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Award Number: DAMD17-99-1-9399

TITLE: Predicting Response to Adjuvant Therapy with Tamoxifen

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REPORT DATE: September 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2000	Annual (1 Sep 99 - 31 Aug 00)

4. TITLE AND SUBTITLE
Predicting Response to Adjuvant Therapy with Tamoxifen

5. FUNDING NUMBERS
DAMD17-99-1-9399

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
This report contains colored photos

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***
It is our hypothesis that multiple patterns of cDNA expression characterize the acquired Tam-resistant phenotype, and that these expression profiles can be ascertained using cDNA microarrays. We propose three technical objectives to identify mechanisms and/or the specific gene profiles contributing to acquired Tam resistance. In Aim 1 we will identify Tam resistance expression profiles using models of acquired resistance. This will be accomplished using two models of resistance and commercially available expression arrays. Representative gene candidates will be confirmed for relative expression using Western blot and RT-PCR analyses. In Aim 2 we will profile resistance patterns in clinical breast cancer samples. We will utilize Affymetrix arrays to profile tamoxifen sensitive and tamoxifen resistant tumors from patients. In Aim 3 we will evaluate specific candidate genes for their involvement in the genesis and progression of Tam resistance. We will evaluate the hormone-responsive phenotype of candidate genes *in vitro* utilizing stable transfection of full-length and antisense cDNAs. Their growth potential, invasive, and metastatic properties will also be examined *in vitro*.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	11
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Main Report

Introduction

Tamoxifen (Tam) is the most frequently prescribed drug for the treatment of breast cancer. But while Tam is initially effective in many breast cancer patients, tamoxifen resistance eventually develops. It is unlikely that one single mechanism can explain Tam resistance in all of these patients because some patients exhibit complete, while some display partial endocrine resistance to second-line therapies. It is our hypothesis that multiple patterns of cDNA expression characterize the acquired Tam-resistant phenotype, and that these expression profiles can be ascertained using cDNA microarrays. It is our eventual goal to build a custom microarray containing genes that are associated with Tam resistance identified from models of acquired resistance to then use for predicting patient response.

Body

Technical Objectives. We originally proposed three technical objectives to accomplish our long-term goal of developing a custom array that can be used clinically to predict tamoxifen response in patients. In **Aim 1** we proposed to identify Tam resistance expression profiles using three models of acquired resistance (a xenograph model, a cell line model, and an ER variant-transfected cell line model). This aim will be accomplished with commercially available cDNA expression microarrays. Representative gene candidates will be confirmed for relative expression in the various models using Western blot and RT-PCR analyses. In **Aim 2** we proposed to assemble a Tam resistance microarray, and utilize this custom array for profiling resistance patterns in clinical breast cancer samples. We proposed to utilize the cDNAs identified in Aim 1 to print a preliminary custom array, and then examine two separate clinical studies for expression profiles associated with the Tam-resistant phenotype using this custom array. Our ultimate clinical question is whether the microarray will be able to identify ER-positive patients with recurrent, Tam-resistant disease who would benefit from different therapies. In **Aim 3** we will evaluate specific candidate genes for their involvement in the genesis and progression of Tam resistance. We will evaluate the hormone-responsive phenotype of candidate genes *in vitro* utilizing stable transfection of full-length and antisense cDNAs. Their growth potential, invasive, and metastatic properties will also be examined *in vitro*.

