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Fibroblast activation protein-alpha, a serine protease that facilitates metastasis by modification of diverse microenvironments

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14. ABSTRACT
Our overarching hypothesis is that FAP functions with other proteases in an extracellular communication network to digest certain proteins, thereby exposing signals stored in peptide regions that enable breast cancer cells to thrive in diverse microenvironments. FAP likely has important functions in two parts of the metastatic cascade: 1) FAP and proteases such as MMP-1 and MMP-9 cooperate to produce fragments of ECM proteins during adjacent tissue remodeling and these derivative peptides promote fibroblast growth, ECM deposition and angiogenesis; 2) cancer cell membrane FAP cleaves precursive A2AP to generate the more effective derivative for protecting and stabilizing fibrin within ECM margins of the expanding neoplastic cell mass as well as fibrin within cancer cell/fibrin/platelet emboli that lead to hematogenous metastasis. We believe that peptides that target and inhibit FAP on FAP-expressing cells can be produced by taking advantage of the substrate/active-site binding specificity of FAP.

This progress report documents the preparation of cell lines needed to complete the aims of the project as well as new discoveries of FAP suppression of DPPIV expression and a putative role for FAP in suppressing the innate immune response to breast cancer cells. Substantial progress has been made and has resulted in production of 2 abstracts presented at international meetings, and 4 grant proposals.

15. SUBJECT TERMS
Seprase, extracellular matrix, microembolism, tumor biology, angiogenesis

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Introduction

Fibroblast **a**ctivation **p**rotein-α (FAP), a prolyl-specific serine proteinase, is a constituent membrane protein of activated fibroblasts that synthesize some of the major components of the extracellular matrix (ECM). FAP is recognized on embryonic mesenchymal tissue and on fibroblasts during wound healing, but it is not expressed by normal, quiescent fibroblasts or benign tumor fibroblasts. Interestingly, FAP is over expressed by stroma within epithelial-derived cancers such as breast [1-3]; however, its precise role is unknown. Although best known on activated fibroblasts, in the tumor FAP is also expressed on epithelial cancer cells [2, 4, 5] and has been linked to invasive, matrix-degrading behavior of malignant melanoma and breast cancer cells [6]. In animal models, over expression of FAP by the malignant cells also stimulates rapid growth of cancer [7-9].

FAP is a member of the dipeptidyl peptidase family and/or structural homologs (DASH) family of prolyl-specific peptidases [10-12]. The FAP protease is a dual function serine protease having both N-terminal prolyl-specific dipeptidase and endopeptidase activities [13-15]. The dipeptidase activity is presumably important for modifying chemokines [10, 12], but the role of FAP's endopeptidase activity (gelatinase) that degrades denatured collagen remains unclear, since the latter activity is not possessed by the closely related enzyme, dipeptidyl peptidase IV (DPPIV) [16]. Recently our collaborator, Dr. Patrick McKee's laboratory identified low molecular weight proteolytic fragments of type I collagen that are produced by FAP cleavage [17]. His group also purified an enzyme present in trace amounts in human plasma, which turned out to be a soluble form (sFAP) of membrane-bound FAP (mFAP) [18]. Except for lacking the first 26 residues of its N-terminus that comprise the intramembrane and cytosolic segments, the soluble FAP's primary structure and proteolytic properties appear identical to naturally-occurring FAP. McKee's group made the unique discovery that sFAP cleaves precursive Met-alpha2-antiplasmin (methionine as the N-terminus: Met-A2AP) to yield a 12-residue N-terminally shortened derivative, Asn-alpha2-antiplasmin [18]; both A2AP forms circulate in plasma and are responsible for >95% of the inhibition of plasmin activity towards fibrin, that is a recognized component of ECM as well as platelet/malignant cell/fibrin microemboli. Importantly, the FAP-cleaved A2AP maintains antiplasim activity and incorporates more readily into fibrin than its uncleaved counterpart. We reasoned that FAP on breast cancer cells might promote metastasis by increasing the formation of microemboli.

This synergy award is designed to determine if FAP proteolytic activity promotes formation of microemboli, tumor growth, and degradation of the extracellular matrix. The second period has been productive in terms of preparing the cells needed to pursue these questions and for working out the conditions for applying inhibitors while evaluating FAP-mediated matrix degradation. Kelly's part of the project involves determining microembolus formation in vivo and in vitro as well as aggressive matrix behavior in vitro.

Body

Last year we reported that plasmid cDNA constructs had been made in order to engineer MDA MB-231 cancer cells to express both FAP and GFP so that we could easily detect them after

injecting them into the venous circulation of mice. Here we report that Avis E. Simms and Yan Huang have produced MDA MB-231 human breast cancer cells that over express both FAP and GFP together and MDA MB-231 control cells that do not express FAP but express high levels of GFP. Flow cytometry reveals that the FAP expression is high on 84% of these WTY-1 breast cancer cells but the GFP expression is seen in only about 25% of the cells. However, 66% of the GFP positive cells are also positive for FAP. We will continue sorting them using FACS technique to improve the percentage of cells expressing both GFP and FAP. At present, with the FAP^{high}/GFP and FAP^{Low}/GFP cells that we have in hand we will start the experiments outlined in the SOW under specific aims 2 & 3 indicating development of in vitro and in vivo models of microemboli formation. This will be accomplished under the one year no-cost extension period that we requested in July, 2010 and got approved in September, 2010.

Avis E. Simms, a graduate student in the lab, has produced data regarding fibrin degradation associated with cells expressing active FAP, mutant FAP, or very little FAP. These data specifically address of aspects of SOW specific aim 1. Initially Ms Simms evaluated the association of α2-antiplasmin (A2AP) with the cells by immunofluorescence microscopy. Ms Simms found no difference in the levels of A2AP associated with cells that don't express FAP (231), cells expressing active FAP (WTY6), or those expressing inactive mutant FAP (S5) (not shown). The fibrin degradation associated with 231, WTY-6, and S5 cells was determined using a commercially available ELISA kit for clinical measurement of the D-dimer product of fibrin breakdown, Asserachrom® D-Di (Diagnostica Stago, Asnieres-Sur-Seine, France). Equal numbers of cells were grown in 6 well plates and serum free medium. The cells received fibrinogen, thrombin and factor XIII (all wells). Some wells received A2AP alone, others received plasmin without A2AP. One group received all elements including plasmin and A2AP.

The results show that there is both plasmin independent and plasmin dependent degradation of fibrin by these cells. Moreover, A2AP is very effective at suppressing plasmin-dependent fibrin degradation regardless of FAP expression by the cells (Fig. 1). Surprisingly, A2AP was also effective at plasmin independent fibrin degradation (Fig. 1, Fibrin, A2AP).

The observed plasmin-independent fibrin degradation suggested that other proteases produced by the cells might be mediating the fibrin degradation. Indeed, earlier we showed that expression of either wild type and active FAP or a catalytic mutant FAP caused elevated expression of matrix metalloproteinase-9 (MMP-9) (see first progress report, submitted manuscript in the Appendix). To determine if MMPs might be mediating the cleavage of fibrin, the cell-mediated fibrin cleavage was investigated in the presence or absence of the broad-spectrum MMP inhibitor BB-94. We found that MMP inhibition greatly decreased the levels of plasmin independent fibrin degradation (Fig. 2, Fibrin; Fibrin, A2AP). Not surprisingly, MMP inhibition does not inhibit the plasmin dependent fibrin degradation (Fig 2, Fibrin, Plasmin; Fibrin, Plasmin, A2AP).

The results are consistent with the idea that FAP expression on MDA MB-231 human breast cancer cells leads to an increase in MMP-9 release (see manuscript in the appendix of last year's progress report), and that this increased MMP-9 actually stimulates dissolution of fibrin

and associated microemboli. If this is true then we would expect the opposite of the hypothesis driving specific aim 2 of the grant. Namely, that FAP should lead to increased incorporation of A2AP into fibrin and thereby stabilize fibrin coating of tumor cells. However, A2AP is still quite effective at blocking the plasmin dependent fibrin degradation and may have a lesser inhibitory effect on fibrin degradation mediated by MMPs. Therefore, the results are inconclusive as to what the net result will be in vivo where there is abundant plasmin in the blood and extracellular fluids. We will test for fibrin accumulation and tumor platelet-cell aggregate formation in the presence or absence of A2AP for FAP expressing and non-expressing breast cancer cells. These experiments will be done in vitro under flow and in vivo as described in the grant proposal.

