AD____________________

Award Number: DAMD17-00-1-0278

TITLE: Involvement of Heparanase in Breast Carcinoma Progression

PRINCIPAL INVESTIGATOR: Israel Vlodavsky, Ph.D.

CONTRACTING ORGANIZATION: Hadassah Medical Organization
Jerusalem, Israel 91120

REPORT DATE: June 2001

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2001	Annual (1 Jun 00 - 31 May 01)

4. TITLE AND SUBTITLE
Involvement of Heparanase in Breast Carcinoma Progression

5. FUNDING NUMBERS
DAMD17-00-1-0278

6. AUTHOR(S)
Israel Vlodavsky, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Hadassah Medical Organization
Jerusalem, Israel 91120

E-Mail: Vlodavsk@cc.huji.ac.il

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

20010926 133

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)*

Observations obtained during the first year of research focusing on the three main tasks of the original application support the proposed involvement of heparanase (heparan sulfate degrading endoglycosidase) in breast carcinoma progression. A straight forward indication is the accelerated growth of tumors produced by MCF-7 cells over-expressing the heparanase gene and enzyme, versus tumors produced by control mock-transfected MCF-7 breast cancer cells. Mammary glands of transgenic mice over-expressing the heparanase enzyme exhibited precocious branching of ducts and early signs of hyperplasia and basement membrane disruption. We have cloned the heparanase promoter region fused to a luciferase reporter gene and demonstrated a significant stimulation by estrogen of both heparanase promoter activity and gene expression. Deletion experiments identified regions in the heparanase promoter necessary for its activity in transfected MDA-435 cells. Experimental approaches for achieving the other objectives of the proposed research were developed. Among these is a system to purify and characterize a proteolytic activity secreted by highly metastatic breast carcinoma cells, involved in processing and activation of the 65 kDa latent heparanase. Potent heparanase-inhibiting fragments derived from laminaran sulfate were prepared. The biochemical nature of these oligosaccharids and their efficacy in experimental models of breast carcinoma are being evaluated.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Heparanase; Heparan sulfate; Metastasis; Angiogenesis; Gene expression	18
	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

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

Cover..........1

SF 298..........2

Table of Contents..........3

Introduction..........4

Body..........4

Key Research Accomplishments..........10

Reportable Outcomes..........11

Conclusions..........11

References..........13

Appendices..........14

Introduction

Metastases formation depends on the ability of tumor cells to invade basement membranes (BM) and tissue barriers in a process involving enzymes capable of degrading extracellular matrix (ECM) components. While the majority of studies focus on proteolytic enzymes and their inhibitors, the involvement of glycosaminoglycan (GAG) degrading enzymes (e.g., heparanase) was underestimated. The overall goal of the proposed research is to study the involvement of heparanase (heparan sulfate degrading endoglycosidase) in breast carcinoma metastasis and angiogenesis. For this purpose, we proposed to determine the effect of increased heparanase expression on breast carcinoma progression, applying both stable and inducible overexpression of the heparanase cDNA in non-metastatic human and mouse breast cancer cells (task 1). An increased expression of the enzyme was also achieved *in vivo* by producing transgenic mice overexpressing the heparanase RNA and protein in all tissues and analyzing the resulting effect on morphogenesis, growth and transformation of mammary epithelial cells. In an attempt to better elucidate the regulation of heparanase gene expression, we proposed to construct a vector composed of the heparanase promoter fused to a luciferase reporter gene and study the effect of estrogenes and anti-estrogens on its expression in transfected breast carcinoma cells (task 2). A related aspect was to study the processing and activation of latent heparanase by highly metastatic breast carcinoma cells, toward characterization and purification of a putative heparanase converting protease (task 1b). Finally, we proposed to prepare and characterize heparanase-inhibiting molecules, primarily define species of laminaran sulfate, and evaluate their effect on heparanase activity *in vitro* and breast carcinoma metastasis and angiogenesis *in vivo* (task 3).

Body

*As recommended by the peer review panel, specific aim #1 (immunostaining, *in situ* hybridization, ELISA) of the original application was deleted. The task # and time-table were corrected accordingly.

Task 1: Involvement of heparanase in breast cancer metastasis and angiogenesis

Processing and activation of latent heparanase: The heparanase gene encodes a latent 65 kDa proenzyme that is processed at the N-terminus into a highly active 50 kDa enzyme. The proteolytic cleavage of the 65 kDa proenzyme is likely to occur in two potential cleavage sites, Glu_{109}-Ser_{110} and Gln^{157}-Lys^{159}, yielding an 8 kDa polypeptide at the N-terminus, a 50 kDa polypeptide at the C-terminus and a linker polypeptide in between them (1). This proteolytic cleavage is crucial for obtaining functional heparanase. Several human breast carcinoma cell lines (MCF-7; MDA-235; MDA-431) were tested for their ability to process and activate the heparanase enzyme. Best results were obtained with the highly metastatic MDA-435 cells, using cell lysates and, to a lesser extent, intact cells. Serum free medium conditioned by these cells was identified as a suitable source for characterization and purification of the putative protease, converting the full-length 65 kDa latent heparanase into an active 50 kDa form. First, we established an appropriate assay system to measure the conversion of inactive 65 kDa recombinant heparanase into a 50 kDa active enzyme, applying Western blot analysis and heparanase activity assay (Fig. 1).

