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AWARD NUMBER: DAMD17-03-1-0102

TITLE: Eicosanoid Regulation of Prostate Cancer Progression: Disruption of Hemidesmosomes and Collaboration in Tumor Invasive Growth

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REPORT DATE: March 2005

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. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-03-2005	Annual	1 Mar 2004 – 28 Feb 2005

4. TITLE AND SUBTITLE

Eicosanoid Regulation of Prostate Cancer Progression: Disruption of Hemidesmosomes and Collaboration in Tumor Invasive Growth

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0102

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Wayne State University
Detroit, Michigan 48202-3622

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. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

A significant achievement in the current reporting period is our ability to immunostain both the 12-LOX protein and β4 integrin in paraffin-embedded prostate tumor tissues circumventing the problems described in the previous report. With the new protocol we have stained about 20 cases so far and the remaining cases are in progress. We have generated several stable transfectants of PC-3 cells expressing mutant forms of the β4 integrin and studied their interaction with 12-LOX. During this study, we have identified that the peptide spanning between amino acids 1126 and 1315 of the cytoplasmic tail of the β4 integrin shows strong interaction with 12-LOX. An important observation is that the full-length cytoplasmic tail of β4 integrin, when expressed ectopically, disrupts the interaction with 12-LOX with β4 integrin in a dominant negative manner. This interaction also resulted in a decrease in the biosynthesis of the enzymatic product, 12-HETE, as well as reduction in the tumor growth rate from subcutaneously injected PC-3 cells in athymic nu/nu mice.

15. SUBJECT TERMS
Prostate cancer, hemidesmosome, 12-lipoxygenase, HGF, SF

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	10	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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INTRODUCTION

During the progression of human PCa hemidesmosomes, adhesion structures that anchor epithelial cells to basement membrane and function as a tumor suppressor, are lost (1, 2). We found that 12-lipoxygenase directly interacts with β4 integrin, an integral part of hemidesmosomes (3, 4). We hypothesized that an increase in 12-LOX activity can cause the disassembly of hemidesmosomes, mobilization of α6β4 integrin from hemidesmosomes to other parts of the cell membrane, and stimulate tumor invasive growth. We proposed to conduct a correlation study to evaluate the relationship between 12-LOX expression and dispersion of β4 integrin in clinical tumor specimens. Our proposal further aims to study whether 12(S)-HETE, the enzymatic product of 12-LOX, can disrupt hemidesmosomes and whether 12-LOX inhibitors promote the formation of hemidesmosomes. Then we will study the underlying signaling pathway, especially PKCα, initiated by 12(S)-HETE, in the disassembly of hemidesmosomes. Next we will overexpress β4 integrin and study the role of the interaction between 12-LOX and β4 integrin in the adhesion, proliferation, migration, and survival, in response to HGF/SF. Finally we will xenograft these transfected cells into mice, to evaluate whether any phenotypic changes of tumor cells in vitro can be recapitulated in vivo. The work will significantly advance our understanding about the complex process of prostate cancer progression as well as the possible role played by dietary fat in the progression of prostate cancer.

BODY OF PROGRESS REPORT

Task 1.

We have attempted several procedures for immunostaining for 12-LOX. In the previous report, the procedure only worked in frozen human prostate tumor tissue. We now worked out the conditions for immunohistochemical analysis of 12-LOX at the protein level in paraffin-embedded human prostate tumor tissues. As shown in the figure 1, 12-LOX immunoreactivity correlated with tumor grade. Neoplastic glands are weakly, moderately or strongly positive for 12-LOX in hyperplatic glands (**Figure 1A**), atropic glands (**Figure 1 B**), and in tumor Glenson score 4-6 (**Figure 1 C,D**).

