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CONTRACTING ORGANIZATION: The Burnham Institute
La Jolla, California 92307

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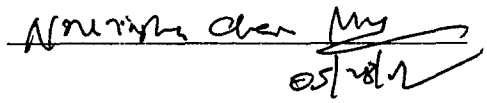

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13. ABSTRACT ***(Maximum 200 Words)***

We proposed to identify peptides that home to the vasculature of prostate. Peptides capable of homing to the prostate vasculature may allow imaging of the prostate for diagnostic purposes. They will also make it possible to direct into the prostate treatments that can reduce the prostate's size and, therefore, reduce the risk of developing prostate cancer. Over the course of this grant, we identified a peptide that homes specifically to mouse prostate tissue (SMSIARL) and we synthesized a chimeric peptide comprised of an anti-bacterial peptide $(KLAKLAK)_2$ in a D-amino acid configuration coupled to SMSIARL. We have shown in other work that, when targeted to tumor angiogenesis, the proapoptotic peptide triggers apoptosis in the angiogenic endothelial cells in tumor vasculature suppressing tumor growth (Ellerby et al., 1999). The prostate-targeted proapoptotic peptide chimera has a similar effect in the prostate. We are continuing this work by attempting to show that reducing the size of the prostate can reduce or delay the incidence of prostate cancer in the prostate-cancer prone transgenic TRAMP mice. In sum, we have discovered an effective prostate-homing peptide. It is specific for the prostate vasculature, and it can direct a therapeutic compound (a proapoptotic peptide) into the prostate and effect the organ's shrinkage.

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8-22-01

PI - Signature Date

This report contains unpublished results.

DAMD17-98-1-8562
Targeting Prostate Vasculature
PI: Erkki I. Ruoslahti, M.D., Ph.D.

This report contains unpublished results.

1. INTRODUCTION

The overall goal of this project was to complete the identification of prostate-homing molecules and characterize their specificity. We will also aimed to determine the ability of the peptides to serve as carriers of materials such as imaging agents, drugs and radioisotopes into the prostate. What we've learned from this project may make it possible to image the prostate for diagnostic purposes. This knowledge will also make it possible to target treatments into the prostate. We will use the targeting to develop an animal model for prostatectomy by selective toxin ablation of the gland. We hope that this model will serve as a starting point for the development of a non-surgical prostatectomy and that reducing the size of the prostate will reduce the risk of developing prostate cancer. These studies may also provide a model for similar approaches to the treatment of premalignant and malignant conditions in other tissues.

Drs. Michele Bernasconi and Erkki Ruoslahti were paid from this award.

2. PROGRESS REPORT

Technical Objective 1. *To use in vivo screening of phage-displayed peptide libraries for identifying peptides capable of homing to prostate vasculature.*

Objective 1 had been completed at the filing of the previous progress report. We isolated prostate-homing peptides, one of which is particularly effective in that it gives a 30-40 fold enrichment of phage homing to prostate vasculature relative to other tissues. Coinjecting the synthetic peptide inhibits the homing of the phage, confirming the specificity of the homing. The peptide is heptapeptide, with the sequence SMSIARL. Because it is short and linear, it is easy to synthesize and fabricate into the conjugates needed in objectives 2 and 3.

Technical Objective 2. *To characterize the tissue and species specificities of the prostate-homing peptides identified under Objective 1.*

Objective 2 has now been completed. Phage homing studies show that the SMSIARL phage homes specifically only to the prostate. Immunostaining reveals phage in the prostate vasculature within minutes of the intravenous injection, but not in many other tissues examined. The phage also specifically homes to a rat prostate. We also have evidence, obtained by using a phage overlay assay with human prostate tissue section, that the SMSIARL phage binds to the vasculature in the human prostate.

Technical Objective 3. *To evaluate the prostate-homing peptides as carriers of materials such as radionuclides into the prostate.*

We have synthesized a chimeric peptide in which an anti-bacterial peptide $(KLAKLAK)_2$ in a D-amino acid configuration is coupled to the SMSIARL peptide.

This report contains unpublished results.

We have shown in other work that, when targeted to tumor angiogenesis, the proapoptotic peptide triggers apoptosis in the angiogenic endothelial cells in tumor vasculature suppressing tumor growth (Ellerby et al., 1999). The prostate-targeted proapoptotic peptide chimera can have a similar effect in the prostate. Moreover, treatment of young mice with the peptide chimera delays the subsequent development of prostate cancer in the prostate-cancer prone transgenic mice (TRAMP mice). This work is still ongoing – we submitted a paper to Science on the results generated under this grant. The reviewers wanted us to add the results of the TRAMP experiment into the paper. These data are now at hand and we resubmitted the manuscript on August 21, 2001. A copy of this manuscript is appended to this report.