Briefly, serum free medium conditioned (48 h) by MDA-435 cells was collected and incubated (1 h, 37°C) with recombinant 65 kDa pro-heparanase. The reaction mixture was then subjected to10% SDS/PAGE and Western blot analysis using anti-heparanase antibodies (mAb130) directed against the N-terminus of the 50 kDa active enzyme; antibodies (anti-hpa3) directed against a synthetic peptide of 20 amino acids in an internal region of the 50 kDa heparanase; or antibodies (mAb 239) directed against the linker region and hence recognizing only the pro-enzyme. As demonstrated in figure 1 (lanes 5-10), the 65 kDa heparanase is readily converted into a 50 kDa form during incubation (1 h) with medium conditioned by MDA-435 cells. This incubation resulted in a gain of heparanase activity (Fig. 1D). The same immunoblot pattern was obtained with mAb130 (Fig. 1A) and the anti-hpa 3 (Fig. 1B) antibodies, recognizing both the latent and active forms of the enzyme. In contrast, only the unprocessed enzyme was detected by mAb 239 (Fig. 1C), again showing an almost complete conversion of the 65 kDa enzyme into a 50 kDa form. In preliminary experiments, proteolytic cleavage of the 65 kDa pro-heparanase was observed in the absence and presence of a cocktail of protease inhibitors (lane 7), DCI, a serine protease inhibitor (lane 8), pepstatin, an aspartic protease inhibitor (lane 9), and phenantroline, a metalloprotease inhibitor (lane 10). Other protease inhibitors directed against known classes of proteases (i.e., serine- and cysteine-proteases; matrix metalloproteinases) are being tested for their ability to inhibit conversion of the 65 kDa heparanase into a 50 kDa active enzyme. We have synthesized peptides corresponding to the potential cleavage region in order to study the cleavage reaction in a competition assay. We also plan to introduce point mutations in the cleavage site (gln^{157}-lys^{158}) and analyze the susceptibility of the modified protein to cleavage by the MDA-435 cell conditioned medium. In related experiments, we found that preincubation of low metastatic B16-melaoma cells with the 65 kDa heparanase is associated with cellular binding, processing and activation of the enzyme. Following binding, the cells acquired a high heparanase activity, suggesting that heparanase cleavage is a cell surface event. Heparanase-treated cells were then washed free of unbound enzyme and injected to C57BL/6 mice. Three weeks later lung colonization was evaluated and compared to mice injected with control cells. In three independent experiments, heparanase treated cells showed a significantly higher number (6-30 fold) of lung metastases, apparently due to an accelerated cell migration induced by the cell-bound enzyme. A similar experiment will be performed with the low metastatic MCF-7 breast carcinoma cells.

Stable transfection of mammary carcinoma cells and analysis of their tumorigenic properties

The human heparanase cDNA was subcloned into the eukaryotic expression plasmid pcDNA3 at the *Eco*RI site. Cultured mouse (DA3) and human (MCF-7) breast carcinoma cells were incubated (48-72 h, 37^0C) with 1-2 µg DNA and 6 µl FuGene transfection reagent followed by selection with 350 µg/ml G418. Stable populations of the transfected cells, evaluated by RT-PCR, were subjected to measurements of heparanase activity. As demonstrated in figure 2, incubation of heparanase transfected DA3 (Fig. 2A) and MCF-7 (Fig. 2B) cells with sulfate labeled ECM resulted in release of low molecular weight heparan sulfate (HS) degradation fragments (peak II) into the incubation

medium, as opposed to nearly intact heparan sulfate proteoglycans (HSPGs) (peak I) released from the ECM during incubation with the corresponding mock transfected cells. Tumorigenicity of the MCF-7 cells was then evaluated. For this purpose, female athymic nude mice at 6-8 weeks of age were pre-implanted subcutaneously with 1.7 mg of 17 β-estradiol pellets (60-day release). Two groups of mice (10 mice each) were then injected bilaterally into the mammary pads with $1x10^7$ MCF-7 cells (pooled population) that were either mock-transfected, or stable transfected with the full length heparanase cDNA. As described above, these cells exhibited little or no vs. a high heparanase activity, respectively (Fig. 2B). Palpable tumors were felt 4-5 weeks after cell inoculation and their size measured twice a week thereafter. Nine-10 weeks after injection, the mice were sacrificed, the primary tumor removed, weighted, fixed and processed for histological examination. As shown in figure 3, tumors produced by cells over-expressing the heparanase gene grew faster and reached a 4-5 fold higher size than tumors produced by mock transfected cells. Macroscopic examination revealed no visible metastatic colonies.
Control studies performed *in vitro* revealed that over-expression of heparanase had no effect on proliferation (doubling time) of the MCF-7 cells. In both cell types, there was a similar stimulation of growth in the presence of low concentrations of estradiol (2×10^{-7}–2×10^{-9} M) and a pronounced anti-proliferative effect at relatively high concentrations (2×10^{-5}) of estradiol, or Tamoxifen (Fig. 4).

Inducible expression of heparanase. MCF7 breast carcinoma cells expressing no metastatic potential and little or no heparanase activity were cotransfected with the heparanase encoding cDNA driven by a tetracycline-responsive CMV promoter (pTET-heparanase) and a vector encoding a transactivator protein (pTET-TAK) (2) that drives heparanase expression only in the absence of doxycycline. Following selection in G418, stable transfected colonies were picked, and tested for tetracycline-regulated expression of heparanase, applying both RT-PCR and heparanase activity measurements. Selected clones expressing a high heparanase activity in the absence of doxycycline vs. little or no activity in its presence (Fig. 5B) were propagated and frozen. Unexpectedly, cells that express high levels of heparanase in the absence of doxycycline, retained a significant activity 3-4 days after its addition (Fig. 5A), suggesting that the heparanase protein is highly stable. Similarly, the *hep* RT-PCR signal persisted, albeit at a low level, for an extended period of time (3-4 days) after addition of doxycycline into the culture medium (Fig. 6C). Hence, the goal of obtaining a readily regulated expression of heparanase in breast cancer cells was not achieved. Nevertheless, tetracycline responsive MCF-7 cells will be tested for tumor growth, angiogenesis and metastatic dissemination in response to up- and down- regulation of the heparanase RNA and protein, as proposed in the original application.

We have recently produced a chimeric construct composed of the chicken heparanase signal peptide preceding the human heparanase cDNA. Cells transfected with this construct exhibited cell surface localization and secretion of the enzyme, as opposed to a mostly intracellular localization and little or no secretion of the human

enzyme (3). Moreover, cells (lymphoma, glioma) transfected with the chimeric construct were highly metastatic and pro-angiogenic *in vivo* (our unpublished results). We propose to compare the tumorigenicity of control MCF-7 cells to cells transfected with the chimeric, secreted form of heparanase. Similarly, MCF-7 cells will be transfected with the chimeric heparanase construct driven by a tetracycline-responsive promoter and analyzed for a regulated involvement of heparanase in angiogenesis and metastasis *in vivo*.