FAP suppresses DDPIV expression.

FAP is closely related to DPPIV and the enzymatic functions of FAP and DPPIV overlap at least partially [19]. Because FAP expression elevated MMP-9 release and altered phosphotyrosine proteins (manuscript in Appendix of first year progress report) we investigated if there were also changes in surface expression DPPIV. The parental MDA MB-231 human breast cancer cells have very little FAP expression but reveal high levels of cell-surface DPPIV (Fig. 3A, Parental^{FAPLow}). On the other hand, these cells engineered to express FAP to high levels reveal very little surface expression of DPPIV (Fig. 3A, WTY-6$^{FAP\ High}$). Moreover, the FAP proteolytic activity is not required to suppress expression of DPPIV as it is reduced in parental MDA MB-231 cells expressing a mutant of FAP that is proteolytically inactive (Fig. 3A, S624A-5$^{FAP\ High}$). This FAP-mediated suppression of DPPIV expression is also observed at the mRNA level by RT-PCR (Fig 3B). These results suggest that FAP-mediated signaling causes a suppression of cell-surface DPPIV. This in turn may correlate with the already published aggressive behavior of FAP-expressing breast cancer cells [9].

In order to help link any observed alterations in microembolus formation to FAP and not to DPPIV, we sought to produce FAP suppressed cells from the WTY-6$^{FAP\ High}$ cells using shRNAs and lentiviral vectors. We purchased five FAP-specific cDNA clones encoding the FAP shRNA (TRCN0000006802, TRCN0000006803, TRCN0000006804, TRCN0000006805, and TRCN0000006806; which we abbreviate 802-806 respectively) from Open biosystems, packaging vector (pSPAX2), and the envelop vector (PCI-VSVG) from Addgene. We successfully grew the plasmid DNA for each of the clones, packaging, and envelop vector (Fig. 4A). Each of the clones was co-transfected with the packaging and envelope vectors into 293 cells to produce separate lentvirus progeny that could cause expression of each one of the five individual clones. After infection, the cells were selected using puromycin.. Subsequently, culture supernatants were harvested containing lentiviruses carrying the FAP-specific clones. These supernatants were used to infect WTY-6$^{FAP\ High}$ cells. After selection with puromycin, RT-PCR analysis of RNA isolated from infected WTY-6$^{FAP\ High}$ cells revealed that clones 802, 803, and 806 did not suppress FAP expression but clones 804 and 805 were effective at suppressing FAP at transcription level (Fig 4B, shows data for clone 804). To determine if cell surface expression of FAP was suppressed the cells were collected from the culture dish using EDTA

treatment and gentle scraping. The cells were stained with F19 monoclonal antibody to FAP and subjected to flow cytometry. Consistent with the RT-PCR results, WTY-6$^{FAP\ High}$ cells infected with lentivirus encoding FAP-specific shRNA clones 802, 803 and 806 did not suppress cell surface expression of FAP (Fig. 5A-C) but clones 805 and 804 (Fig. 5D & E) did suppress surface expression of FAP relative to WTY-6$^{FAP\ High}$ cells (Fig. 5F). It was observed that MDA MB-231 cells that do not express FAP have high levels of DPPIV and that DPPIV is suppressed when these cells were engineered to express FAP (Fig. 3). We therefore investigated the possibility that suppression of FAP might result in an increase in DPPIV expression. Indeed, flow cytometry reveals that WTY-6$^{FAP\ High}$ cells have high levels of FAP expression (>82% cells positive) and low levels of DPPIV expression (0.38% positive cells) (Fig. 6A & C) while, WTY-6$^{FAP\ High}$ cells infected with lenti virus carrying clone 805 FAP-specific shRNA have suppressed FAP expression (7.9 % positive as compared to WTY-6$^{FAP\ High}$ cells where 91.5% cells are positive) but increased DPPIV expression (14.83 % positive cells versus the MDA MB-231 cells that are negative for FAP and have 47.21% cells positive for DPPIV) (Fig. 6B & D).

Clearly, in this section we show that FAP expression suppresses DPPIV expression on breast cancer cells. Moreover, suppression of FAP leads to increased expression of DPPIV. This indicates that relative amounts of the two molecules on the cell surface may determine the overall phenotype of the cell. For example, we have shown that parental MDA MB-231 human breast cancer cells produce tumors that grow relatively slowly in immune compromised mice as compared to fast growing tumors formed by WTY-6$^{FAP\ High}$ cells and other populations of breast cancer cells that express FAP to high levels. Thus, DPPIV may be at one end of the spectrum promoting slower growth whereas FAP may be at the other end promoting rapid tumor growth. Given that FAP and DPPIV can form complexes it is not hard to envision such a system that is similar to the function of bcl-2 and bax proteins. These apoptotic proteins determine if the cell will survive or go into apoptosis by their relative amounts where bcl-2 like proteins favor survival and bax like proteins favor cell death.

FAP may suppress innate immunity.

The overall theme of this synergy grant is that FAP may promote survival of breast cancer cells in diverse microenvironments. One such environment is the blood stream where microembolus formation may be facilitated by FAP expression. Ability to evade the immune system may be important in several microenvironments that breast cancer cells encounter as they progress and metastasize. Micro-emboli themselves may promote tumor cell evasion of the immune system. Because of the homology of FAP to DPPIV and the fact that the substrate specificities of FAP and DPPIV share some overlap, we became interested in investigating a possible role for FAP in suppressing innate immunity by cleaving chemokines that attract NK cells. For the initial experiments, Ms Anna Mazur, a graduate student in the lab, used RT-PCR to evaluate the expression of a panel of chemokines known to be chemoattractive for NK cells.. She found that parental MDA MB-231 human breast cancer cells express IL-1β, IL-8, MCP1, and RANTES (Fig. 7, Parental). It was noted that IL-1β and IL-8 were expressed much more abundantly than the others (Fig 7, Parental). Similarly WTY-6$^{FAP\ High}$ cells also expressed IL-1β, IL-8, MCP1, and RANTES, but in addition they had detectable levels of IP-10 (Fig. 7, WTY-6$^{FAP\ High}$). Again the

highest levels of expression were observed for IL-1β and IL-8 (Fig. 7, WTY-6$^{FAP\ High}$). The S624A$^{FAP\ High}$ cells that express high levels of an inactive FAP had high levels of IL-1β and IL-8 but the other chemokines were not detected (Fig. 7, S624A$^{FAP\ High}$).

We wondered if FAP might degrade chemokines? To begin to answer this question Ms Mazur partially purified FAP from detergent extracts of WTY-6$^{FAP\ High}$ using Wheat Germ Agglutinin (WGA) Agarose beads as we reported earlier [15]. This preparation is not FAP-specific in fact there many other proteins including DPPIV present in this preparation [15]. Nevertheless, the preparation does eliminate MMPs. We incubated the WGA Agarose beads containing our partially purified FAP with recombinant and homogeneous IL-8. We pelleted the WGA-Agarose and subjected the supernatant to analysis by Surface Enhanced Laser Desorption Time of Flight Mass Spectrometry (SELDI-TOF MS) as described by our collaborator, Dr. Larry Suva [20-22]. There is apparent increased degradation of IL-8 in WGA-Agarose beads containing active FAP from WTY-6$^{FAP\ High}$ cells (Fig. 8, WTY-6 & IL-8, blue trace). This as compared to no peaks in the bead control (Fig. 7, bead control, black trace) and just the intact peak of the IL-8 control (Fig. 7, IL-8, red trace). Low levels of degradation were observed with the extracts of MDA MB-231 parental cell mixed with IL-8 (Fig. 7, MDA MB-231 & IL-8, magenta trace). Surprisingly there is also somewhat elevated degradation of IL-8 from beads containing inactive FAP isolated from S624A$^{FAP\ High}$ cells (Fig. 7, S5 & IL-8, green trace). These results suggest that membrane extracts of cells overexpressing FAP can mediate degradation of IL-8. Future studies will be designed to confirm or deny that FAP is involved in this proteolytic degradation.