3. KEY RESEARCH ACCOMPLISHMENTS

- Identified a heptapeptide, SMSIARL, that homes to specifically (and only) to prostate vasculature in mice
- Shown that biotinylated SMSIARL injected intravenously accumulates in prostate
- Shown by phage overlay on human prostate tissue sections that SMSIARL binds to human prostate vasculature
- Shown that a proapoptotic peptide fused to the SMSIARL peptide causes extensive apoptosis in prostate, but not in other tissues
- Shown that treatment of male prostate cancer-prone TRAMP mice with the proapoptotic peptide--SMSIARL fusion peptide—early in life delays the development of prostate cancer in these mice.

REPORTABLE OUTCOME

This work has been discussed in the context of the principal investigator's ongoing vascular targeting research at scientific meetings, including the following:

Meetings

CApCURE Annual Prostate Meeting – October 1999 (Lake Tahoe)
CapCURE Special meeting on apoptosis – August 1999 (Santa Monica)
International Conference on Prostate Cancer, U. Iowa - June 1999, speaker
Tuusula, Finland, International Conference of Finnish Prostate Society – February 1999, speaker

This report contains unpublished results.

Patent issued

U.S. Patent 6,232,287 issued May 15, 2001.
"Molecules that Home to Various Selected Organs or Tissues"

Publication submitted

Arap, W., Haedicke, W., Kain, R., Rajotte, D., Krajewski, S., Ellerby, H.M., Bredesen, D.E., Pasqualini, R., and Ruoslahti, E. Targeting the prostate for desctruction through a vascular address. Submitted for publication.

5. CONCLUSIONS

We have an effective prostate-homing peptide. It is specific for the prostate vasculature, it recognizes mouse, rat and human prostate vasculature; it can direct a therapeutic compound (a proapoptotic peptide) into the prostate and effect the organ's shrinkage and delay the onset of prostate cancer. These results complete each of the technical objectives of the grant.

6. REFERENCES

Arap, W., Haedicke, W., Kain, R., Rajotte, D., Krajewski, S., Ellerby, H.M., Bredesen, D.E., Pasqualini, R., and Ruoslahti, E. Targeting the prostate for desctruction through a vascular address. Submitted for publication.

7. APPENDIX

Arap, W., Haedicke, W., Kain, R., Rajotte, D., Krajewski, S., Ellerby, H.M., Bredesen, D.E., Pasqualini, R., and Ruoslahti, E. Targeting the prostate for desctruction through a vascular address. Submitted for publication.

Submitted

[redacted]

Targeting the Prostate for Destruction Through a Vascular Address

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Summary

Organ specific drug targeting was explored in mice as an alternative to surgery to treat prostate diseases. Peptides that home specifically into the prostate were selected from phage libraries and used to target a pro-apoptotic peptide to the prostate. The compound caused selective destruction in the mouse prostate. The homing peptide bound also to vasculature in the human prostate. These results suggest that it may be possible to develop an alternative to surgical prostate resection that may also reduce future cancer risk.

Diseases affecting the prostate have gained tremendous clinical and economic significance due to the increasing average age of the male population in the industrialized countries. Benign prostate hyperplasia affects to some degree most elderly men. Even more serious, the prostate is a frequent site of cancer. Some autopsy studies find that most men older than 70 have occult or overt cancer in the prostate (1). The surgical therapies of prostate hypertrophy and prostate cancer are associated with serious side effects, such as incontinence and impotence.

We have sought to develop a strategy that would provide a less traumatic treatment for prostate disease than is currently available. Our strategy is based on identification of peptides that home to specific sites in the vasculature by *in vivo* screening of intravenously injected phage libraries. These studies have revealed a surprising degree of specialization in the endothelia of various normal tissues (3). Screening phage libraries for tumor homing has yielded a collection of peptides that home to tumor vasculature (4). We and others have used these tumor-homing peptides to direct therapies into tumors in mice (4,5). We report here the identification of peptides that home to the vasculature of the prostate and the use of one of these homing peptides to deliver a pro-apoptotic peptide to the prostate.

