) FITC conjugate. Following the addition of the secondary antibody the nipple(s) are washed thoroughly (4x for 15 minutes) with saline at room temperature.

Fluorescent Procedure Defined:

Mouse Anti-human Epithelial membrane(EMA) is a monoclonal antibody which

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reacts preferentially with glandular epithelium upon histological analysis.

Goat Anti-mouse IgG is a polyclonal antibody which reacts with both the heavy and light chain of mouse IgG.

Visualization of the Ductal openings:

Fluorescein Isothiocyanate (FITC) is a molecular probe which has an excitation peak at 494.2nm and a peak of emission at 514.0nm. To visualize the FITC probe, the following system is used: xenon lamp (150W) with a blue light filter (488nm), argon laser (protective) goggles (514nm).

Results: This fluorescent procedure has only resulted in diffuse fluorescence to date. Histological examination demonstrated this to be due to diffuse nonspecific binding. The ductal epithelium did bind the fluorescent antibody, however. We are working on a blocking approach to attenuate this problem. In summary, we have made some progress in developing a technique to identify the ductal orifices. We believe this approach will be successful with further work.

Project 1: 2. Confirming the anatomy

a) cadaveric studies

Background: Although the analysis of archival single ductograms has been invaluable in determining the pattern of the ducts, it has not been able to show us the interaction between ductal systems. For this aspect we have been employed cadaveric breasts. We are trying to confirm the data derived from Phase IB with sequential multi-duct contrast ductograms of fresh cadaveric mastectomy specimens

Materials and methods: Cadavers have been purchased through the UCLA Willd Body Program. Bilateral radical mastectomies are performed on the freshly thawed cadavers. The breast specimen includes the overlying skin (to sternum, clavicle, rectus fascia and latissimus dorsi muscle) and pectoralis muscles. Sutures are used to orient the specimen. The breast specimens are be refrozen and stored at -10 degrees C until several specimens have been accumulated. The breast specimens will be partially thawed before use. Sequential ductograms on the specimen will be obtained after harvest as follows. Duct orifices will be identified with the aid of a dissecting microscope with a 20x-80x magnification. All identified ducts will be cannulated with a commercial galactography kit (Taber-Rothschild Galactography Kit #M030895-13, Manan Medical Products Inc, Northbrook IL) or a .014" guide wire (Boston Scientific). A double lumen catheter is then threaded over the guide wire into the duct. After fixation of all of the catheters into place with a suture, each catheter will be sequentially injected with 0.20-0.50 cc of renograffin contrast followed by radiographs taken in the AP view. The duct will then be washed out with saline. This sequence will be repeated for each duct which was cannulated.

Results: In our series of cadavers, our ability to blindly catheterize ductal orifices was variable demonstrating further the importance of developing a technique to identify the ductal orifices. In some cases we had catheterized a sebaceous cyst rather than a duct. In other cases there was extravasation of renograffin . It was not clear whether this was a

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consequence of using cadaver breasts or whether they had been ruptured in the process of doing the cannulation. We were able to conclude that the ductal systems are separate. We also noted occasional cysts in continuity with the ductal systems (a finding previously described by Sartorius).

Casting

Materials and methods: Following the imaging studies, we tried leaving the cannulas will be left in place for corrosion casting. A methylnmethacrylate resin (Batson's No. 17 Plastic Replica and Corrosion Kit, Polysciences, Warrington PA) was used for casting of the ductal system. The solution consists of a base, a catalyst and a promoter with an additional methylnmethacrylate monomer added to reduce viscosity as necessary. The volume of contrast needed to fill the duct was estimated from the volume of contrast that was needed to fill the duct as seen on the ductogram, approximately 0.2-0.5cc. Each duct was injected with a resin containing a different coloring agent (color pigment, Batson's anatomical corrosion Kit, Polysciences). After injection the breast was be allowed to polymerize overnight at 25 degrees. The tissue surrounding the ducts will be corroded with 30% potassium hydroxide over several months at 50 degrees. Any remaining fibrous tissue was carefully dissected away.

Results: This procedure has not as of yet been successful. It takes several months to corrode the surrounding tissue with potassium hydroxide. In our initial efforts, part of one duct was casted with evidence of extravasation distally. A second attempt was completely unsuccessful. We are now waiting for the perfect breast (several ducts catheterized and documented with flouroscopy) before making another attempt.