Task 2: Control of heparanase gene expression

Transcriptional regulation of the heparanase promoter. The human heparanase gene promoter (1.8 kb in length) was sub-cloned into pGL2 Basic vector (Promega) upstream to the luciferase reporter gene. The activity of the promoter was tested by transient co-transfection of the pGL2 vector together with CMV-LacZ reporter vector. The CMV-LacZ vector has been used to overcome differences caused by different transfection efficiencies. The *hep*-promoter activity was measured by a luciferase assay kit (Promega) in which an external substrate (luciferin) is added to the lysed transfected cells. In the presence of luciferase activity the substrate is degraded, emitting light (4). This light is monitored by a fluorimeter. Twenty four-48 hours after transient transfection with the promoter construct, the cells are lysed and subjected to luciferase assay. Differences in promoter activity were tested by transfection of the 1.8 kb human *hep*-promoter into three different cells types: C6 Glioma and MCF-7 breast carcinoma, which normally exhibit very little or no heparanase activity, and MDA-435 breast carcinoma cells, expressing a high heparanase activity. Transfection of C6-Glioma and MCF-7 cells with the *hep*-promoter resulted in a slight increase in luciferase activity over transfection with the empty pGL2- basic vector (without promoter). In contrast, luciferase activity was highly stimulated following a similar transfection of MDA-435 cells (Fig. 6A). These results suggest that heparanase activity may be regulated at the transcriptional level. Several effector molecules (EGF, bFGF, IL-1, IL-8, LPS, PMA, RA, IFN) that may regulate heparanase activity were tested in MCF-7, MDA-435 and C6-Glioma cells transiently transfected with the 1.8 kb *hep*-promoter. None of the above agents exerted a significant effect on the heparanase transcription level.

In order to identify regions in the *hep*-promoter that may regulate heparanase transcription, several deletions were introduced in the 1.8 kb *hep*-promoter. Each of these deletions (see diagram, Fig. 6B) has been sub-cloned into the luciferase reporter vector, followed by transient transfection into MDA-435 cells. As expected, the highest luciferase activity level was detected by using the full- length promoter (1.8 kb). A 2-fold decrease in luciferase activity was observed when the cells were transfected with fragment #1. Fragments 2-5 yielded a marked decrease in luciferase activity. Fragment # 2 was 15 folds less active compared to fragment # 3. Fragments # 4 and 5 yielded about the same activity as fragment # 3 (Fig. 6B). On the basis of these results it appears that three major regions in the *hep*-promoter may regulate its activity: region A may act as a positive regulator, increasing (2 fold)

fragment # 1 activity. Region B may serve as a major positive regulator, markedly increasing the activity of the promoter. Region C may act as a repressor, reducing (15 fold) the activity of fragment #3.

Three putative estrogen receptor-binding sites [i.e., AAGGGTCACTGCAACC (+strand); GTTGGTCAGAACTTTTCTTC (-strand); GGCGGTCACGTTCACTTAC (-strand)] were identified in the human heparanase promoter, showing 89-92% homology to the consensus sequence. A reproducible 3-fold stimulation of *hep* promoter luciferase activity was obtained in transfected MCF-7 cells that were pretreated for 24 h with 1 nM estrogen. There was no effect to preincubation with higher concentrations of estrogen (Fig. 7A). Quantitative RT-PCR revealed a 3-fold increase in the heparanase mRNA following treatment of estrogen-depleted MCF-7 cells with 1 nM estrogen. This increase was abolished in the presence of the estrogen antagonist ICI128 (4) (Fig. 7B). These experiments were performed in collaboration with Dr. Michael Elkin (NIDR, NIH).

Task 3: Heparanase inhibitory molecules

Laminaran sulfate: Laminarin sulfate (1-3 β-glucan) was found to efficiently inhibit experimental metastasis in both melanoma and breast carcinoma animal models (5). This polysaccharide is isolated from the cell wall of sea weed and subjected to chemical O-sulfation (5). We have focused on laminaran sulfate as a starting material for preparation of defined heparanase-inhibiting oligosaccharides. For this purpose, we applied enzymatic (laminarase) and chemical methods to produce fragments of laminaran sulfate. These were separated by gel filtration (Sephadex G-50) and anion exchange chromatography (DEAE) to determine the minimal length of oligosaccharide and optimal level of sulfation required for efficient inhibition of heparanase activity. Our preliminary results with 2 of these laminaran sulfate fragments (HP2; HP4) are presented in figure 8, indicating an almost complete inhibition in the presence of 0.5 μg/ml HP2 or 2 μg/ml HP4, containing 16-18 and 10-12 sugar units, respectively. These experiments are expected to yield a more define low molecular weight β-glucans that specifically inhibit heparanase activity. The same fragments may also compete with heparan sulfate and hence inhibit the angiogenic and growth promoting activity of bFGF and possibly other heparin-binding growth factors. Selected fragments of laminaran sulfate will be tested for an effect on MDA-435 tumor metastasis and angiogenesis.

Involvement of heparanase in branching morphogenesis and transformation of the mammary gland (this project was not proposed in the original application, but is yielding results that are higly relevant to the proposed research).

An intact basement membrane (BM) is essential for the proper function, differentiation and morphology of many epithelia. Disruption or loss of this BM occurs during normal development of the mammary gland as well as during breast carcinoma progression. The mammary gland of prepubertal female mouse is essentially quiescent. To investigate the involvement of heparanase in mammary gland remodeling, differentiation and transformation, we

have generated transgenic mice overexpressing the heparanase cDNA and protein in all tissues (6). For this purpose, the full-length human heparanase cDNA under the constitutive control of the chicken β-actin promoter, was microinjected to fertilized eggs of C57BL x Balb/c mice. Isolation of fertilized eggs, injection of DNA and transplantation of blastocytes were performed according to established protocols. Mice developed from the injected blastocytes were tested for the presence of the human *hpa* transgene in their genome. For this purpose, genomic DNA was extracted from tail tips of the mice and the human *hpa*-transgene sequence was amplified using human *hpa*-specific PCR primers. The integration of the human heparanase cDNA in the mouse genome was verified by PCR using two sets of primers. The first set of primers, designed to amplify the 5' region of the transgene, included a β-actin promoter specific primer and a human *hpa*-specific primer. The second set of primers, designed to amplify the 3' region of the transgene, included a human *hpa* specific primer, and a primer specific to the 3'-untranslated region of the plasmid. Four G_0 founder mice were obtained, harboring the human *hpa*-cDNA in their genome as revealed by PCR reaction specific for the human *hpa*-cDNA. Founders were mated with C57BL mice to create F1 mice and those were mated among themselves to create F2 mice. Homozygous F2 mice from each G_0 line were identified by Southern blot analysis and quantitative PCR (6). All founder transgenic mice were back crossed with C57BL mice in order to establish C57BL transgenic mice with a pure genetic background. Western blot analysis and measurements of heparanase activity in tissue extracts indicated a much higher expression of the heparanase enzyme in the transgenic vs. control mice, in all tissues examined. Immunohistochemical staining of tissue sections revealed a high expression of the human heparanase protein in tissues derived from the transgenic mice, but not control mice (not shown, beyond the scope of the original application).