In vivo screening of a fUSE5 phage heptapeptide library for prostate-homing peptides (6) yielded two phage that accumulated selectively in the prostate. One of these phage, displaying the peptide SMSIARL (single letter code), homed to the prostate 15- to 30-times more than non-recombinant control phage (Fig. 1a). The other prostate-selected phage (VSFLEYR) gave a prostate-homing ratio of approximately 10. The homing of this phage to prostate tissue was inhibited when synthetic SMSIARL peptide was injected together with the phage, but not when an unrelated peptide was injected (Fig. 1a). The SMSIARL phage homed also to the rat prostate tissue (not shown). The SMSIARL peptide when cloned into the T7 phage (6) showed a similar homing specificity for the prostate. Phage expressing a scrambled variant of this peptide (LAMSRIS) showed no homing to

the prostate. The T7 SMSIARL phage was not enriched in the brain (Fig. 1b), salivary gland, kidney, testis, thymus, pancreas, skeletal muscle, or lung (not shown). We also confirmed the homing specificity by co-injecting SMSIARL phage and non-recombinant phage; the ratio of the two types of phage in the prostate was determined by PCR. The SMSIARL phage homed to the prostate 12.5 times more than the non-recombinant phage. The recovery of the SMSIARL phage was enriched more than 5-fold relative to co-injected non-recombinant control phage when the tissue was extracted with detergent rather than buffer alone. The brain as a control organ showed no enrichment with or without detergent (Fig. 1b). The greater phage recovery after lysis of the tissue with detergent suggests that the SMSIARL phage may have been taken up into cells.

Antibody staining of the phage in tissue sections from mice injected intravenously with the T7 SMSIARL phage revealed staining in the prostate 5 minutes after an intravenous injection (Fig. 2). The phage staining co-localized with staining for the blood vessel marker CD31, indicating homing to blood vessels in the prostate. No specific staining was seen control organs, or in prostate or control organs of mice injected with a non-recombinant control phage. The phage staining appeared to be intracellular, supporting the detergent extraction results shown in Fig.1b.

We next studied the ability of the SMSIARL peptide to deliver a biologically active compound to the prostate. $_{D}$(KLAKLAK)$_2$ is an amphipathic D-amino acid peptide that binds selectively to bacterial, but not eukaryotic cell membranes (7). It has anti-bacterial activity, but is relatively non-toxic to eukaryotic cells. We have previously shown that $_{D}$(KLAKLAK)$_2$, if delivered into mammalian cells, disrupts mitochondria (mitochondrial membranes resemble those of bacteria), initiating apoptosis (8). Conjugated through a G-G linker to a homing peptide that homes to tumor vasculature, $_{D}$(KLAKLAK)$_2$ yields a chimeric compound that is selectively cytotoxic to angiogenic endothelial cells and has anti-tumor activity *in vivo* (8). We used the same strategy to prepare a prostate-targeting

pro-apoptotic chimera, and studied its ability to cause selective tissue destruction in the prostate.

Mice were injected with 250 μg of the SMSIARL-GG-$_D$(KLAKLAK)$_2$ targeted chimeric compound and the prostates were collected after 1, 4, 8, 12, 16, 24, 48 hours and 7 days. Control groups received $_D$(KLAKLAK)$_2$ coupled to a non-homing scrambled peptide (LAMSRIS), SMSIARL and $_D$(KLAKLAK)$_2$, as an uncoupled mixture, or buffer alone. A total of 62 mice treated with SMSIARL-GG-$_D$(KLAKLAK)$_2$ were evaluated. In prostates collected 16 hours or later after the injection histology revealed an unevenly distributed destruction of the prostate glandular epithelial cells that in some areas included epithelial shedding and destruction of entire glandules (Fig. 3 a,b). These changes were still present 7 days after the treatment and no mitotic figures were observed, suggesting sustained damage and poor of regeneration (not shown). Electron microscopy showed extensive destruction of intracellular organelles in the SMSIARL-GG-$_D$(KLAKLAK)$_2$-treated, but not control-treated, mice (Fig. 3 c,d).

Tissue damage was also evident from an increase in TUNEL-positive (9) vascular and glandular cell nuclei in the prostates of mice treated with SMSIARL-GG-$_D$(KLAKLAK)$_2$. The prostates of control animals displayed only rare degenerating epithelial cells and all other organs examined (brain, heart, liver kidney, lung, urothelium) were histologically normal during or after treatment with each of the compounds (Fig 3 e-h).

To effect sustained levels of the compounds used in the treatments, we used an implanted peristaltic pump for controlled release (10). In another control experiment, we also implanted subcutaneous testosterone pellets to eliminate any variation in the sensitivity of prostate tissue caused by possible fluctuations in endogeneous androgen levels (11). SMSIARL-GG-$_D$(KLAKLAK)$_2$ consistently produced damage in the prostate (not shown).