Key Research Accomplishments

- **Produced MDA MB-231 breast cancer cells expressing high levels of FAP and GFP**
- **Demonstrated plasmin dependent and plasmin independent fibrin degradation by FAP expressing cells**
- **Showed that even plasmin independent fibrin degradation is reduced by A2AP**
- **Demonstrated that MMPs mediate much of the plasmin independent fibrin degradation – This infers a role for FAP because we previously showed causes up-regulation MMP-9**
- **Produced lenti viruses for 5 different clones encoding shRNAs specific for FAP**
- **Two FAP-specific clones were successful in suppressing expression of FAP**
- **Demonstrated that FAP expression suppresses DPPIV expression**
- **Conversely, FAP suppression induces increased DPPIV expression – these findings support the notion that FAP stimulates cell signaling**
- **Identified several chemokines that attract innate immune cells and are expressed by MDA MB-231 cells and these cells engineered to express wild type or mutant FAPs.**
- **Found that partially purified FAP extracts can proteolytically degrade IL-8.**
- **Presented two abstracts at international meetings.**

Reportable Outcomes

Abstracts/Presentations:

1. “Fibroblast activation protein-α: A potential modulator of chemokines that regulate natural killer recruitment to breast tumors” Yan Huang, Anna Mazur, Avis E. Simms and Thomas Kelly, Cell Adhesion and Stress Fibers: A symposium in honor of Keith Burridge, August 27-29, 2010, Chapel Hill, NC (Included in the appendix)

2. “Alpha 2-Antiplasmin (α2AP) Can Promote Fibrin Coating of Tumor Cells through Inhibiting Fibrin Degradation by Matrix metalloproteinases (MMPs) and Plasmin” Avis E. Simms and Thomas Kelly, Jackson Heart Study Scientific Conference. 9/23-24/2010. Jackson, MS. (Included in the appendix)

Funding applied for:

1) USAMRDC-BCRP Pre-doctoral fellowship BC100366 “Fibroblasts protect breast cancer cells from innate immunity”, PI Anna Mazur (Not Funded, Overall score 1.8, Excellent)

2) NIH, NCI, 1R21CA155057 Fibroblasts protect breast cancer stem cells from innate immunity, PI Thomas Kelly (Not Funded, Unscored).

3) USAMRDC-BCRP Idea grant application BC100281 “Matrix remodeling by cancer associated fibroblasts promotes breast tumor growth”, PI Thomas Kelly, (Pending)

4) UAMS, Medical Research Endowment, Fibroblasts protect breast cancer stem cells from innate immunity, PI Thomas Kelly (Pending)

Ongoing development of cell lines

FAP-GFP-luc cells in production that is originally derived from MDA MB-231 human breast cancer cells. FAP-pluc cells in production that is originally derived from MDA MB-231 human BrCa cells

Research opportunities

Avis Simms, PhD candidate in the Interdisciplinary Biomedical Sciences (IBS) program, presented her research at the Jackson Heart Study Scientific Conference. Anna Mazur, graduate student in the IBS program has joined the laboratory and is making significant laboratory progress.

Conclusion

In summary we have made significant and important progress by positioning ourselves to complete the experiments that will directly test the possibility that FAP promotes formation of microemboli and that these facilitate metastasis of breast cancer. Moreover, we have discovered that FAP expression apparently suppresses expression of DPPIV and conversely when FAP is suppressed, DPPIV is induced. This suggests that FAP may control the surface protease phenotype of breast cancer cells. Finally, we have data suggesting a novel role for FAP in promoting the survival of tumor cells by suppressing the innate immune response to the tumor cells.

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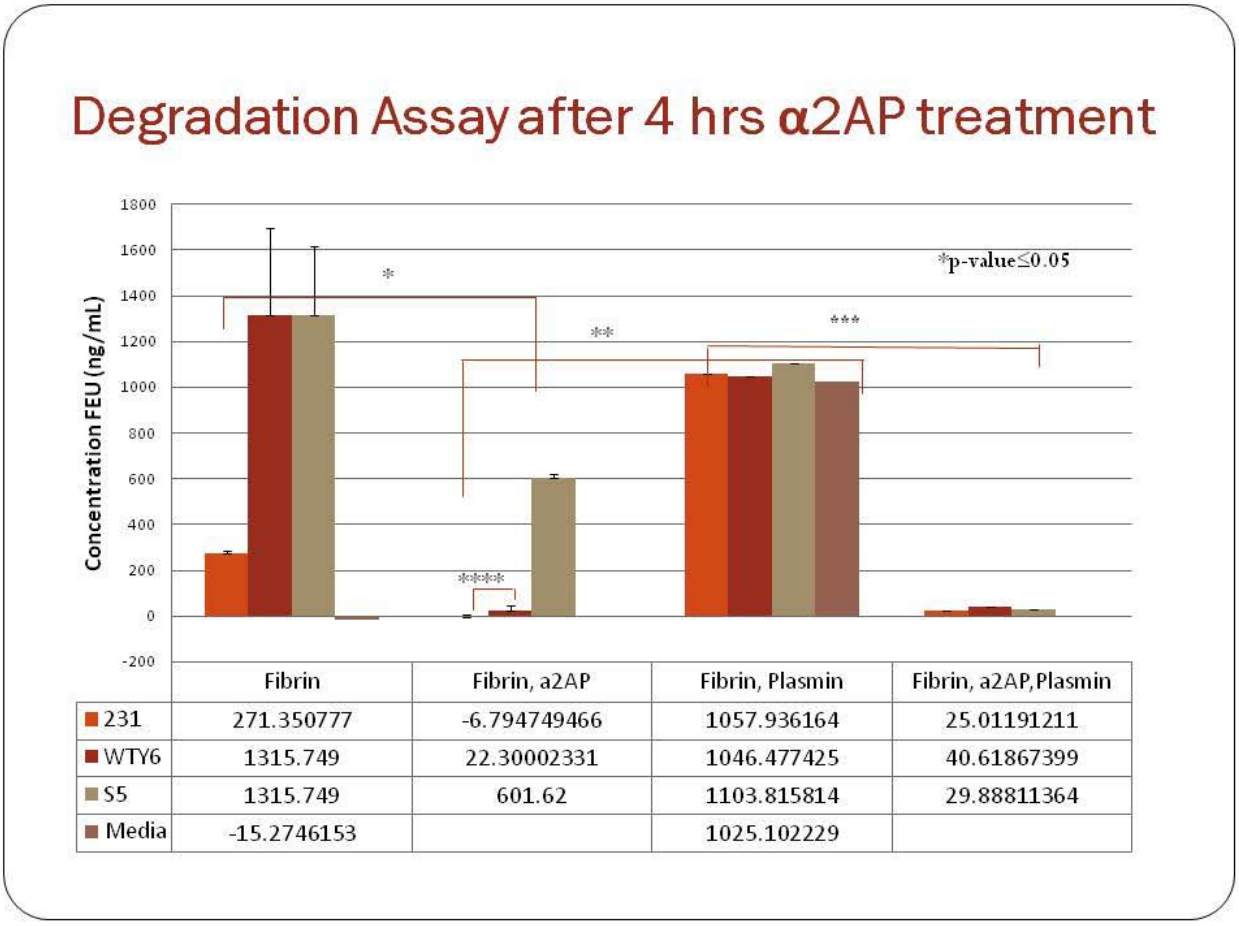
Figures and Figure Legends



	Fibrin	Fibrin, a2AP	Fibrin, Plasmin	Fibrin, a2AP,Plasmin
231	271.350777	-6.794749466	1057.936164	25.01191211
WTY6	1315.749	22.30002331	1046.477425	40.61867399
S5	1315.749	601.62	1103.815814	29.88811364
Media	-15.2746153		1025.102229	

Figure 1. After 4h treatment with α2-AP plasmin independent and dependent fibrin degradation was decreased. After 4h incubation in the absence of plasmin fibrin degradation was slightly elevated by 231 cells, greatly increased by WTY-6 and S5 cells and not detected in medium without cells (Fibrin group). When treated with A2AP but not plasmin the fibrin degradation was greatly diminished even in the absence of plasmin (Fibrin, A2AP group). When treated with plasmin and not with A2AP, fibrin degradation was high for all conditions (Fibrin, Plasmin Group) and was suppressed for all conditions by A2AP (Fibrin, A2AP, Plasmin, group).