Morphological appearance. Examination of mammary glands taken from normal virgin females revealed the typical branching pattern. Mammary glands were excised from 2-3 month old virgin homozygous transgenic mice originating from 3 independent transgenic events. Morphological evaluation of whole-mount preparations, revealed, in all 3 cases, abnormally abundant side branches and precocious alveolar structures with primary and secondary ducts, similar to a normal 9-12 day pregnant gland. It was also noted that the circumference of many of the mammary ducts was wider in the transgenic mice compared to non-transgenic ones. The same effect of over-branching and wider ductal circumference was noted in heterozygous transgenic mice, albeit to a lesser extent. The effect was taken to the extreme at an older age. The mammary gland ducts and alveoli of one-year old virgin transgenic mice were very dense, the alveoli were abnormally clustered and abundant and the ductal circumference was at least 10 fold wider (Fig. 9A, right) than that of normal non-lactating mammary ducts of C57/Bl mice at this age (Fig. 9A, left). Similar alterations were reported in the mammary gland of transgenic mice over-expressing the matrix metalloproteinase stromolysine 1 (7). Overexpression of stromelysin-1 is associated with a high incidence of

phenotypic abnormalities characteristic of premalignant and malignant transformation and ranging from severe hyperplasia to adenocarcinoma (7, 8).

The histology of the mammary glands was evaluated in tissue sections stained for BM components. Disruption of the BM surrounding the mammary ducts and alveoli of the transgenic mice was noted by several methods. Disappearance of collagen was evident in young (2-3 months old) (Fig. 9B right, arrows) and old (~1 yaer old) mice. Staining with anti-laminin antibodies revealed diminished intensity of laminin around the mammary epithelium and adipocytes of the transgenic compared to the non-transgenic tissue (not shown). These results suggest that heparanase in the transgenic mammary gland is actively digesting the sub-epithelial BM/ECM and may thus allow branching, dense alveolar formation and widening of the ducts. It is conceivable that cleavage of BM HSPGs by heparanase can unmask ECM molecules and liberates HS-bound growth promoting factors that may stimulate cell proliferation and differentiation (6).

Transformation. In subsequent studies, *hpa* over-expressing transgenic mice were crossed with MMTVneu homozygous mice (The Jackson Laboratory). The littermates were divided to neu^+/hpa^+ and neu^+/hpa^- mice, and the mammary glands from both types of progeny were taken for histological evaluation. In neu^+/neu^+ mice, focal mammary tumors begin to appear at 4 months of age, with median incidence of 205 days (9). At two months of age mammary glands of neu^+/hpa^+ (Fig. 9C, left) mice showed higher levels of side branches compared to neu^+/hpa^- (Fig. 9C, right), suggesting a hyperplastic, possibly preneoplastic phenotype of the mammary epithelium in neu^+/hpa^+ mice which may result in tumor development earlier than in neu^+/neu^+ mice. We are currently evaluating older mice for their mammary gland phenotype.

Key research accomplishments

i) Serum free medium conditioned by MDA-435 breast carcinoma cells was identified as a suitable source for characterization and purification of the putative protease, converting the full-length 65 kDa latent heparanase into an active 50 kDa enzyme. An assay to detect this conversion was developed.

ii) Mammary tumors produced by heparanase over-expressing MCF-7 cells grew faster and were 4-5 fold bigger than tumors produced by control mock-transfected MCF-7 cells.

iii) An inducible (tetracycline responsive) expression of heparanase was obtained in transfected MCF-7 cells. The estimated half-life of both the heparanase mRNA and protein was 3-4 days.

iv) The heparanase promoter was sub-cloned upstream of a luciferase reporter gene. A much higher expression was obtained in MDA-435 vs. MCF-7 cells. Both heparanase promoter activity and gene expression were stimulated by estrogen. Deletion experiments identified three regions necessary for *hep* promoter activity in transfected MDA-435 cells.

v) Oligosaccharide fragments of laminaran sulfate, retaining a high heparanase inhibiting activity, were prepared

vi) Transgenic mice over-expressing the heparanase cDNA and protein were generated. Abnormally abundant side branches, precocious alveolar structures and wide ducts were observed in the transgenic mammary gland. Early signs of hyperplasia and phenotypic transformation were seen in transgenic mice bred with MMTVneu mice.

Reportable outcomes

Manuscript: Zcharia, E., Metzger, S., Friedmann, Y., Pappo, O., Aviv, A., Elkin, E., Pecker, I., Peretz, T., and Vlodavsky, I. Molecular properties and involvement of heparanase in cancer progression and mammary gland morphogenesis. Mammary Gland Biology & Neoplasm. 6: 311-322, 2001.

Presentation: VIIIth Int. Congress, Metastasis Research Society (London, Sep. 2000): Molecular properties and involvement of heparanase in cancer metastasis and angiogenesis.