Progress. We have made substantial progress on the project in Year 1. We have also made several strategic decisions to modify our approach to increase our likelihood of success to complete both our technical objectives and our long-term goal. Our Year 1 progress and our new experimental strategies are described below:

Aim 1. Our first strategy was to utilize Clontech filter arrays, and this proved to be an informative first approach. We compared the Tam-sensitive (TS) and Tam-resistant (TR) MCF-7 xenograph tumors from our model of acquired resistance, and developed a statistical method for analysis of the data (1). From this screen we discovered that there were two profiles of expression that were associated with Tam resistance, eg. 7 genes were found to be downregulated by Tam, and 19 genes were upregulated with Tam treatment and significantly increased in the TR tumors. Importantly, 7 of the genes in these two profiled groups were genes involved in tumor angiogenesis. The angiogensis genes included: vascular endothelial growth factor (VEGF), VEGF receptor 1 (flt-1), beta fibroblast growth factor receptor (bFGFR),

transforming growth factor III, TGF receptor III (TGFR III), endothelin receptor, and Tie-2. This finding was exciting because it is known that breast cancer is an angiogenesis-dependent tumor. We have thus far focused our efforts on two of these gene candidates, VEGF and its receptor flt-1, for a number of reasons. First, it has been shown that VEGF can be produced by breast tumors and its overexpression is prognostic of a poor outcome (2). Secondly, VEGF is known to be one of the most potent angiogenic factors produced by tumors, and thus was an excellent candidate. And third, a number of groups have targeted the VEGF receptors to effect tumor angiogenesis and growth, thus we thought that targeting this pathway would be a viable approach for the biological characterization of candidates in **Aim 3** of this proposal.

Our array results predicted that VEGF RNA would be decreased with Tam. Antibodies to VEGF were commercially available and we next used these for Western blot analysis of MCF-7 xenograph tumors to confirm the relative levels of VEGF in the tumor groups from our xenograph model. It is known that VEGF is both transcriptionally and translationally regulated, as well as having several different protein isoforms (3). Thus, VEGF levels are under complex regulation. As shown in Figure 1 (included in the Appendices), our data suggests that Tam resistance is accompanied by a shift in expression of VEGF isoforms in the TR tumors. As a positive control, we show that hypoxia induced by cobalt chloride results in a similar shift of expression from the higher VEGF forms to VEGF 121 (panel A, MCF-7 cells). In tumors, we found this same shift, resulting in higher VEGF121 levels in the TR tumors (panel B). These results suggest a preferential role for the VEGF121 isoform in our model of Tam-resistant growth. We thus hypothesize that angiogenesis, and specifically the VEGF pathway, may be critical for the development of Tam resistance, and we will directly test this hypothesis in **Aim 3**.

We next wanted to confirm that the VEGF receptor flt-1 was indeed upregulated in the TR tumors as predicted by the RNA array profiling experiments. We would like to suggest that a coordinate upregulation of the receptor in the vasculature surrounding or within the tumor for VEGF could greatly complement the increase in VEGF121 from the tumors and its resultant effect. Confirmation of flt-1 has been more of a challenge though, since commercially available antibodies, and antibodies that we have obtained from other investigators do not work well in Western blot analysis (data not shown). Thus we have developed *in situ* hybridization technologies in our laboratory for candidates that we identify from the arrays for whom there aren't available antibodies for use. To this aim, we obtained expression constructs for flt-1 and flk-1 (a second related VEGF receptor), and are using these for *in situ* analysis of tumors from our xenograph model. These experiments are underway, but we have been able to obtain good localization of signal to the vascular endothelium of tumors using our flt-1 and flk-1 probes (data not shown). These experiments will continue in the next granting period, and we will similarly develop *in situ* probes for the other candidates that we identified from this first round of array profiling experiments.

We are also using the CD31 antibody to visualize the vasculature and determine vascular density measurements for comparison to the signal obtained from the flt-1 and flk-1 hybridizations. Preliminary immunostaining with the CD31 antibody is shown in Figure 2 (included in the Appendices). Panel A illustrates a "hot spot" of staining in the peripheral edge of a xenograph tumor. Panel B shows vascular staining within a tumor, and Panel C shows staining of the normal endothelium in mammary gland of the mouse for comparison. We have also recently prepared a tissue array of 5 tumors each of TS and TR, and we will be obtaining quantitative microvessel density measurements in the xenograph tumors.