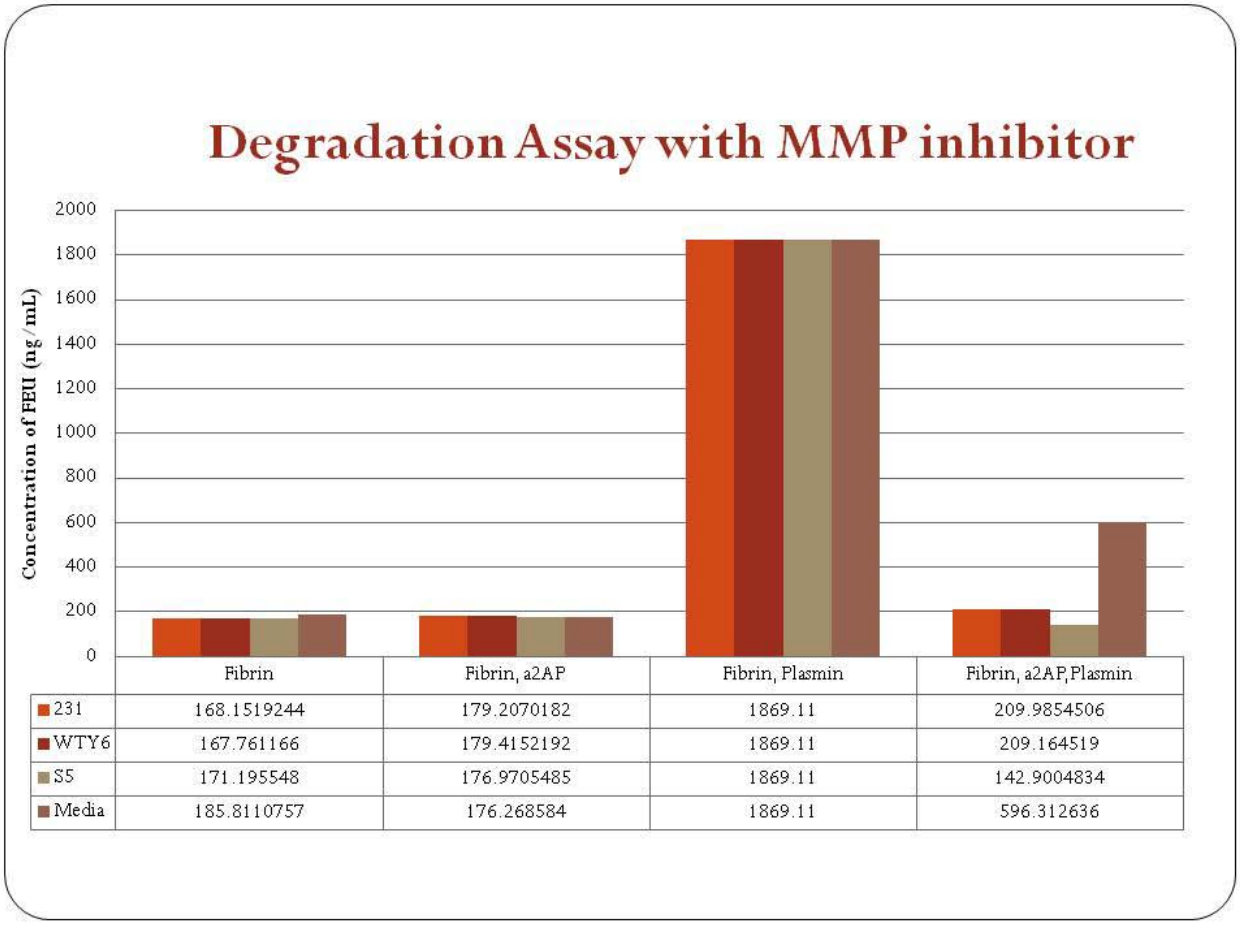


Figure 2. MMP inhibitors drastically decreased the plasmin independent fibrin degradation. Plasmin independent fibrin degradation greatly decreased when treated with BB94 (MMP inhibitor) regardless of whether or not A2AP was present (Fibrin group, Fibrin, A2AP group). Fibrin degradation still occurred in the presence of BB94 (1 μM) when treated with plasmin (Fibrin, Plasmin group). The plasmin dependent fibrin degradation was decreased when treated with α2-AP (Fibrin, A2AP, Plasmin).

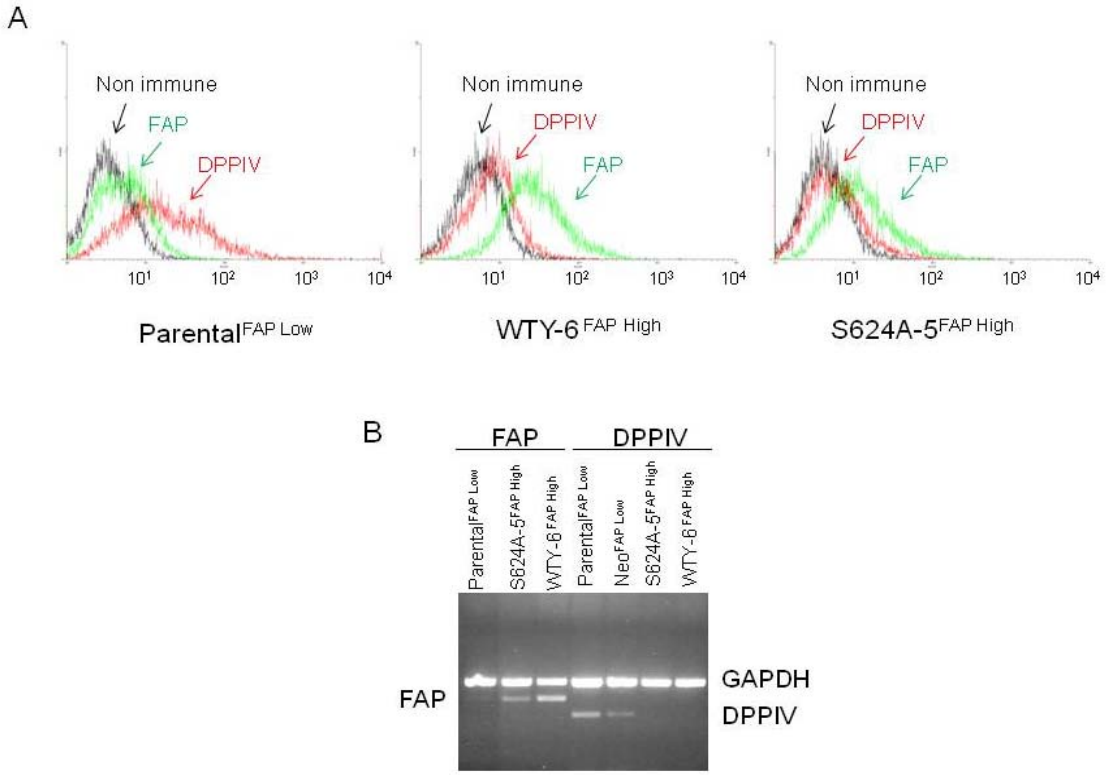


Figure 3. FAP expression suppresses DPPIV expression
A. Cell surface expression of DPPIV (red trace), FAP (green trace) and non immune IgG (black trace) on MDA-MB-231(Parental$^{FAP\ Low}$) cells and these engineered to express active (WTY-6$^{FAP\ High}$) or inactive FAP (S624A-5$^{FAP\ High}$). **B.** FAP (left panel) or DPPIV (right panel) message signalsv were detected in cellular extracts by RT-PCR using specific primers. GAPDH serves as internal control.