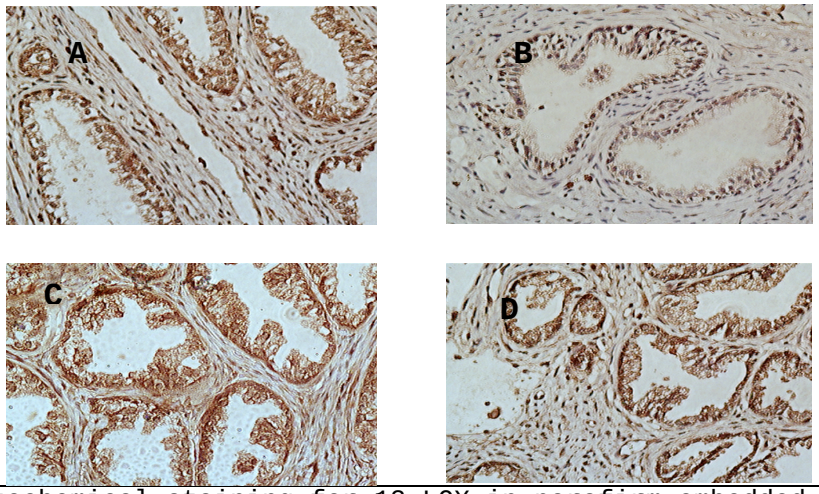


Figure 1. Immunohistochemical staining for 12-LOX in parafirm-embedded prostate tumor tissues. Sections of specimens were probed with a12-LOX polyclonal antibody (Oxford Biomedical Research Inc, Oxford, MI). Positive immunoreactivity is indicated by staining with brownish color. A, hyperplasia gland; B, atropic; C/D, tumor with Glenson score 4-6.

We have also attempted several protocols of immunostaining for β4 integrin in paraffin-embedded material. As shown in figure 2, positive staining was found in tumors and correlated with tumor grade.

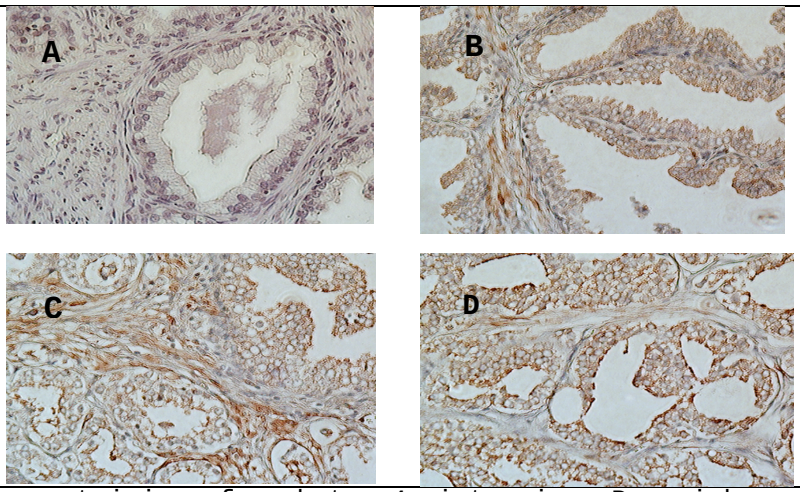


Figure 2. Immunostaining for beta 4 integrin. Brownish color indicates positive staining. Note the pattern of beta 4 integrin positivity. A, normal epithelium; B, tumor with Glenson score 4; C, tumor with Glenson score 6; D, tumor with Glenson score 8.

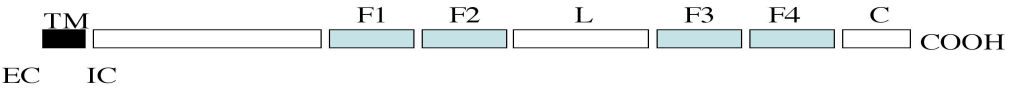
We have procured 100 cases of prostate tumor specimens. We are working on the protocol for double staining, the study will be completed in the next year.

Task 2. Study the effects of 12-LOX inhibitors and 12(S)-HETE on hemidesmosome in prostate epithelium. Months 1-18:
This specific aim was partially achieved during the previous reporting period and it is in the final stages.

Task 3. Study the signal transduction pathways that underlie the disassembly of hemidesmosomes by 12(S)-HETE or an increase in 12-LOX activity, Months 12 -24:
The studies proposed have been initiated and are ongoing.