In addition, in a collaborative effort with Phil Thorpe, who is an expert in vascular targeting, we have obtained an antibody (designated T014) prepared to flk-1 that also demonstrates cross-reactivity to flt-1 (4). IHC with the T014 antibody in a TR tumor is shown in Figure 3 (included in the Appendices). The panels on the left demonstrate staining along the periphery of the tumor, and the right upper panel shows staining within the inside of a tumor. For comparison, normal mammary tissue staining is shown in the lower right hand panel. These results demonstrate that we can measure total levels of VEGFR in our model. We will next be comparing the levels of the receptors in the xenograph model to determine if flt-1, as measured by *in situ* hybridization and IHC, is indeed upregulated coordinately with Tam resistance. Also available are antibodies to activated VEGFR; these antibodies only recognize VEGF when bound to receptor (4). These unique reagents will also be used to further evaluate the VEGF pathway as candidates in our xenograph model.

We have also used Clontech arrays to evaluate the expression profile differences between the LCC1 (TS) and LCC2 (TR) cell lines (5) as originally proposed in our IDEA grant. (This was the second model of resistance that we proposed). We found that there were 32 genes upregulated and 8 genes downregulated in the TR LCC2 cell line. We decided our strategy for data exploration would be to first examine those genes altered in expression in common with our first model of acquired resistance, eg. the xenograph tumors. There were two upregulated genes in common between the two models (clusterin and acyl-CoA-binding protein), as well as two downregulated genes in common (TGFR III and CCAAT displacement protein). We have obtained antibodies to clusterin and are presently examining its level by Western blot analysis and IHC. We propose that clusterin is a good candidate based upon reports that it can be regulated by Tam (5) and that it is possesses antiapoptotic functions (6). If its level is indeed altered in the TR tumors, then we will add it to our list of candidates to evaluate in **Aim 3.**

We now feel that we have mined the Clontech cDNA arrays to our best advantage, but we also feel that the technology has significantly "moved on" past the less dense Clontech array format. Thus, we have recently purchased an Affymetrix GeneChip system using funding from Baylor and our Breast Cancer SPORE. This was a large investment and commitment on our part to the Affymetrix technology, but we believe that it will greatly increase our ability to obtain useful expression profiling from large datasets such as that proposed in this IDEA award. We will utilize the Affymetrix Human Gene 95A slide arrays containing 12,000 known genes and we will profile both the xenograph Tam resistance model, and the LCC1/2 cell line models in Year 2 of this award. We have decided that we will not pursue a third model of resistance (the exon 5 ER variant transfectants) with Affymetrix arrays, mainly because of their high cost (the arrays are $1000 each), and because of our increasing concern that overexpresison of this ER variant is not critically important for clinical resistance.

Aim 2. We originally proposed to prepare a custom array of gene candidates obtained in Aim 1, isolate RNA from two clinical sets of tumors (total number =1235), and then do array profiling of the tumors. However, we now have learned that there are two major obstacles to this approach. First, we are not able to obtain sufficient RNA from archival samples for adequate array analysis. Hopefully, amplification strategies will be developed in the near future to overcome this barrier, but at present this approach is just not feasible. A second major barrier is the prohibitive cost of manufacturing custom arrays. Therefore we have elected to profile 10 TR and 10 TS patient samples on the Affymetrix arrays, to obtain candidate markers of resistance as

described in Aim 1. We have already identified tumor samples where we have sufficient frozen tumor material to isolate RNA, and we will begin profiling of these RNAs in Year 2 of the proposal. Candidate genes will be compared to those obtained in the two resistance models, and functional characterization of selected candidates will be examined in **Aim 3**.