Project 2: cannulation of the duct orifices

Background: In our IDEA grant we devised a double lumen catheter which would allow a continuous flow of saline through out the ductal system. The prototype catheter was a 3 French double lumen catheter. The proximal lumen is smaller in diameter and is used for instilling saline; the distal lumen is larger and is used for aspiration. We initially studied detached breasts, or breasts which had just been removed surgically but not yet sent to pathology. We assumed that these fresh breasts would still have intraductal cells which could be retrieved through washings. We had determined that cells could be retrieved and preserved in 75% of the 8 breasts we had studied. We have studied two additional detached breasts with good cell retrieval bringing our percentage to 80%.

Materials and methods: The next phase was to use one of these prototype catheters in "attached breasts" prior to mastectomy to confirm that we can get viable cells through the distal lumen of the catheter. After general anesthesia had been obtained the patient's breast was prepped and draped. Mild suction was applied to the nipple to try and elicit discharge. A dissecting microscope or loupes were used to magnify the nipple. A map was made of the identified orifices. Starting with the most promising orifice (i.e., most amount of discharge, largest) we attempted to cannulate it using either a standard set of metal dilators (galactography set by Mahan), a very small glide wire (type used in angiography). Once the duct had been cannulated and dilated to approximately 0.7-1.0

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cm, the double lumen catheter was threaded into the duct. Saline was instilled setting up a continuous flow until 10cc have been collected. This procedure takes approximately 15 minutes. If we were unable to complete the procedure within the 15-minute limit, we stopped prematurely. The washings were then sent to cytology for analysis.

Results: We have studied six breasts. The first one gave good cell retrieval consistent with carcinoma. The next three samples were sent to San Francisco for analysis and were not well preserved. Nonetheless there were cells obtained. The final case is still pending. In this mastectomy series we are still working on the optimal technique for cell preservation.

CONCLUSIONS

This year's effort has moved us ahead toward the development of an intraductal approach to breast cancer.

1. We are close to having a reliable technique for identifying the ductal orifices using an antibody and fluorescence technique.
2. We have demonstrated our ability to reliably catheterize the ducts and retrieve cells.

Both of these findings are important in the development of an intraductal approach to the breast. We are optimistic that we will have the duct identification technique worked out by the first of 1998. We will then be able to proceed with the next steps confirming the anatomy of the ductal systems and exploring the utility of this new technique.

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1. Love SM, Barsky SH. Breast-duct endoscopy to study stages of cancerous breast disease. *The Lancet* Oct 12, 1996 Vol 348 No 9033 pages 997-999.
2. Love SM, Barsky S, Chou J, Offodile R, Rao JY, Hirschowitz S, Schmit P, Grosser S. Demonstration of ductal anatomy and the feasibility of an intraductal approach to the breast. Abstract presented at Department of Defense Breast Cancer Research Program 'Era of Hope,' Washington DC, Oct 31-Nov 4, 1997.

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Appendix: Table 1

"Detached breasts" (3000) series and "attached breasts" (4000) series

<u>patient #</u>	<u>breast</u>	<u>mass location</u>	<u>duct location</u>	<u># duct</u>	<u>pathology</u>	<u>cytology</u>
Detached breasts						
3000	R	RLQ	central	1	Ductal ca comedo	acellular
3001 a	R	No ca		6:00 3		ductal cells
3001 b	L	L breast 7:00		9:00 3	Micropapillary DCIS	ductal cells
3002	L	LLQ		12:00 3	infiltrating ductal ca	ductal cells
3003	R	RUOQ		8:00 4	DCIS pagetoid	ductal cells
3004	L	LUOQ		11:00 5	Intraductal ca in situ	ductal cells
3005 a	R	no ca		9:00 2		acellular
3005 b	L	3:00,9:00		6:00 4	Invasive ductal ca, DCIS	acellular
3006	L	LUOQ		6:00 4	Infiltrating ductal ca, DCIS	(1) acellular, (2) ductal cells
3007	L	LOQ,3:00	central	2	infiltrating lobular ca, infiltrating ductal	ductal cells
Attached breasts						
4001	R	RUOQ		12:00 2	adeno ca	carcinoma cells
4002	L	retroaerolar	central	1	poorly diff. Ductal ca	deteriorated cells
4003	R	RUOQ		3:00 3	tubular ca, micropapillary	deteriorated cells
4004	L	infraaerolar		9:00 2	invasive ductal ca	deteriorated cells
4005	R	N/A (abcess)	central	1	lactiferous duct	pending



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