Task 4. Overexpress β4 integrin in PC-3 cells, in the presence or absence of 12-LOX expression, and evaluate the capacity of transfected cells to form hemidesmosomes and whether an increase in surface expression of α6β4 alters cell proliferation, adhesion, migration, and survival, in response to HGF/SF, Months 18 - 30:

Generation of PC-3 cells expressing various β4 mutants: We constructed a panel of bacterial expression plasmids and mammalian expression constructs that express various β4 mutants of the cytoplasmic tail as shown in Figure 3. Using pGR30~47, we have generated a panel of stable transfectants that express various β4 mutants as GFP fusion proteins through fluorescence activated



Name	Position (aa)	N-Terminal HIS-tag, T7 promoter	C-terminal myc-tag, CMV promoter	C-terminal EGFP, CMV promoter
$ß_4$-$F_{1,2}$	1126-1315	pGR1	pGR16	-
$ß_4$-$F_{1,2}$L	1126-1457	pGR36	-	pGR45
$ß_4$-$F_{1,2}$L(F_3)	1126-1485	pGR5	-	pGR40
$ß_4$-F_1	1126-1218	pJP1	-	-
$ß_4$-F_2	1219-1314	pJP2	-	-
$ß_4$-L	1316-1457	pGR2	pGR17	pGR38
$ß_4$-F_2L	1219-1457	pGR4	pGR18	-
$ß_4$-F_3	1457-1546	pJP3	-	-
$ß_4$-F_4	1570-1662	pJP4	-	-
$ß_4$-$F_{3,4}$	1457-1662	pJP5	-	-
$ß_4$-$F_{(3),4}$C	1486-1752	pGR3	pGR13	pGR39
$ß_4$-L$F_{3,4}$C	1316-1752	pGR37	-	pGR41
$ß_4$-$F_{3,4}$C	1458-1752	pGR44	-	-
$ß_4$- $F_{1,2}$L$F_{3,4}$C	1126-1752	pGR6	pGR20	pGR30
$ß_4$-TM-F_2	708-1315	pGR42	-	pGR46
$ß_4$-TM-C	708-1752	pGR43	-	pGR47

F... Fibronectin type3-like repeat
L... Linker region between fibronectin pairs
C... C-terminal region (C-terminal of second fibronectin pair)
TM... Transmembrane domain

Figure 3. Schemes of the cytoplasmic tail of β4 integrin and bacterial and mammalian expression constructs. TM, transmembrane; EC, extracellular; IC, intracellular

cell sorting (FACS) and G418 selection for the transfectants.

The expression of β4 mutants as GFP-fusion proteins is shown in figure 4. Co-immunoprecipitation was performed to determine the interaction of 12-LOX with various β4 mutants. As shown in the

figure 5, 12-LOX is able to bind to the fusion proteins expressed by pGR40, pGR45, pGR46, and pGR47, but not to the fusion protein with only the linker region (pGR38). 12-LOX weakly binds to the fusion protein encoded by pGR39. Collectively, the data suggest a strong binding site(s) for 12-LOX located between 1126–1315 of β4 cytoplasmic tail.

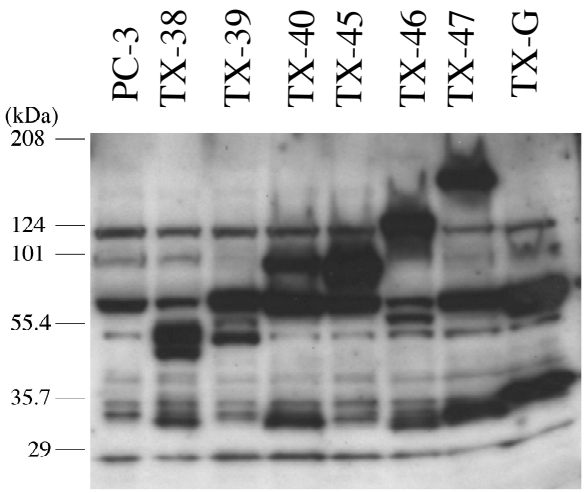


Figure 4. Western blot analysis for the expression of β4 mutants in PC-3 transfectants with an antibody against GFP. Note the expression of GFP fusion proteins of various MWs. The calculated MW for fusion proteins for pGR47 (Tx47) is about 146. For pGR46 (Tx46), 97.4; pGR45 (Tx45), 66.8; pGR40 (Tx40), 70; pGR39 (Tx39), 59.6; and pGR38 (Tx38), 45.7. The MW for pEGFP (TxG) is approximately 30.