Conclusions

During the first year of research we addressed each of the tasks stated in the original application, toward a better understanding of the involvement of heparanase in breast carcinoma progression. The experimental systems and tools established during this year provide a strong basis for continuation of the proposed research. Among these is the identification of MDA-435 cell conditioned medium as a source for purification of a putative protease, converting the latent heparanase into an active enzyme (task 1b). An appropriate assay to detect this processing was developed. We have also obtained and characterized an inducible (Tet off) system for heparanase expression in breast cancer cells (task 1c) and plan to utilize it in an animal model. Using this system, we have found that the half-life of both the heparanase mRNA and protein is long (3-4 days), so that the inducible system may not provide the originally desired, readily regulated heparanase expression system, as previously expected. Transcriptional regulation of heparanase gene expression in breast cancer cells is being studied using the heparanase promoter fused to a luciferase reporter gene (task 2). Heparanase gene expression in MCF-7 cells was up-regulated in response to estrogen, but there was no effect to a number of cytokines and growth factors tested in the same system (task 2b). In other studies, MCF-7 tumor growth in the mammary fat pad of nude mice was accelerated 4-5 fold as a result of over-expression of the heparanase enzyme (task 1c), demonstrating a significant role of heparanase in breast cancer progression. As proposed in the original application, oligosaccharide fragments

of laminaran sulfate were prepared and found to retaine a high heparanase-inhibiting activity (50% inhibition at 1 μg/ml) *in vitro* (task 3). The inhibitory effect of these fragments and other polyanionic molecules on tumor growth and metastasis *in vivo* will be investigated during the 2nd and 3rd years of the research.

Transgenic mice over-expressing the heparanase cDNA and protein were generated and found to exhibit precocious alveolar structures and over-branching. These mice were bred with MMTVneu mice and the newborn mice are being evaluated for the incidence of neoplastic transformation. Although this approach was not addressed in the original application, our preliminary results suggest that it may provide important information relevant to the main goal of the proposed research. In recent experiments, beyond the scope of the proposed research, we have found that the involvement of heparanase in tumor metastasis and angiogenesis depends to a large extent on its cellular localization and secretion properties (3). Transfected lymphoma cells expressing the enzyme on their cell surface were highly metastatic and elicited a more pronounced angiogenic effect than cells expressing the enzyme primarily in the endosomal/lysosomal compartment. On the basis of this result, we now propose to transfect MCF-7 cells with a chimeric cDNA encoding for the secreted form of heparanase (3) and compare their pro-angiogenic and metastatic properties to those of the currently available MCF-7 cells over-expressing the intracellular form of heparanase. We believe that translocation of the enzyme from the cytoplasm to the cell surface plays an important regulatory role which should be investigated in order to better address the overall objective of elucidating the role of heparanase in breast cancer progression.

"So what": The results obtained thus far clearly support the originally proposed involvement of heparanase in breast carcinoma progression. A conclusive indication is the accelerated growth of tumors produced by MCF-7 cells over-expressing the heparanase cDNA. Of high significance is the potential regulatory effect of estrogen on heparanase promoter activity and the observed, seemingly pre-malignant alterations in the architecture and morphology of transgenic mammary glands over-expressing the heparanase gene. Experimental tools for achieving the other objectives of the proposed research were developed. Among these is a system to purify and characterize a proteolytic activity involved in processing and activation of latent heparanase. This protease will then serve as a complementary target for identification and development of inhibitory molecules. Heparanase-inhibiting fragments of laminaran sulfate were prepared. Their exact biochemical nature and efficacy in experimental models of breast carcinoma are being evaluated, using both the previously described molecular constructs and the newly identified secreted form of heparanase.

References

1. M. B. Fairbanks, A. M. Mildner, J. W. Leone, G. S. Cavey, W. R. Mathews, R. F. Drong, J. L. Slightom, M. J. Bienkowski, C. W. Smith, C. A. Bannow and R. L. Heinrikson (1999). Processing of the human heparanase precursor and evidence that the active enzyme is a heterodimer. J Biol Chem 274, 29587-29590.

2. Benjamin, L.E. and Keshet, E. (1997). Conditional switching of vascular endothelial growth factor (VEGF) expression in tumors: Induction of endothelial cell shedding and regression of hemangioblastoma-like vessels by VEGF withdrawal. Proc. Natl. Acad. Sci. USA 94: 8761-8766.

3. Goldshmidt, O., Zcharia, E., Aingorn, H., Guatta-Rangini, Z., Atzmon, R., Michal, I. Pecker, I., Mitrani, E., and Vlodavsky, I. Cloning, molecular properties and developmental expression of chicken heparanase. J. Biol. Chem. In press, 2001.

4. O'Neil JS, Burow ME, Green AE, McLachlan JA, Henson MC. (2001) Effects of estrogen on leptin gene promoter activation in MCF-7 breast cancer and JEG-3 choriocarcinoma cells: selective regulation via estrogen receptors alpha and beta. Mol Cell Endocrinol. 176:67-75

5. H-Q. Miao, M. Elkin, H. Aingorn, R. Ishai-Michaeli. C. A. Stein and I. Vlodavsky (1999). Inhibition of heparanase activity and tumor metastasis by laminarin sulfate and synthetic phosphorothioate oligodeoxynucleotides. Int J Cancer 83, 424-431.

6. Zcharia, E., Metzger, S., Friedmann, Y., Pappo, O., Aviv, A., Elkin, E., Pecker, I., Peretz, T., and Vlodavsky, I. (2001) Molecular Properties and Involvement of Heparanase in Cancer Progression and Mammary Gland Morphogenesis. Mammary Gland Biology & Neoplasm. 6: 311-322.

7. C. J. Sympson, M. J. Bissell and Z. Werb (1995). Mammary gland tumor formation in transgenic mice overexpressing stromelysin-1. Seminars in Cancer Biol 6, 159-163.

8. M. D. Sternlicht, A. Lochter, C. J. Sympson, B. Huey, J-P, Rougier, J. W. Gray, D. Pinkel, M. J. Bissell and Z. Werb (1999). The stromal proteinase MMP3/Stromelysin-1 promotes mammary carcinogenesis. Cell 98, 137-146.

9. Guy CT, Webster MA, Schaller M, Parsons TJ, Cardiff RD, Muller WJ. (1992) Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. Proc Natl Acad Sci USA 89:10578-10582.

Appendix (figures & figure legends)

Figure legends

Figure 1. Proteolytic conversion of the 65 kDa latent heparanase into an active 50 kDa anzyme. Serum free medium collected from MDA-435 cell cultures was incubated with recombinant 65 kDa pro-heparanase at 37°C for 1 hour. The reaction mixture was subjected to10% SDS/PAGE and Western blot analysis using **A.** mAb 130 directed against the recombinant 65 kDa heparanase; **B.** anti-hpa3 directed against a synthetic peptide of 20 amino acids in the 50 kDa active form of heparanase; and **C.** Ab 239 directed against the linker region. In lanes 5-10 the 65 kDa heparanase is converted into a 50 kDa form. The same pattern was seen with mAb130 and anti-hpa3. Proteolytic cleavage of the 65 kDa pro-heparanase was observed even in the presence of a cocktail of protease inhibitors (lane 7), DCI, a serine protease inhibitor; pepstatin, an aspartic proteases inhibitor (lane 9); and phenantroline, a metalloprotease inhibitor (lane 10). **D.** Heparanase activity. Incubation of the 65 kDa pro-heparanse in the presence of medium conditioned by MDA- 435 cells resulted in stimulation of heparanase activity.