Aim 3. We will begin studies examining the functional consequences of sense overexpression of specific candidates, such as VEGF121 starting in Year 2. We have obtained an expression vector for VEGF121 from Phil Thorpe and will stably overexpress it into MCF-7 cells. These transfectants will be characterized as originally proposed to determine if overexpression alters hormone responsiveness to Tam. What is apparent though from our Tam resistance array candidates is that stromal components, such as flt-1 expressed in the vasculature, may also play a very important role in acquired resistance. Thus, overexpression of candidates expressed in the stroma into MCF-7 cells is not an experimental option, and since it has also been demonstrated that inhibition of VEGF-induced angiogenesis can significantly impact tumor growth (8), we also propose to utilize different therapeutic agents, such as antibodies to candidate genes, or small molecule inhibitors of specific endothelial receptors, in our transfectants to formally test whether inhibition of potential stromal/paracrine pathways can reverse or delay the development of resistance.

Key Research Accomplishments

- Discovered that signaling pathways important for angiogenesis may be involved in the acquisition of Tam resistance.
- Identified that the VEGF121 isoform is increased in Tam-resistant tumors.
- Identified 4 candidate genes to explore as a common mechanism between two different models of Tam resistance

Reportable Outcomes

- Manuscript in preparation for Cancer Research: Schiff R, Osborne KO, Thorpe P., and Fuqua, SAW. The VEGF angiogenic pathway is altered in tamoxifen-resistant MCF-7 tumors.
- Grant submitted June, 2000 to the DOD Breast Cancer program by my collaborator on this project, R. Schiff, entitled "The Role of the VEGF Angiogenic Pathway in Tamoxifen Resistance of Breast Tumors" to examine the use of antiangiogenic therapies in the MCF-7 xenograph model.

Conclusions

We have utilized two models of tamoxifen resistance for expression profiling. From the first year of study we have identified that angiogenesis may be important for the development of resistance. This finding is of importance because the contribution of the stromal compartment to resistance is novel, was not anticipated, nor has it been previously evaluated. We hypothesize that the tumor may exhibit alterred expression of the VEGF 121 isoform; coordinte upregulation of the VEGFR flt-1 would also accompany the angiogenic process. These are testable hypotheses that will be addressed in the next grant period. In addition, with the acquisition of the Affymetrix technology, we can greatly increase the number of genes that we can profile, using both our models of resistance and tumor samples from patients with tamoxifen resistance. We anticipate that we will identify key intermediaries of antiestrogen resistance with our continued studies.

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Appendices

Fig. 1. VEGF Expression in MCF-7 Cells and Xenograph Tumors. Top Panel: Western blot analysis of MCF-7 cells grown in either fetabl bovine serum (FBS)or serum free (SF). VEGF expression was induced with cobalt chloride for 6 and 24 hours. The four VEGF isoforms are indicated with small arrows, and VEGF121 is indicated with a large arrow. Bottome Panel: Four tumors from estrogen stimulated tumors (ES), tamoxifen sensitive (TS) after 2 weeks of treatment, TS after 2 months of treatment, and tamoxifen resistance (TR). An extract of MCF cells is included in the first lane as a positive control.

Fig. 2. Endothelium staining in MCF-7 Xenograph Tumors. Immunostaining of a xenograph tumor (left panels) and normal adjacent tissue (N) with the endothelium specific antibody CD31. Staining along the periphery of the tumor is shown in the top left panel, and staining within the interior of the tumor is shown in the bottom left panel. The normal tissue staining is shown in the panel on the right. CD31 staining is demarcated with arrows.

Fig. 3. VEGF Receptor Expression in MCF-7 Xenograph Tumors. Immunostaining of xenograph tumors and normal adjacent tissue with the T014 antibody which recognizes both VEGF receptors flt-1 and flk-1. Staining along the periphery is shown in the panel on the left, staining in the interior of the tumor is shown in the top right panel, and normal staining is shown in the bottom right panel. T014 staining is demarcated with arrows