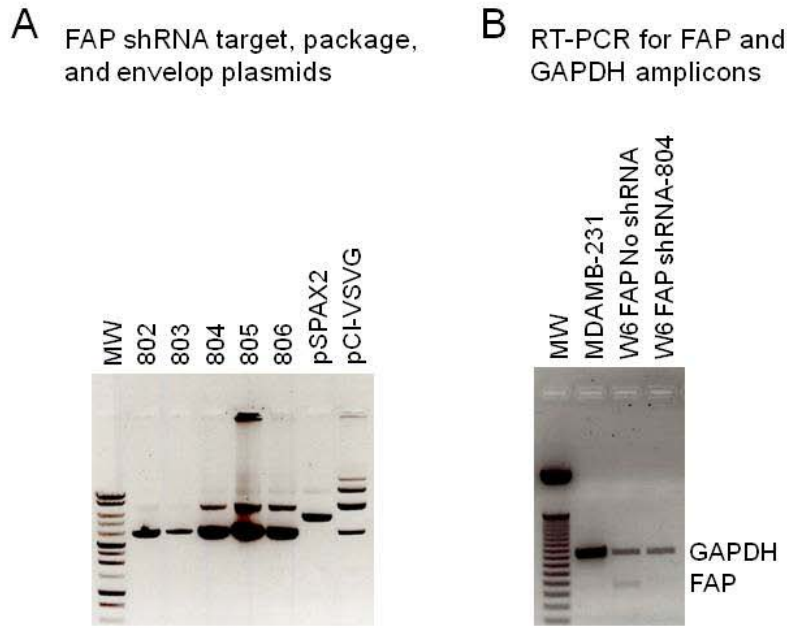


Figure 4. Supression of FAP expression by lenti viral vectors A. This gel shows the isolated DNA of the plasmids encoding FAP-specific shRNAs (802-806), the plasmid packaging vector DNA (pSPAX2) and the plasmid encoding the envelope DNA (pCI-VSVG) molecular weight standards are loaded in the left (MW). **B.** GADPH or FAP specific amplicons were detected by RT-PCR using primers specific for FAP and GAPDH. The MDA MB-231 cells do not express FAP and no FAP amplicon was detected (MDA MB-231). The WTY-6 cells express FAP to high levels and an amplicon was detected in uninfected cells (W6 FAP No shRNA) but not in cells infected with lenti viruses encoding FAP-specific shRNA-804 (W6 FAP shRNA-804).

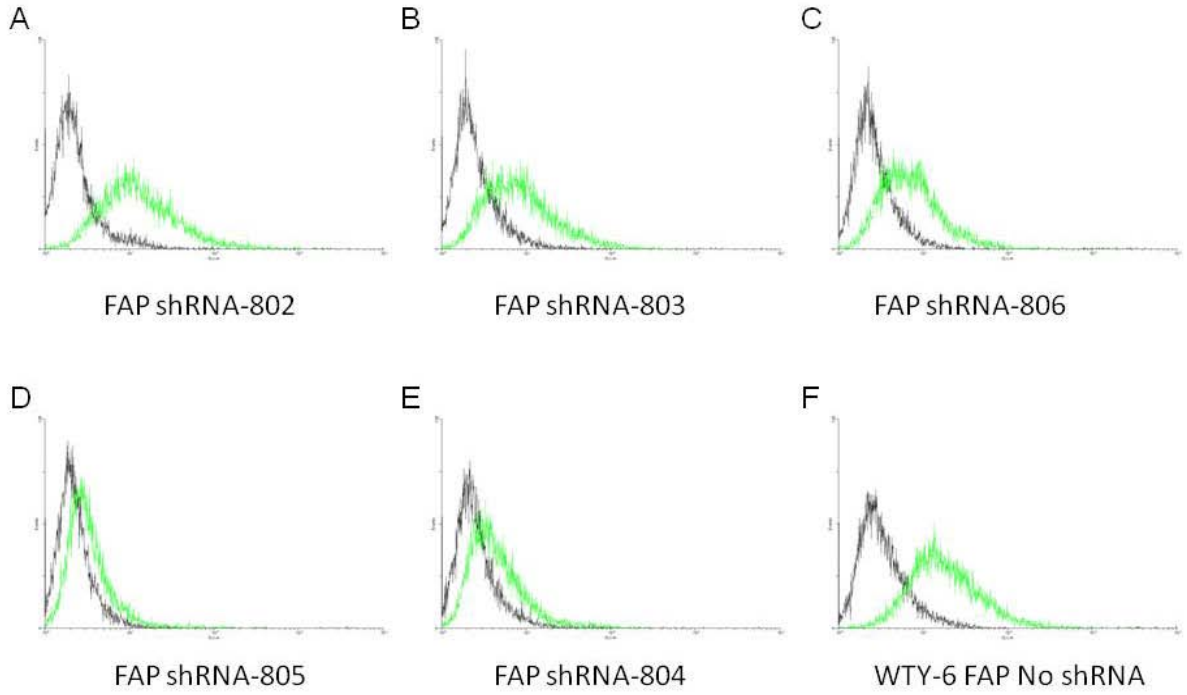


Figure 5. Suppression of FAP expression by lenti viral vectors containing clones 805 and 804 of FAP-targeted shRNA In histogram, the black trace is non immune IgG and the green trace is cell surface FAP expression **A-C.** WTY-6 cells were infected with lentiviruses encoding FAP-specific shRNAs 802 (A), 803 (B), and 806 (C). None of these were effective in reducing cells surface FAP expression. However WTY-6 cells infected with lentiviruses encoding FAP-specific shRNAs 805 (D) and 804 (E) showed suppression of FAP expression relative to the WTY-6 cells that were not infected with lentivirus (F).

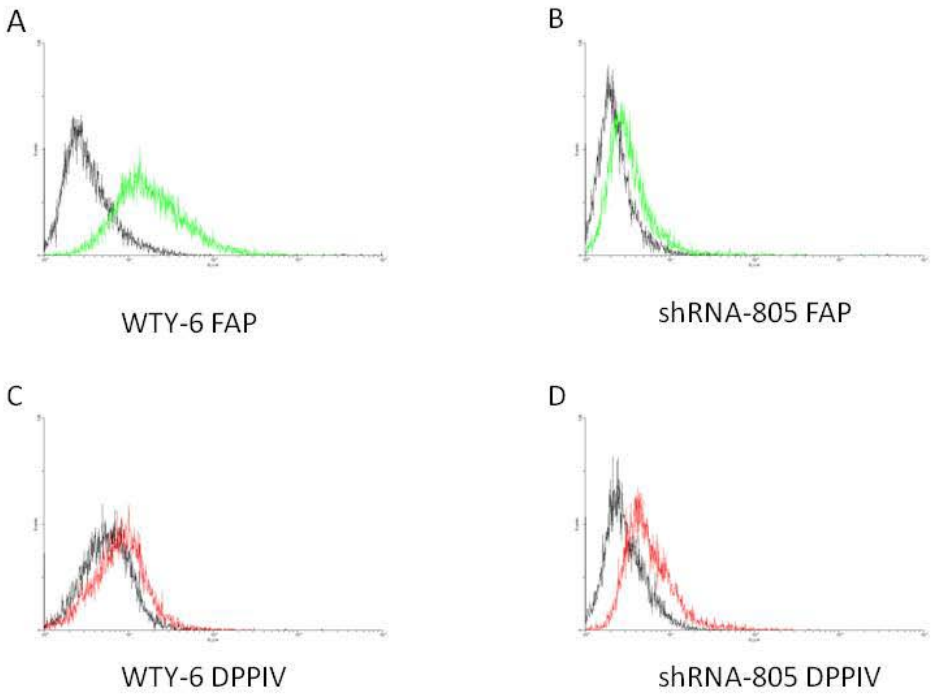


Figure 6. FAP suppression induces increased expression of DPPIV. A) FAP expression (green trace) is high on over 90% of the WTY-$6^{FAP\ High}$ cells. The black trace in all panels is the non-immune control. **B)** FAP expression (green trace) is suppressed in WTY-$6^{FAP\ High}$ cells by the shRNA from clone 805. **C)** DPPIV expression (red trace) on WTY-$6^{FAP\ High}$ cells is very low. **D)** DPPIV expression (red trace) is induced in cells where FAP is suppressed.