Figure 2. Expression of heparanase activity by transfected breast carcinoma cells. Non metastatic mouse DA3 (Fig. 2A) and human MCF-7 (Fig. 2B) breast carcinoma cells were stable transfected with the human heparanase cDNA (▲), or control plasmid alone (Δ). The cells were incubated with sulfate labeled ECM and labeled degradation products released into the incubation medium were subjected to gel filtration on Sepharose 6B. HS degradation fragments elute in peak II (fractions 20-35).

Figure 3. Tumor growth of *hep*-transfected vs. mock-transfected MCF-7 cells in nude mice.
Female athymic nude mice at 6-8 weeks of age were pre-implanted subcutaneously with 1.7 mg of 17 β-estradiol pellets (60-day release). Two groups of mice (10 mice each) were then injected bilaterally into the mammary pads with $1x10^7$ MCF-7 cells (pooled population) that were either mock-transfected, or stable transfected with the full length heparanase cDNA. **A.** Tumor size was measured at various times with a caliper and calculated (volume = $\frac{D1 \times D2 \times D3}{6}$). Nine-10 weeks after injection, the mice were sacrificed and the primary tumor removed, fixed and processed for histological examination. **B.** Representative mice injected with mock- (top) vs. *hpa*- (bottom) transfected MCF-7 cells.

Figure 4. Effect of estradiol and tamoxifen on proliferation of *hep*-transfected (**A**) vs. mock-transfected (**B**) MCF-7 cells. Cells were seeded into wells of 96 well plates (1×10^3 cells/well) in the absence and presence of increasing concentrations of estradiol (2×10^{-5}–$2 x10^{-9}$ M), or tamoxifen (2×10^{-5}–$2 x10^{-7}$ M). Three days after seeding, the cells were fixed (3.8% formaldehyde), washed and stained with 1% methylene blue. After extensive washing, the methylene stain was eluted with 1M HCl and the color intensity was measured at a wavelength of 620 nm.

Figure 5. Inducible expression of heparanase. MCF-7 cells were cotransfected with the full-length *hpa*- cDNA driven by a tetracycline-responsive CMV promoter (pTET-heparanase) and a vector encoding a transactivator protein (pTET-TAK) that drives heparanase expression only in the absence of doxycycline. Following selection in G418, stable transfected cells were tested for tetracycline-regulated expression of heparanase, applying heparanase activity (**A & B**), and RT-PCR (**C**). Doxycycline (0.5 μg/ml) was added every other day (□) to cells that were maintained in its absence, or withdrawn (○) from cells that were first maintained in its presence for 3 weeks. At various time periods, cells were tested for heparanase activity (**A & B**), and *hpa*-mRNA expression (RT-PCR). Heparanase activity (release of sulfate labeled HS degradation fragments) expressed by cells (clone 3) maintained in the absence (○) or presence (●) of doxycycline is shown in **B**.

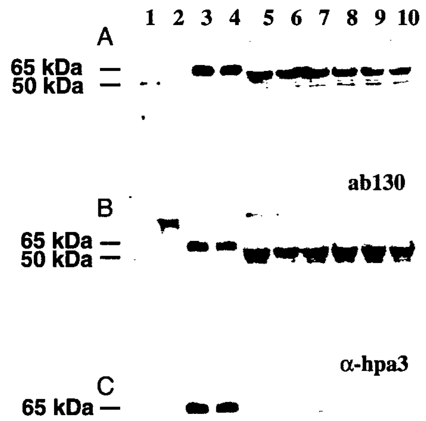
Figure 6. Heparanase promoter activity. **A.** Luciferase activity expressed by *hpa*-promoter transfected MCF-7 vs. MDA-435 breast carcinoma cells. **B.** MDA-435 cells were transfected with the full length *hpa*-promoter and with various deleted species of the *hep*-promoter. Promoter activity (luciferase emission units) was then measured. Inset: Diagramatic presentation of five deleted species of the promoter.

Figure 7. Effect of estrogen on heparanase promoter activity and mRNA expression. **A.** promoter activity. MCF-7 cells were transfected with pGL2 vector containing the *hep*-promoter. Cells were then treated (24 h, 37°) with increasing concentrations of estrogen and tested for luciferase activity. The results are expressed in relative emission units. **B.** RT-PCR. Estrogen depleted MCF-7 cells were treated (24 h, 37°C) with estrogen (1 x 10^{-9}M) in the absence and presence of the estrogen specific antagonist ICI128 (1 x 10^{-7}M). The cells were then subjected to RNA extraction and quantitative RT-PCR, using heparanase and GAPDH specific primers.

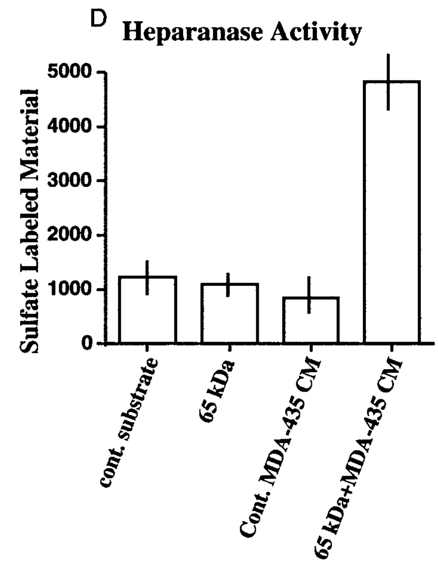
Figure 8. Inhibition of heparanase activity by fragments of laminaran sulfate. Recombinant human heparanase was incubated (24 h, 37°C, pH 6.2) with sulfate labeled ECM-derived HSPGs in the absence and presence of oligosaccharide fragments (HP2; HP4) derived from laminaran sulfate. The reaction mixture was then subjected to gel filtration over Sepharose 6B column. Heparanase activity (peak II) was inhibited by HP2 (16-18 sugar units) at 0.5 μg/ml **(A)** and by HP4 (10-12 sugar units) at 2 μg/ml **(B)**.