The tolerated dose of SMSIARLGG-$_D$(KLAKLAK)$_2$ was limited by acute toxicity of the compound; the dose could be increased significantly by giving the injection slowly over several minutes. Mice injected with SMSIARL-GG-$_D$(KLAKLAK)$_2$as well as those injected with equivalent amount of non-conjugated mixture of the homing peptide and pro-apoptotic peptide showed marginal elevation of serum parameters of liver (ALT, AST, GGT) and kidney (creatinine and blood urea nitrogen) function. The levels returned to normal one week after the treatment. In one experiment, 4 mice that had been treated with 4 weekly injections of SMSIARL-GG-$_D$(KLAKLAK)$_2$ were allowed to mate. Vaginal plugs showed that that mating had occurred and litters were born in each case. These results suggest that SMSIARL-GG-$_D$(KLAKLAK)$_2$ causes damage in the prostate, while other tissues are spared and the mice remain fertile.

We next analyzed the effect of a systemic SMSIARL-GG-$_D$(KLAKLAK)$_2$ treatment on the longevity of transgenic prostate cancer-prone mice (TRAMP mice; 12). Two independent experiments gave similar results; one of the experiments is shown in Figure 4. The SMSIARL-GG-$_D$(KLAKLAK)$_2$ survived significantly longer than the control groups that received the uncoupled peptides or buffer ($P<0.01$ for both; Log Rank test).

Overlay of tissue sections from human prostate with the SMSIARL phage indicated that this phage also binds to the endothelium of human prostate blood vessels the same way it binds to the mouse prostate vessels (Fig. 5). Significantly, vessels in hypertrophic human prostate tissue bound the SMSIARL phage. No binding of this phage was detected in the blood vessels in several other human tissues (data not shown).

Our findings show that, like the vasculature of many other tissues analyzed in previous work (2-4), the prostate vasculature expresses tissue-specific features. The biochemical nature of this vascular specialization is incompletely understood.

We have identified the receptor for a peptide that homes to lung vasculature as membrane dipeptidase (13). Others have shown that a modified von Willebrand factor promoter is activated in endothelial cells in a tissue-specific manner under the influence of the surrounding parenchymal tissue (14), providing one possible regulatory mechanism for the expression of tissue-specific endothelial markers. Perhaps prostate tissue induces receptors for SMSIARL in the resident endothelium. While the molecule the SMSIARL peptide binds to in the prostate vasculature remains to be identified, our findings represent a step toward the development of a medical prostatectomy procedure. This procedure may provide an alternative treatment for prostate hypertrophy and the resulting reduction in the number of target cells available for malignant transformation can delay the development prostate cancer.

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5. F. Curnis, et al., Nature Biotech. 18: 1185 (2000).
6. Phage libraries for the screening were prepared in the fUSE5 vector as described [E. Koivunen et al. Meth. Enzymol., 245:346 (1999)] in the fUSE 5 vector [(G.P. Smith and J.K. Scott, Meth. Enzymol., 217:228 (1993)]. The primary library contains about 5×10^9 individual recombinant phage. CD-1 mice (2-4 month-old males; Jackson Laboratories, Bar Harbor, ME) were anesthetized with Avertin (0.015 ml/g) and injected intravenously (tail vein) with phage libraries containing 10^9 transducing units diluted in 200 μl DMEM. The phage was rescued from tissues by bacterial infection (2), and about 300 individual colonies were grown separately. The bacterial cultures were then pooled and the amplified phage were injected into mice as described above. To test individual phage for homing, 10^9 CFU (fUSE5) or 10^{10} PFU (T7), diluted in 200 μl PBS were injected. The SMSIARL insert and its scrambled variant were cloned to the T7 phage (T7select415 – 1 vector; Novagen, Madison, Wi), and the resulting phage was tested as described [(J. Hoffman et al., in H. Lowman and T. Clarkson,(Ed.)]. Phage display, a practical approach; Oxford, 2001).
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10. Testosterone pellets (12.5 g; Innovative Research of America, Sarasota, FL) were surgically implanted in the peritoneal area (10). Placebo pellets were used as controls. After 7 days, controlled release pumps (Alzet, Mountain View, CA) were implanted on the peritoneal area opposite the pellets. Each

pump was loaded following the manufacturer's instructions with either SMSIARL-$_D$(KLAKLAK)$_2$ or an uncoupled mixture of SMSIARL and $_D$(KLAKLAK)$_2$. The animals were sacrificed after one week, and their organs processed for histology.

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16. We thank Dr. Norman Greenberg for providing TRAMP mice and Eva Engvall for comments on the manuscript. This work was supported by grants DAMD17-99-1-8164 (WA), DAMD17-98-8581 (DB), and DAMD17-98-1-8562 (ER) from the Department of Defense, research awards from CaPCURE (WA and ER), grants CA74238 (ER), CA82713 (ER) and Cancer Center Support Grant CA30199 from the National Cancer Institute.