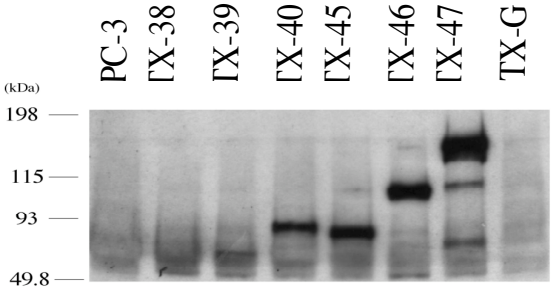


Figure 5. Co-immunoprecipitation of 12-LOX with the fusion proteins expressed by pGR40, pGR45, pGR46, and pGR47, but not to pGR38 and pEGFP (TxG). It is deduced that a binding site or sites are located between 1126 – 1315 region of the β4 cytoplasmic tail.

Cytosolic β4 cytoplasmic tail binds to 12-LOX and blocks the interaction between 12-LOX and full length β4 integrin: As shown above, we have constructed a panel of expression constructs encoding various mutants for the β4 cytoplasmic tail. The construct pGR30 encodes the cytosolic β4 cytoplasmic tail, with TM domain deleted, as a GFP-fusion protein (Figure 3). When ectopically expressed in 12-LOX transfected PC-3 cells, the cytosolic β4 cytoplasmic tail interacts with 12-LOX (Figure 6, the IP: 12-LOX / IB: GFP panel). Interestingly the ectopically expressed cytosolic β4 tail blocks the interaction of 12-LOX with full-length β4 integrin (Figure 6, the IP: 12-LOX / IB: β4 panel). The blockade of the interaction between 12-LOX and full-length β4 integrin is not due to the lack of β4 expression (Figure 6, the IB: β4 panel), but due to the presence of the cytosolic β4 cytoplasmic tail (Figure 6, the IB: GFP panel). The results suggest that the cytosolic β4

mutant, encoded by pGR30, is able to block the interaction between 12-LOX and full-length β4 integrin in a dominant negative manner.

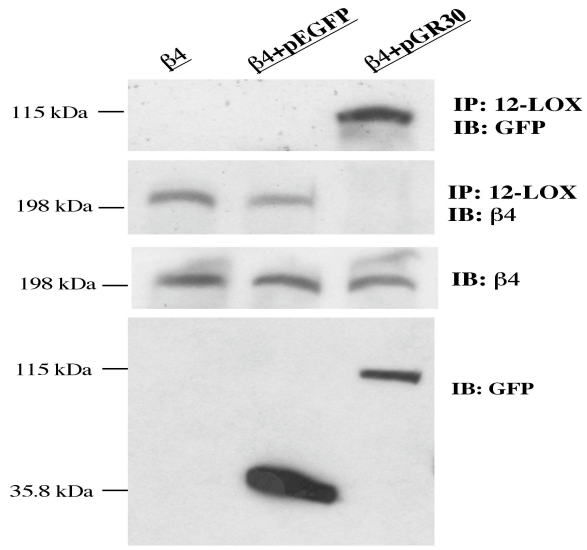


Figure 6: Cytosolic β4 tail, encoded by pGR30, binds to 12-LOX and blocks the interaction between 12-LOX and full length β4 integrin. 12-LOX transfected PC-3 cells (PC3/12LOX) were transiently transfected with an expression construct for full-length β4, in the presence or absence of pGR30 or its vector, pEGFP. Cell lysates were immunoprecipitated with a polyclonal antibody against 12-LOX and immunoblotted for GFP (JL8 mAb, Clontech) or full-length β4 integrin (AB1922, Chemicon International. Target band, around 205 kDa). Total lysates were also analyzed for the expression of full-length β4 integrin and GFP or GFP-fusion protein by immunoblot. Note the co-immunoprecipitation of 12-LOX and the cytosolic β4 cytoplasmic tail and the blockade of the interaction between 12-LOX and full-length β4 integrin by co-transfection with pGR30.