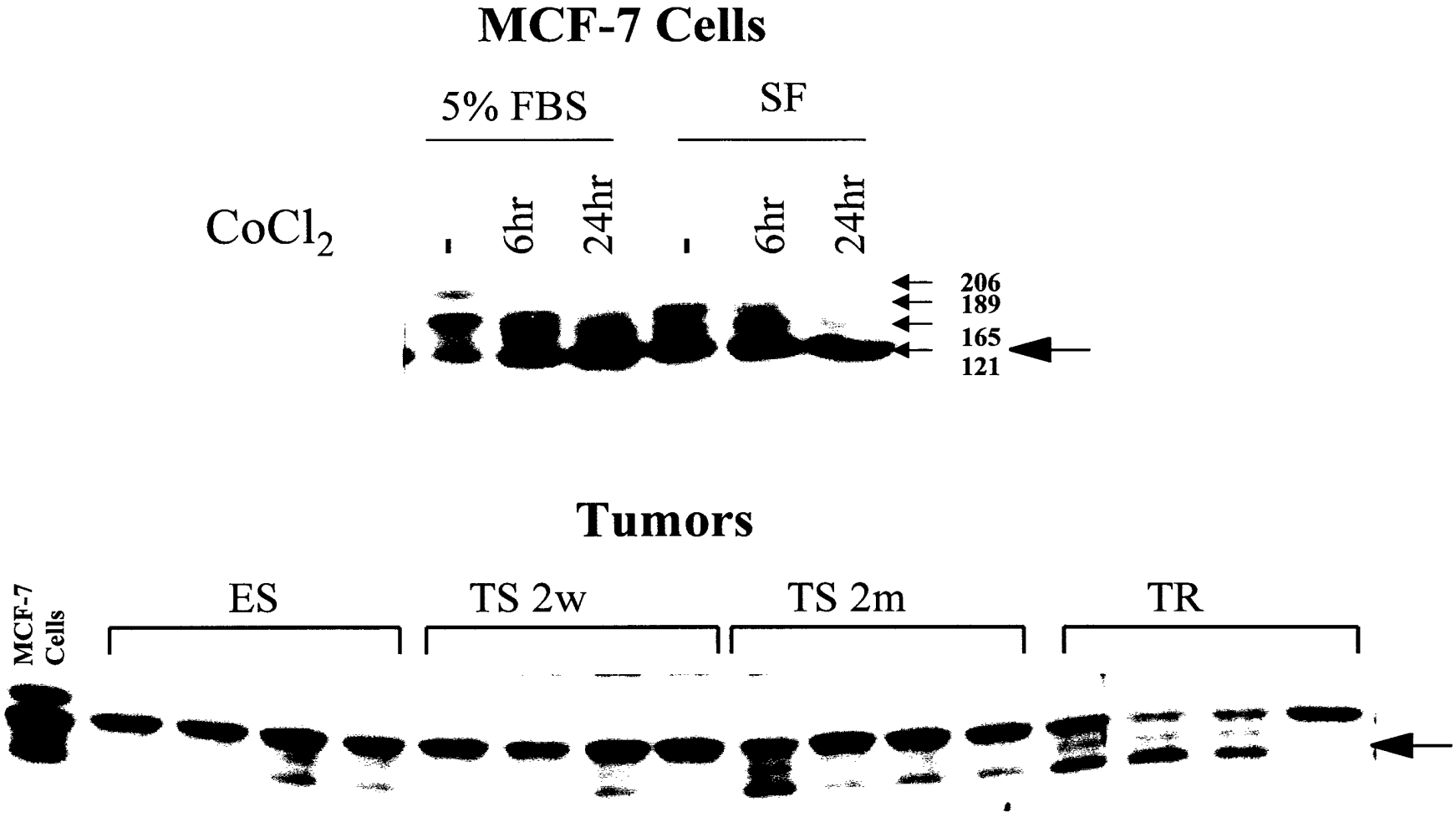
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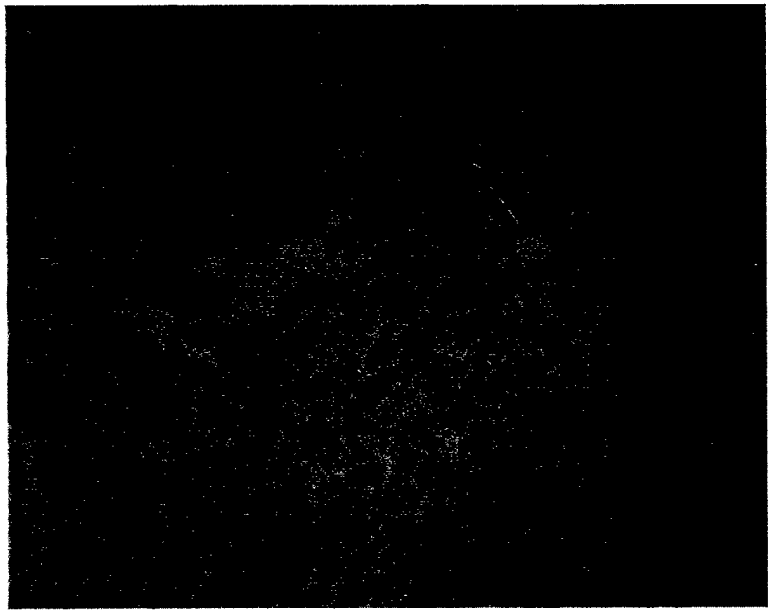
Fig. 1