Figure legends

Fig. 1. Specific homing of phage to the prostate. *a. Phage selected for prostate homing accumulates specifically in the prostate and the homing is inhibited by soluble peptide.* The SMSIARL fUSE5 phage, identified by in vivo screening, was tested for prostate homing. This phage and an irrelevant control phage were injected intravenously to male mice (10^9 colony forming units; [CFU] per mouse) and the phage were rescued from various tissues based on their ability to infect a host bacteria. As indicated, 200 μg of the SMSIARL peptide or a control peptide (CARAC), were included in the injection to test inhibition of SMSIARL phage homing. *b. The SMSIARL peptide directs specific homing of T7 phage to the prostate.* The SMSIARL sequence was cloned to the coat protein of the T7. A 1:10 mixture of SMSIARL and non-recombinant control T7 phage (10^{10}PFU) was injected and allowed to circulate for 7 min. Phage were extracted from tissue with buffer (PBS) or a detergent solution (0.5% NP-40 in PBS) prostate and brain, plated and 32 colonies were randomly chosen for PCR. The PCR products of SMSIARL and control phage DNA were distinguished on the basis of a size difference in a 4% agarose gels.

Fig 2. Immunohistochemical staining of phage within prostate endothelial cells after intravenous injection into mice. SMSIARL-phage were injected intravenously into mice. After 7 min circulation, animals were perfused with PBS, the prostate (a,b,c), brain (d,e,f) and various control organs were removed, processed for frozen sectioning and stained with a polyclonal antibody against T7 phage (FITC; a,d) and CD31 (Rhodamin; b,e) . Merge with nuclear counterstain with DAPI (e,f). Control organs (kidney, spleen, , lung; not shown) were negative for the phage, except for liver and spleen, where the reticuloendothelial tissue traps phage non-specifically (4). Magnification: 400x.

Fig. 3. Targeted proapoptotic peptide to mouse prostate vasculature causes tissue damage in prostate but not in other tissues. Mice received an intravenous 250 μg injection of the SMSIARL-GG-$_D$(KLAKLAK)$_2$or an equivalent dose of

SMSIARL and $_D$(KLAKLAK)$_2$ as uncoupled peptides (control-treated mice) The mice were sacrificed 24 hours after the injection.. Prostates were fixed in paraformaldehyde or plastic and processed for light microscopy by staing with hematoxylin-eosin (H&E) or electron microscopy. Light microscopy showed focal loss of cell borders and epithelial shedding in the ventral lobe of prostates from the SMSIARL-GG-$_D$(KLAKLAK)$_2$ group. (a) H&E-stained micrograph shows massive glandular destruction with nearly complete shedding of the glandular epithelial cells into the lumen. (b) A representative migrograph of normal prostate tissue from a mouse treated with the uncoupled peptide mixture. (Magnification 400x). (c) An electron microscopic images of a single epithelial cell from a SMSIARL-GG-$_D$(KLAKLAK)$_2$ group prostate. The cell has sloughed off into the glandular lumen and massive destruction of its organelles is seen. (d) A representative micrograph of normal prostate shows intact cellular structure. (Magnification in c and d, 1:6,000). Light microscopy shows no damage bladder (e; 1:200), heart (f; 1:400), kidney (g; 1:400), liver (h; 1:400).

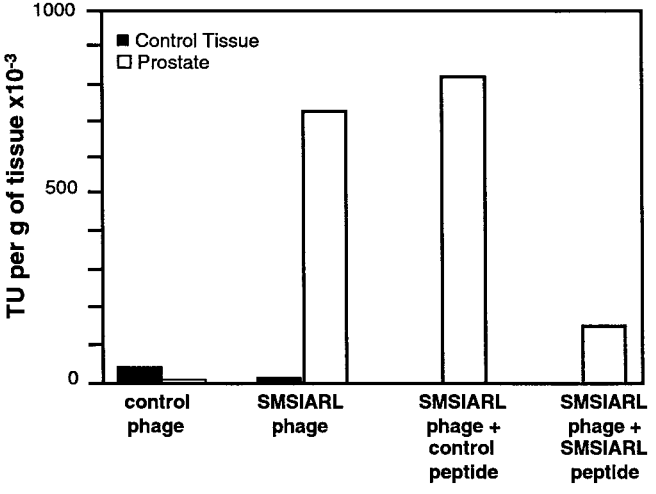
Fig. 4. Survival of transgenic prostate cancer-prone mice (TRAMP mice) treated with SMSIARL-GG$_D$(KLAKLAK)$_2$ or control materials. The treatment was initiated at 12 weeks of age. Male mice (10 per group) received intravenous injections of SMSIARL-GG-$_D$(KLAKLAK)$_2$ peptide (200 μg per dose), or an equivalent dose of SMSIARL and (KLAKLAK)$_2$ as uncoupled peptides (control-treated group) . The injections were given every other week for a total of 10 doses. The mice in the SMSIARL-GG-$_D$(KLAKLAK)$_2$ group survived significantly longer than the control mice treated with the uncoupled peptide mixture or with buffer.