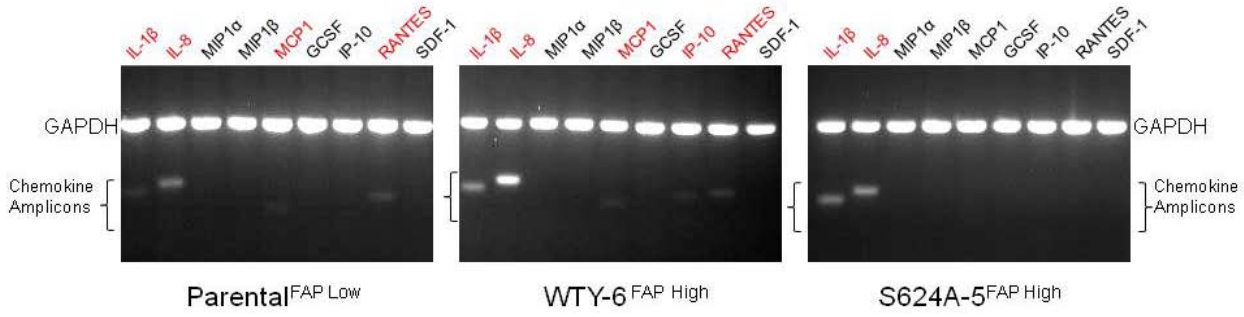


Figure 7. **IL-8 and other NK-cell attracting chemokines are expressed by the human breast cancer cells used in this study.** Chemokines were detected by RT-PCR from Parental$^{FAP\ Low}$, WTY-6$^{FAP\ High}$, or S624A-5$^{FAP\ High}$ cells using specific primers to produce amplicons between 180-220 bp. Reactions included primers for a 450bp GAPDH amplicon. Gel samples labeled red are positive for expression of the chemokine

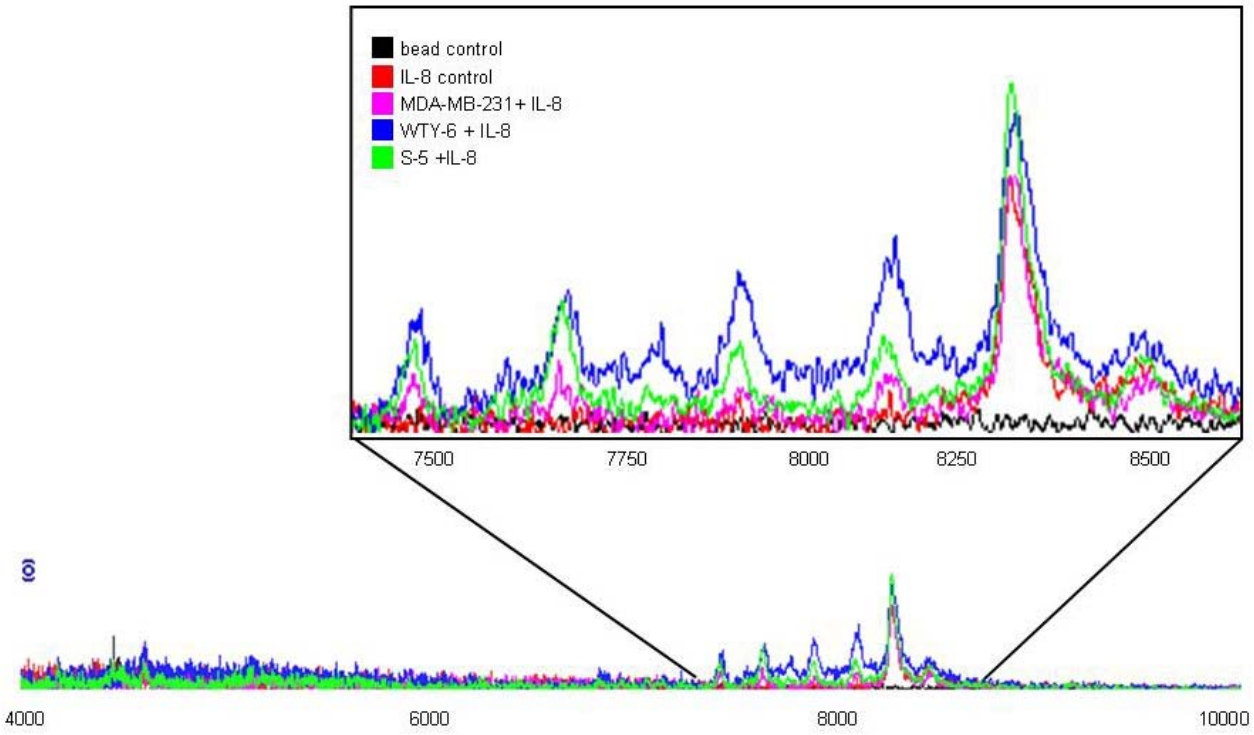


Figure 8. Increased proteolytic degradation of IL-8 is observed in cells that express FAP. Surface enhanced laser desorption time of flight mass spectrometry (SELDI) analysis of IL-8 proteolysis in the 4-10 kDa range. FAP from extracts of cells expressing active FAP to high (WTY-6$^{FAP\ High}$) or low (Parental$^{FAP\ Low}$) levels or mutant (S624A-5$^{FAP\ High}$) FAP was bound to wheat germ agglutinin agarose beads, incubated with purified IL-8, and then the supernatant subjected to SELDI. Lower molecular weight peaks in high FAP (blue trace) and less extensively, mutant FAP samples (green trace) indicate proteolysis of IL-8. IL-8 break down is low in parental cells (magenta trace). Intact IL-8 occurs at 8.2 kDa (red trace) and no peaks are from the beads (black trace).

Appendix

Poster presentations

1. “Fibroblast activation protein-α: A chemokine modulator regulating natural killer cell recruitment to tumors” Yan Huang, Anna Mazur, Avis E. Simms and Thomas Kelly, Cell Adhesion and Stress Fibers: A symposium in honor of Keith Burridge, August 27-29, 2010, Chapel Hill, NC (Included in the appendix)

2. “Alpha 2-Antiplasmin (α2AP) Can Promote Fibrin Coating of Tumor Cells through Inhibiting Fibrin Degradation by Matrix metalloproteinases (MMPs) and Plasmin” Avis E. Simms and Thomas Kelly, Jackson Heart Study Scientific Conference. 9/23-24/2010. Jackson, MS. (Included in the appendix)

Fibroblast activation protein-α: A chemokine modulator regulating natural killer recruitment to tumors?

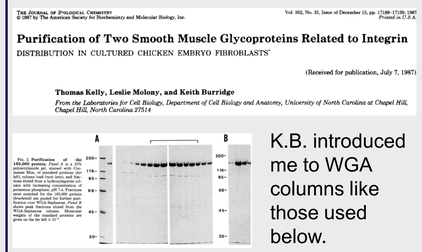
Yan Huang[1], Anna Mazur[1], Avis Simms[1], Charity Washam[2], Larry J.Suva[2], and Thomas Kelly[1]

[1]Department of Pathology, [2]Center for Orthopedic Research, Departments of Orthopedic Surgery and Physiology and Biophysics, Winthrop P. Rockefeller Cancer Institute, and University of Arkansas for Medical Sciences, Little Rock, AR

ABSTRACT

Dipeptidyl peptidases are a family of proteases that are implicated in the regulation of diverse biological processes. One member of the family, fibroblast activation protein-α (FAP), is a cell surface, post-prolyl serine protease that is over-expressed in human breast cancer but is not expressed in normal adult tissues. FAP has 50% amino acid sequence homology to another family member, dipeptidyl peptidase IV (DPPIV), and there is overlap in substrate specificities as both enzymes cleave NH_2-XAA-Pro peptides such as Gly-Pro-AFC. Importantly, DPPIV has been shown to cleave a number of chemokines including RANTES and SDF-1 that are known to be important in attracting innate immune cells such as natural killer (NK) cells. Proteolytic cleavage of RANTES and SDF-1 inactivates them and eliminates their attractive power for NK cells. Consistent with this, others have shown that inhibition of FAP by Val-boroPro, a broad spectrum inhibitor of post-prolyl proteases causes activation of immune responses of the host (Jones et al, 2003, Blood *102*:1641-8), perhaps through preventing the inactivation of chemokines. Previously, we showed that fast-growing tumors were produced by cells expressing proteolytically active FAP whereas parental MDA MB-231 do not express FAP and produce slow-growing tumors (Huang et al, 2004 Cancer Res. *64*:2712-2716). Here we investigate the tumor growth rates and differential expression of FAP, DPPIV, and chemokines in MDA MB-231 cells that do not express FAP and those engineered to express FAP to high levels. We extend our earlier findings by showing that cells expressing a catalytically inactive FAP produce rapidly growing tumors like cells expressing active FAP. We analyzed surface expression of FAP and DPPIV by flow cytometry to determine if DPPIV substitution for FAP results in the rapid growth of tumors produced by cells expressing a catalytically inactive FAP. We confirm that MDA MB-231 cells express little or no FAP and find that they express DPPIV to high levels. Conversely, these cells engineered to express wild type FAP or a catalytic mutant of FAP express FAP to high levels but reveal little DPPIV expression. These findings are confirmed by RT-PCR gene expression analysis. The results show that FAP expression correlates with rapid tumor growth and DPPIV expression correlates with slow tumor growth. Parental cells express DPPIV and the chemokines IL-1β, IL-8, MCP-1 and RANTES but little FAP; cells expressing proteolytically active FAP express these chemokines as well as IP-10 and little DPPIV; cells expressing inactive FAP express IL-1β and IL-8 only and little DPPIV. The findings are consistent with active FAP cleaving one or more of these chemokines and suppressing NK cell recruitment into the tumor thereby leading to rapid tumor growth. This work was supported by USAMRC, CDMRP, BCRP Synergistic Idea Award W91ZSQ7343N640 to TK and the Carl L Nelson Chair of Orthopaedic Creativity to LJS.