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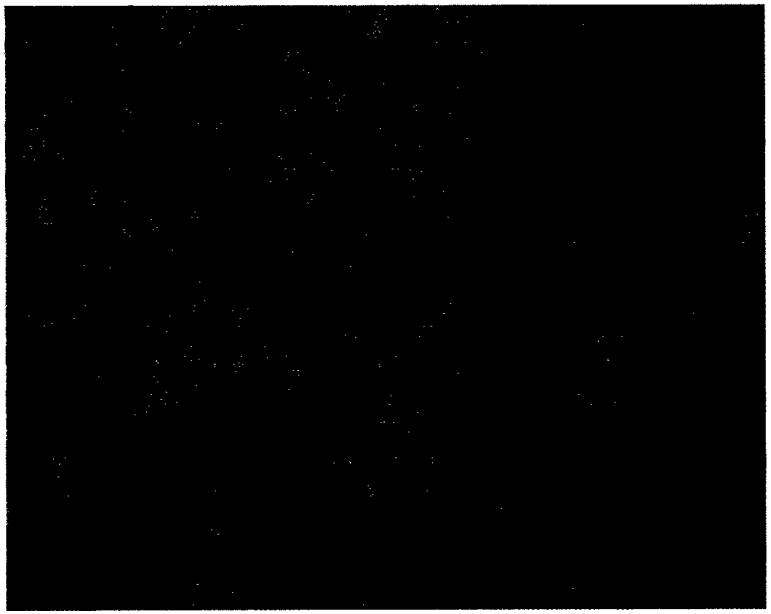

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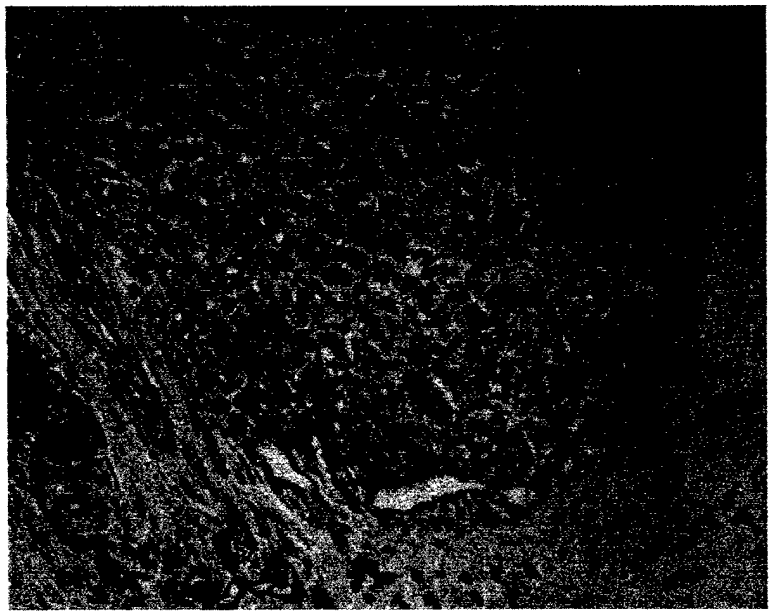
Fig 2