Figure 9. Morphological appearance of mammary glands from control vs. transgenic mice, overexpressing the heparanase gene. **A.** Whole mount of mammary glands derived from 1-year old mice (left, control; right, transgenic); **B.** tissue sections. Basement membrane collagen stained blue with Masson-trichrome reagent (left, control; right, transgenic); **C.** Whole mount of a mammary gland from neu^{+}/hpa^{-} (left) vs. neu^{+}/hpa^{+} (right) mice.

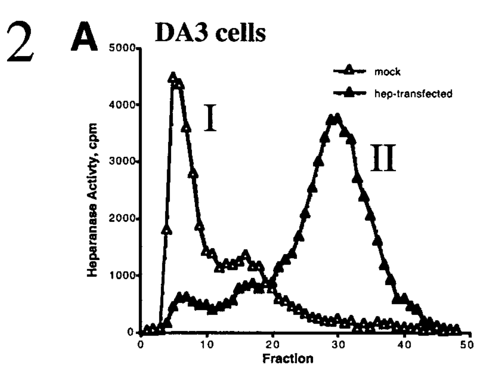
1

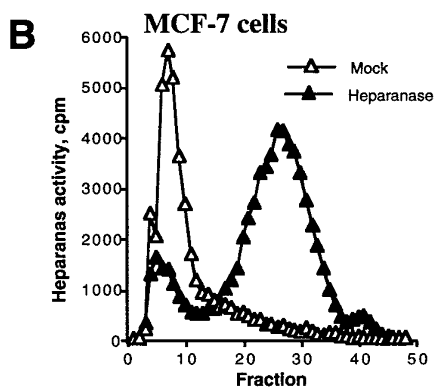


239

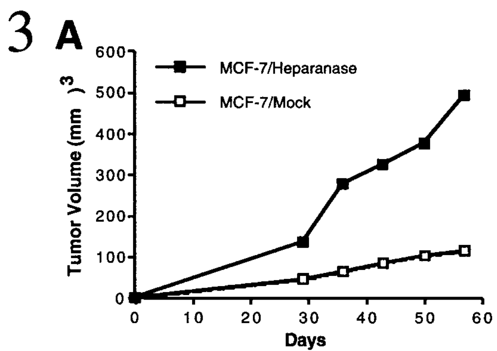


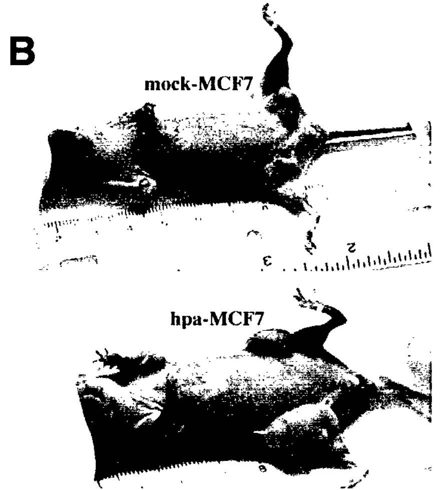