Fig. 5. SMSIARL phage binds to endothelium in human prostate. A human prostate tissue section containing both normal and cancerous tissue was overlaid with the SMSIARL phage (10^9 TU/ ml) and the binding of the phage was detected with anti-M13 phage antibody and peroxidase staining. (a) shows an overview (x200) and (b) a detail from *a* at a higher magnification (x400). Staining of the endothelium is seen. (c) Overlay with phage that contains no peptide insert

produces no endothelial staining. (d) The SMSIARL-phage staining is inhibited when soluble SMSIARL peptide is included in the overlay at 0.3 mg/ml.

Fig. 1

a


1000
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control phage
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SMSIARL phage + control peptide
SMSIARL phage + SMSIARL peptide

b

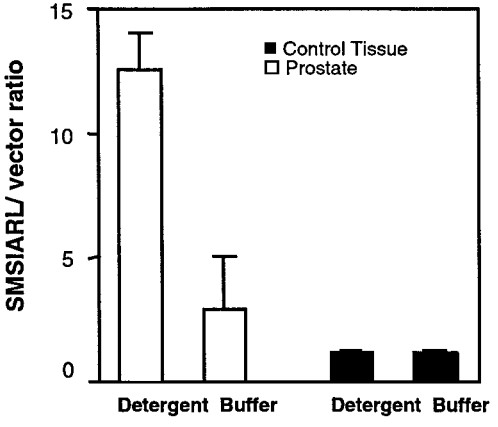

15
10
5
0
SMSIARL/ vector ratio
Control Tissue
Prostate
Detergent
Buffer
Detergent
Buffer