THIS STORY STARTED LONG AGO IN DR. KEITH BURRIDGE'S LAB



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Purification of Two Smooth Muscle Glycoproteins Related to Integrin
DISTRIBUTION IN CULTURED CHICKEN EMBRYO FIBROBLASTS*

(Received for publication, July 7, 1987)

Thomas Kelly, Leslie Molony, and Keith Burridge
From the Laboratories for Cell Biology, Department of Cell Biology and Anatomy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27514

K.B. introduced me to WGA columns like those used below.

Keith taught me so much about science and the wonder of discovery but while we worked hard….

….we also had a lot of fun!!

QUESTION

Does FAP expression suppress chemokines and recruitment of NK cells to tumors?

RESULTS

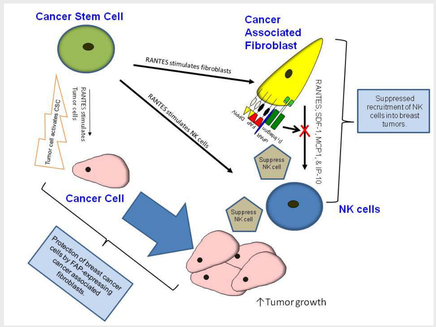


Figure 1. Cellular interactions in the tumor microenvironment. Cancer Associated Fibroblasts (CAFs, yellow) express FAP (red surface molecules) that may inactivate chemokines that attract NK cells and thereby suppress NK cell recruitment. The FAP activity of the CAFs may also decrease the tumor-killing function of NK cells.

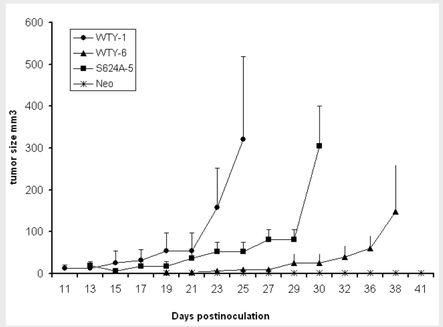


Figure 2. FAP promotes tumor growth. Growth of tumors of MDA MB-231 cells expressing wild type FAP (WTY-1 & WTY-6), inactive FAP (S624A-5) or no FAP (Neo). Growth curves for Neo, WTY-1 and WTY-6 were published previously (Huang et al, (2004) Cancer Research *64*:2712-2716. These human breast cancer cells were grown as tumors in the mammary fat pads of female SCID mice.

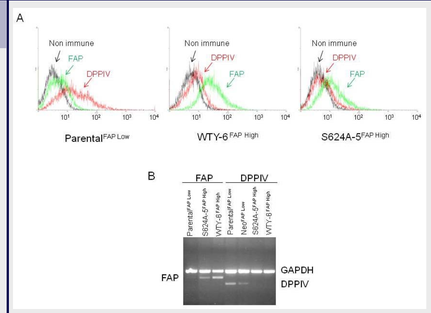


Figure 3. FAP expression suppresses DPPIV expression A. Cell surface expression of DPPIV (red trace), FAP (green trace) and non immune IgG (black trace) on MDA-MB-231(Parental$^{FAP\ Low}$) cells and these engineered to express active (WTY-6$^{FAP\ High}$) or inactive FAP (S624A-5$^{FAP\ High}$). **B.** FAP (left panel) or DPPIV specific (Right panel were detected by RT-PCR using primers specific for FAP and for DPPIV as well as GAPDH.

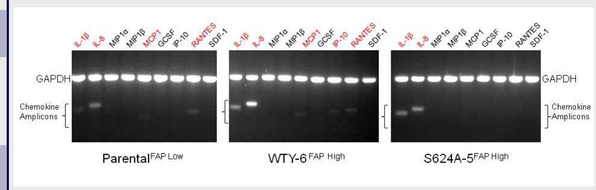


Figure 4. IL-8 and other NK-cell attracting chemokines are expressed by the human breast cancer cells used in this study. Chemokines were detected by RT-PCR from Parental$^{FAP\ Low}$, WTY-6$^{FAP\ High}$, or S624A-5$^{FAP\ High}$ using specific primers to produce amplicons between 180-220 bp. Reactions included primers for a 450bp GAPDH amplicon. Gel samples labeled red are positive for expression of the chemokine.

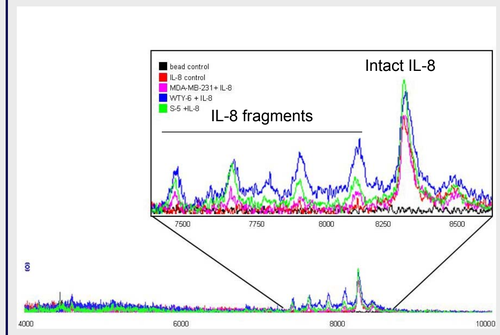


Figure 5. Increased proteolytic degradation of IL-8 is observed in cells that express FAP. Surface enhanced laser desorption time of flight mass spectrometry (SELDI) analysis of IL-8 proteolysis in the 4-10 kDa range. FAP from extracts of cells expressing active FAP to high (WTY-6$^{FAP\ High}$) or low (Parental$^{FAP\ Low}$) levels or mutant (S624A-5$^{FAP\ High}$) FAP was bound to wheat germ agglutinin agarose beads, incubated with purified IL-8, and then the supernatant subjected to SELDI. Lower molecular weight peaks in high FAP (blue trace) and less extensively, mutant FAP samples (green trace) indicate proteolysis of IL-8. IL-8 break down is low in parental cells (magenta trace). Intact IL-8 occurs at 8.2 kDa (red trace) and no peaks are from the beads (black trace).

CONCLUSIONS

1. Expression of FAP coincides with suppression of DPPIV and is independent of FAP proteolytic activity.

2. FAP expression correlates with rapid tumor growth while DPPIV expression correlates with slow tumor growth in an animal model of human breast cancer.

3. FAP may facilitate enhanced cleavage of IL-8 and perhaps other chemokines that attract NK cells. FAP may cooperate with other proteases to degrade chemokines.