Task 5. Evaluate the growth rates of s.c. tumors derived from α6β4 expressing PC-3 cells, in the presence or absence of stable 12-LOX expression, and compare with that of control PC-3 cells, Months 24-36:

We have conducted a preliminary study to determine whether ectopic expression of β4 mutants, especially those that can bind 12-LOX, can effect 12-LOX activity. As shown in Figure 7, there was a reduction in 12(S)-HETE biosynthesis in PC-3 cells expressing two 12-LOX binding β4 mutants (pGR40 and pGR47). We are still in the process of determining whether 12-LOX cellular localization and interaction with the full-length β4 integrin are altered as result of the presence of β4 mutants encoded by pGR40 and pGR47. When injected into athymic nu/nu mice, it was found, as shown in Figure 8, that there were a reduction in tumor growth rate in PC-3 cells expressing 12-LOX binding β4 mutants (Tx40 and Tx47, see figure 5), when compared to those from vector control (TxG) or a β4 fragment that does not interact with 12-LOX (Tx38, see Figure 5).

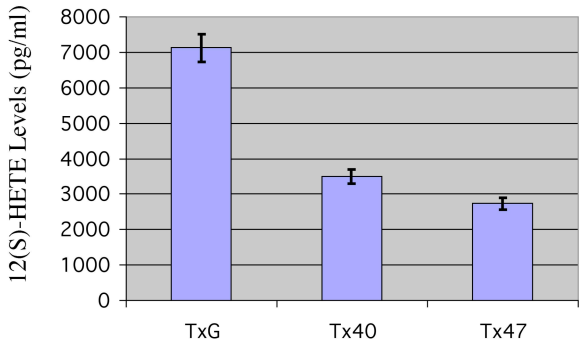
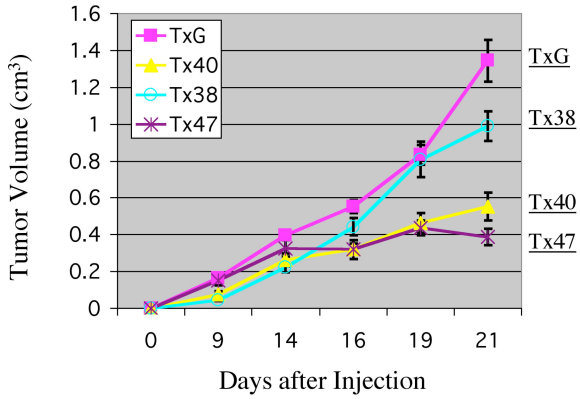


Figure 7. Decrease in 12(S)-HETE biosynthesis in PC-3 cells that express 12-LOX binding β4 mutants. 12(S)-HETE was isolated from cell lystaes and measured by EIA. N = 6 for each type of cells. Bar, SEM.



Figigure 8. Alteration in PC-3 tumor growth as a result of ectopic expression of 12-LOX binding β4 mutants (Tx40 and Tx47), not non-12LOX-bind mutant (Tx38). TxG is the vector control. Each datum point, the average tumor volume from 4 mice. Bar, SEM. Note the correlation of the reduction in tumor growth with reduced 12(S)-HETE biosynthesis shown in figure 7.

Key Research Accomplishments:

- Optimized the conditions for immunohistochemical analysis of 12-LOX and β4 at the protein level in paraffin-embedded human prostate tumor tissues. This standardized method will enable us to continue with the correlation between 12-LOX expression and distribution of β4 integrin with Gleason score.
- Constructed several β4 cytoplasmic tail mutants and identified the sequence of amino acids on β4 integrin that interacts with 12-LOX.
- Preliminary experiments conducted to show the feasibility of disruption of the interaction of 12-LOX with β4 integrin in PC-3 cells with ectopic expression of the cytoplasmic tail sequence of β4 integrin. This resulted in the reduction of the biosynthesis of 12-HETE as well as tumor growth from PC-3 cells in experimental animals.

Reportable Outcomes:
NONE

Conclusions:
Interaction of 12-LOX with the cytoplasmic tail of β4 integrin results in the disruption of hemidesmosomes. Our data presented here demonstrates the potential to exploit the interaction between

12-LOX and β4 integrin using ectopically expressed cytoplasmic tail sequence of the integrin to modulate tumor growth.

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Appendices:

NONE