Fig. 2

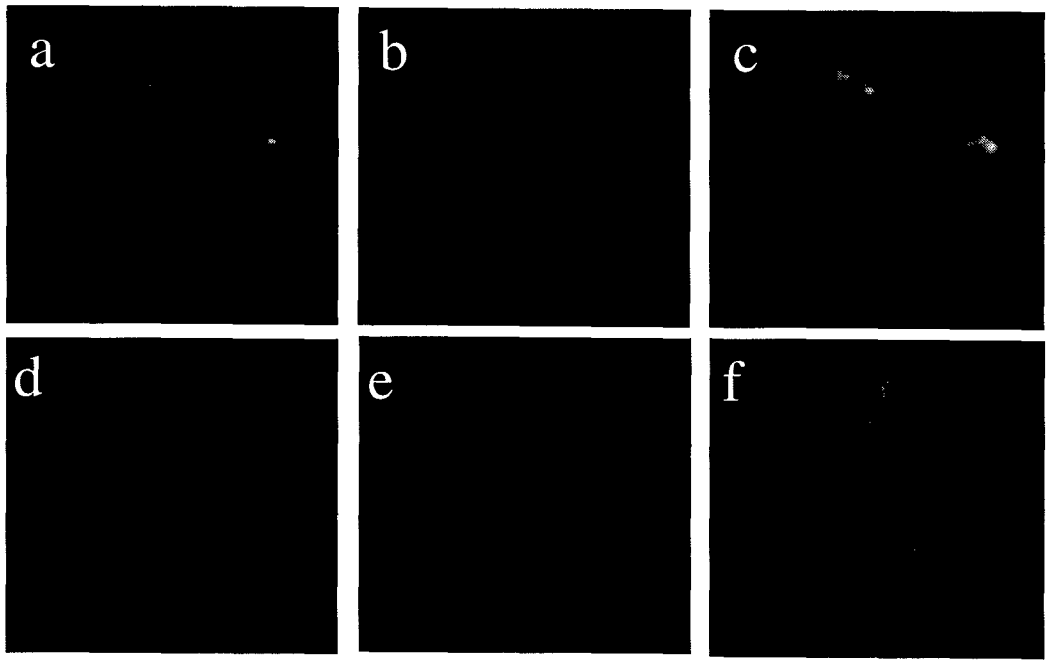


Fig. 3

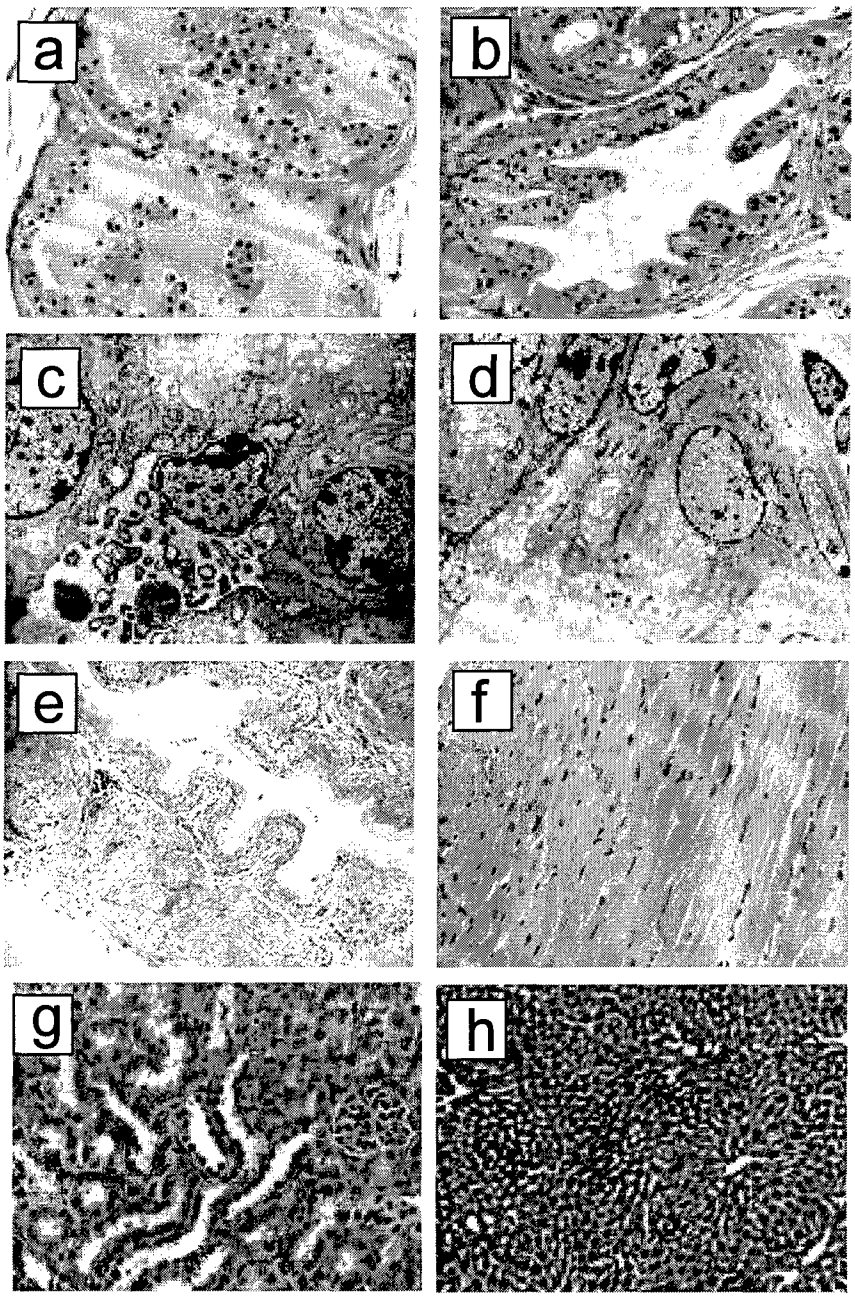


Fig. 4

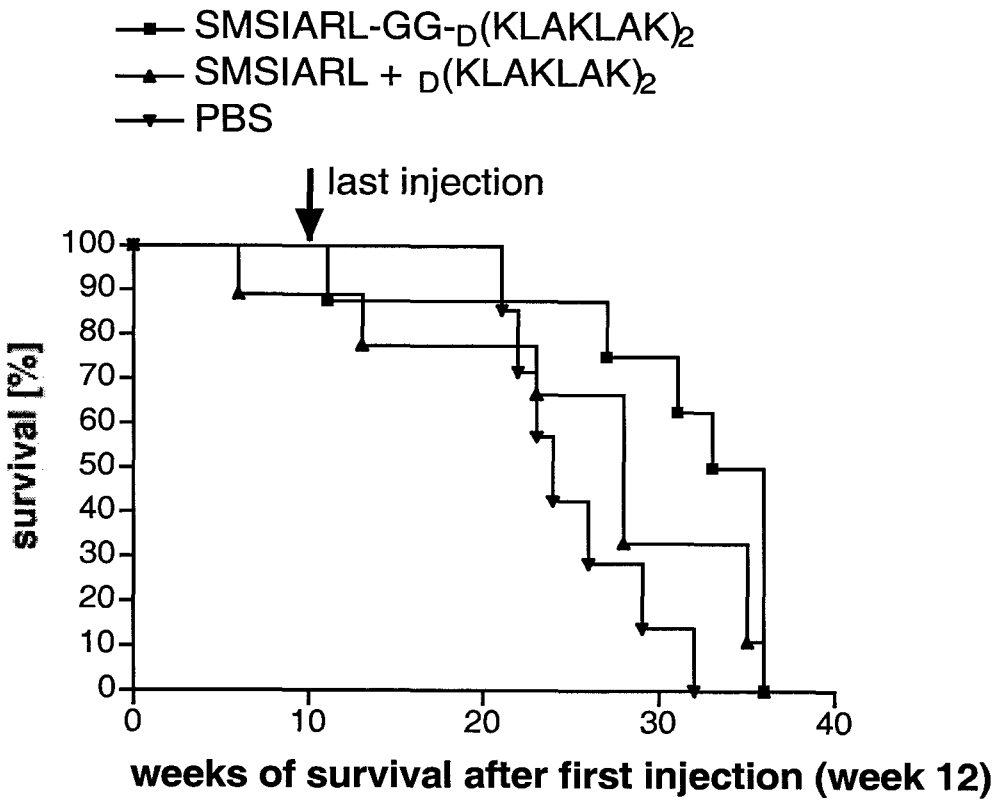
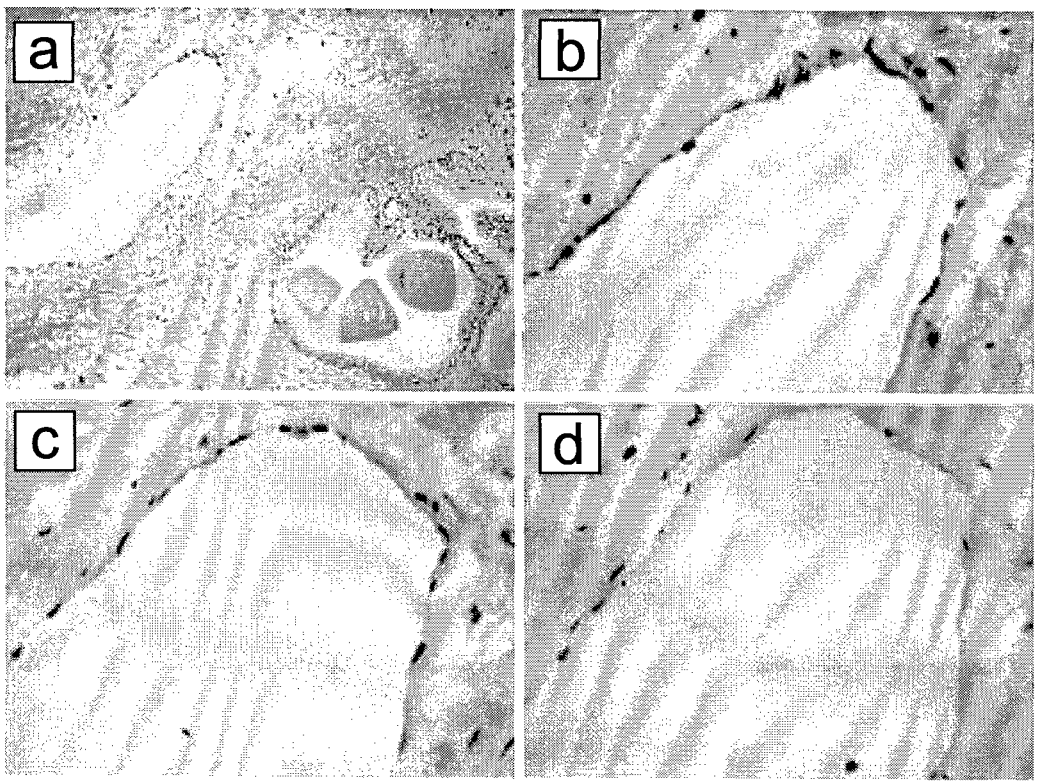

SMSIARL-GG-D(KLAKLAK)2
SMSIARL + D(KLAKLAK)2
PBS
last injection
survival [%]
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
weeks of survival after first injection (week 12)

Fig. 5



DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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REPLY TO
ATTENTION OF

MCMR-RMI-S (70-1y) 15 May 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
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