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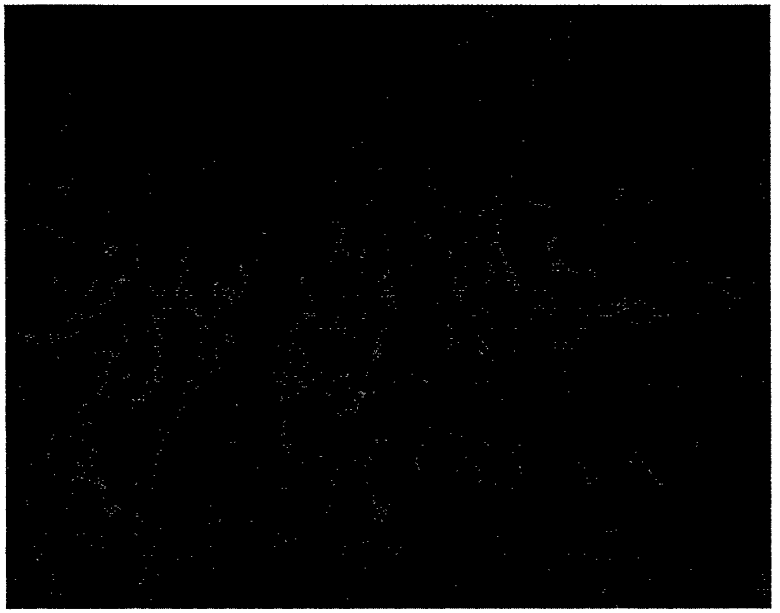

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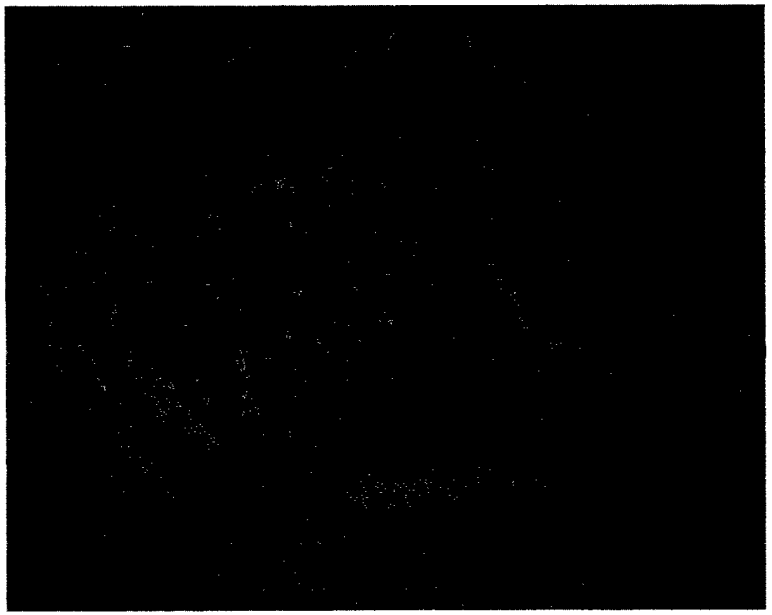

T periphery (X 400)

N (X 400)

Fig 3