2



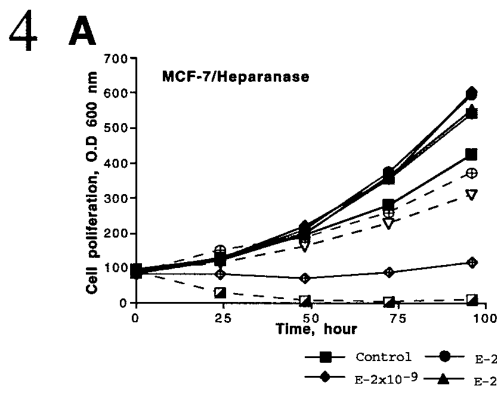


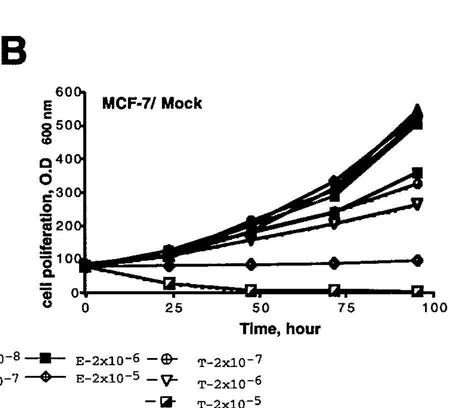
3





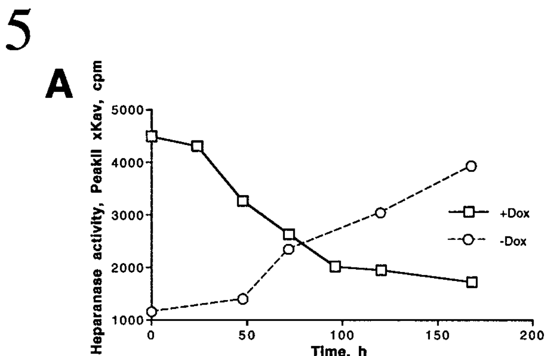
4



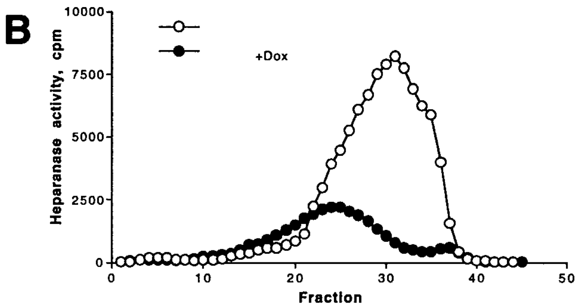


5

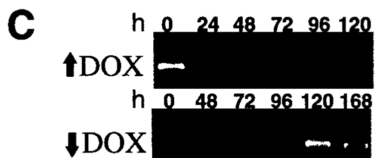
A



B

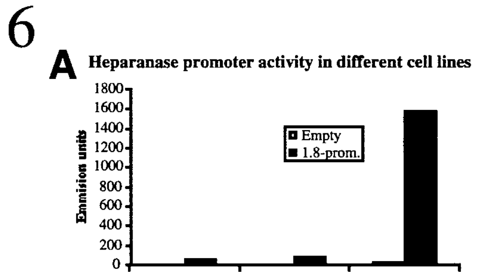


C

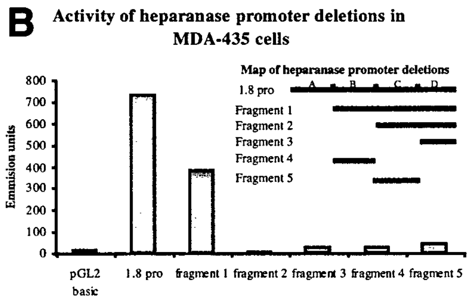


6

A

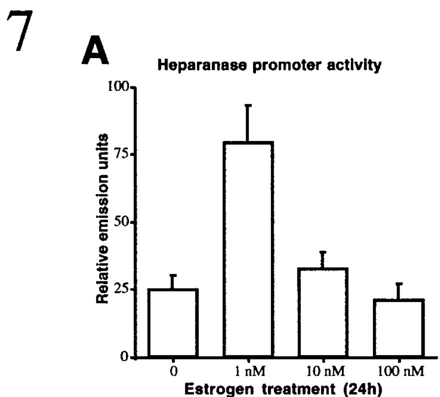


B

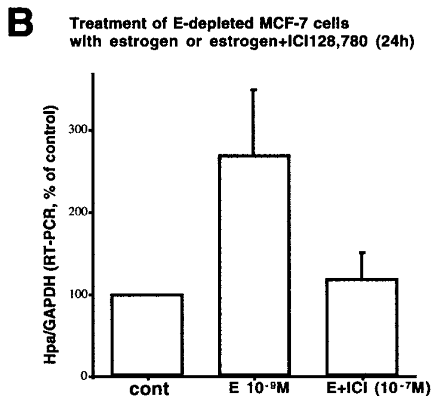


7

A

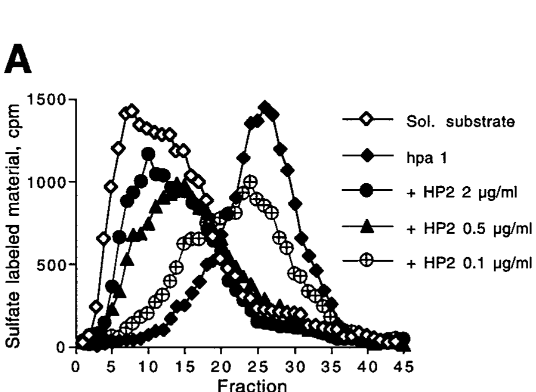


B

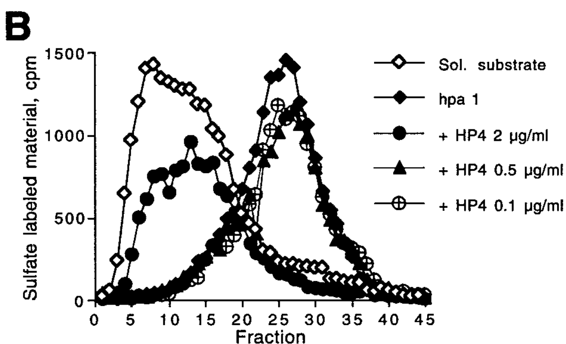


8

A

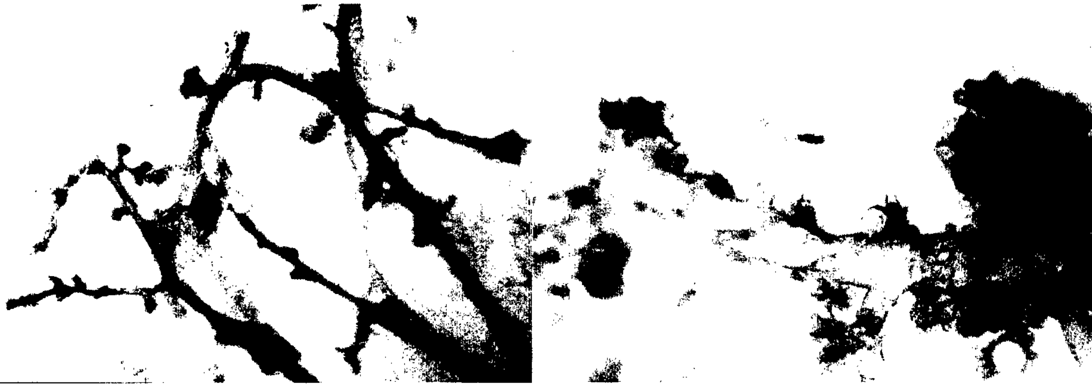


B

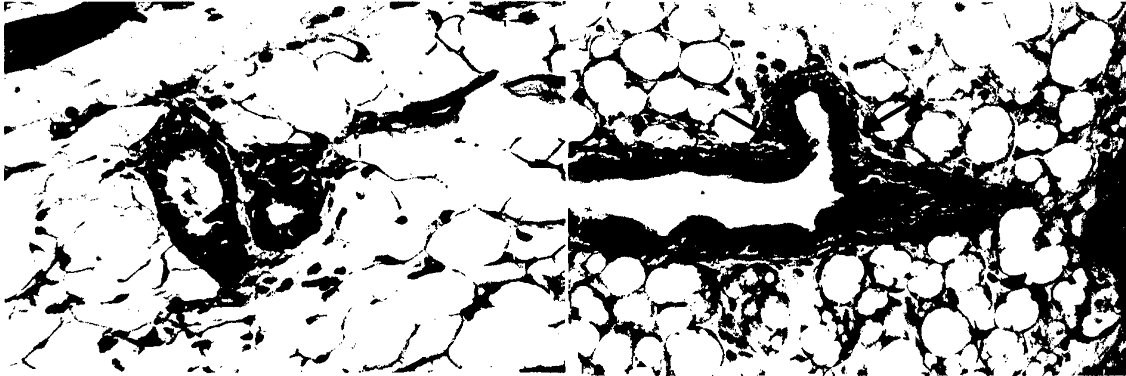


9

A



B



C