Alpha 2-Antiplasmin (α2-AP) Can Promote Fibrin Coating of Tumor Cells through Inhibiting Fibrin Degradation by Matrix metalloproteinases (MMPs) and Plasmin

Avis E.Simms, and Thomas Kelly

Department of Pathology, Interdisciplinary Biomedical Sciences Program, Winthrop P. Rockefeller Cancer Institute, and University of Arkansas for Medical Sciences, Little Rock, AR

BACKGROUND

Metastasis is the most life-threatening aspect of cancer and the ability of tumor cells to form aggregates or microemboli composed of platelets, fibrin, and tumors cells apparently facilitates the metastatic process. Fibrin is a critical promoter of metastasis because when fibrin is present it increases the number of stable secondary tumor foci (metastases) arising from circulating tumor cells. Normally, fibrin is degraded and dissolved by the serine protease plasmin, but may also be degraded by matrix metalloproteinases (MMPs). α2-Antiplasmin (α2-AP) is a serine protease inhibitor that is the primary inhibitor of plasmin and serves to stabilize fibrin by protecting it from degradation by plasmin. Inhibition of fibrinolysis is dependent on the amount of α2-AP cross-linked to fibrin and when α2-AP cross-linking is impaired, inhibition of fibrinolysis is greatly decreased.The inhibition of fibrinolytic activity will allow for stabilization of polymerized fibrin in the blood and increase likelihood of coagulation and embolus formation.Interestingly, α2-AP has been identified as a substrate for fibroblast activation protein -α (FAP) a cell surface serine protease, which is found in elevated levels in human breast cancer. FAP-cleaved α2-AP remains a potent inhibitor of plasmin but is cross-linked to fibrin by the fibrin stabilizing factor (FXIIIa) 13 times faster than un-cleaved α2-AP during fibrin polymerization. Thus, FAP may facilitate metastasis by promoting the stabilization of fibrin and tumor microemboli through rapid recruitment of α2-AP into cell-associated fibrin. Our objective is to determine if FAP-cleaved or un-cleaved α2-AP will promote the metastasis of tumor cells by decreasing fibrin degradation around the cells. We hypothesize that FAP-cleaved α2-AP may prolong the life of fibrin by preventing degradation and promote encasement of FAP-expressing breast cancer cells in a fibrin sheath. To test this hypothesis, we used human breast cancer cells that do not express FAP (MDA- MB-231) or these engineered to express proteolytically active FAP (WTY-6) or a catalytically inactive mutant FAP (S5) as well as a mouse fibroblast line that should express active mouse FAP (NIH 3T3). To observe how α2-AP interacted with tumor cells; we did this through immunofluorescent microscopy. A D-Dimer assay was also done to observe α2-AP inhibited of fibrin degradation in the presence of tumors and in serum free media. This work was supported by USAMRC, CDMRP, BCRP Synergistic Idea Award W91ZSQ7343N640 to TK and Winthrop P. Rockefeller Cancer Institute Predoctoral Fellowship in Breast Cancer to AES.

METHODS

α2-AP Interaction with Cells

Immunofluorescence microscopy was used

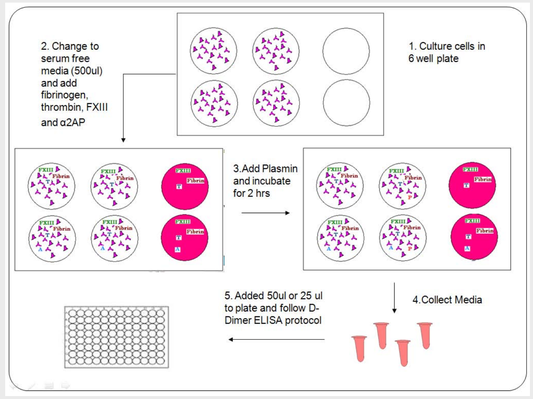
- Cells were cultured in 8 well chamber slide
- They were treated with α2-AP and incubated for 1h on ice or 5h in 37^{0}C .
- Cell were then fixed with paraformaldehyde and then permeabilized or not
- The were treated with a primary antibody for α2-AP and a fluorescent secondary antibody
- Immunofluorescent microscope was used to view slides and take pictures

METHODS cont.

D-Dimer Fibrin degradation assay

The D-Dimer assay was used to look at to the proteolytic degradation of fibrin to determine if α2-AP inhibited fibrin degradation increased in the presence of the different cells.

- Each cell type was grown in 6 well plates before experiment media was changed to serum free media and treated with fibrinogen, FXIIIa, and thrombin to produce fibrin.
- The experimental groups were treated with α2-AP and control groups were not.
- After an incubation period, the cells were either further treated with plasmin for 2 h or not.
- Media was collected and D-Dimer Assay performed

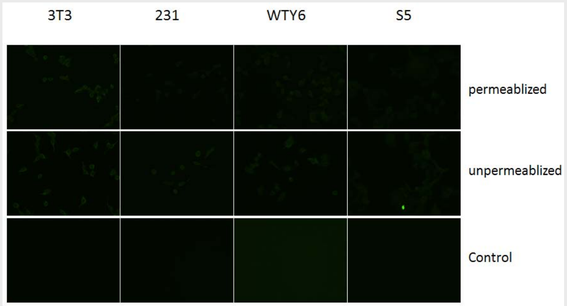


QUESTION

Can FAP-cleaved or un-cleaved α2-AP decrease fibrin degradation around breast cancer cells by inhibition of plasmin and/or other proteases?

RESULTS

A. α2-AP treatment 1h on ice



B. α2-AP treatment 5h 37^{0}C

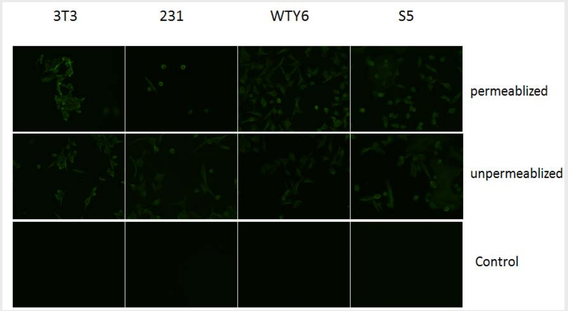
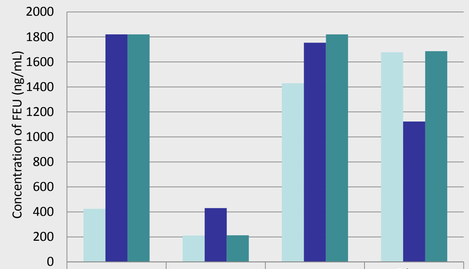
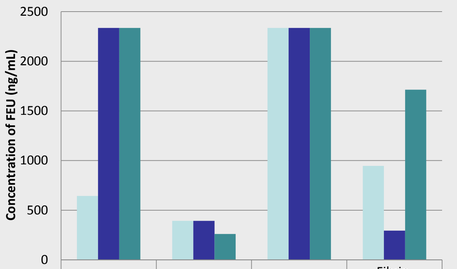


Figure 1. α2-AP attaches to the outer membrane of human breast cancer and mouse fibroblast cells. A. and B. α2-AP does interact with cancer and fibroblast cells by bind to the outer membrane of the cell. There is no visual difference in fluorescent staining of α2-AP in the permablized and unpermablized cells suggesting that α2-AP is not taken up by the cells (if so in small amounts). There is also no visual difference among the cell types. α2-AP binds to the cells whether FAP is present or not.

Concentration of Degraded Fibrin after 24h α2-AP treatment



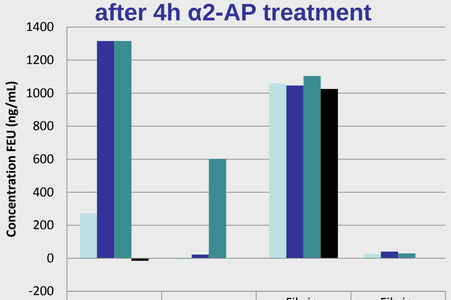
	Fibrin	Fibrin, a2AP	Fibrin, Plasmin	Fibrin, a2AP,Plasmin
Media	425.2115076	210.907952	1429.499335	1677.174938
231	1820.33	430.8356881	1753.334162	1123.360353
WTY6	1820.33	212.9716333	1820.33	1686.215093



	Fibrin	Fibrin, a2AP	Fibrin, Plasmin	Fibrin, a2AP,Plasmin
231	643.1889886	392.1680946	2335.23	945.9183955
WTY6	2335.23	392.1680946	2335.23	294.1750612
S5	2335.23	260.4850052	2335.23	1713.511226

Figure 2. After 24h treatment with α2-AP plasmin independent and dependent fibrin degradation was decreased. After 24h incubation in the absence of plasmin and in the presence of the proteolytically active FAP (WTY-6) or a catalytically inactive mutant FAP(S5) fibrin degradation greatly increased. When treated with α2-AP The degradation was inhibited. When plasmin was introduced we were able to see increased degradation in the FAP negative (231) and this degradation was decreased by α2-AP. (**A**. Concentration could not be calculated higher than 1820ng/mL so concentration may actually be higher. **B.** Concentrations could not be calculated higher than 2335ng/mL) (D-dimer are expressed in (FEU) fibrinogen equivalent units)

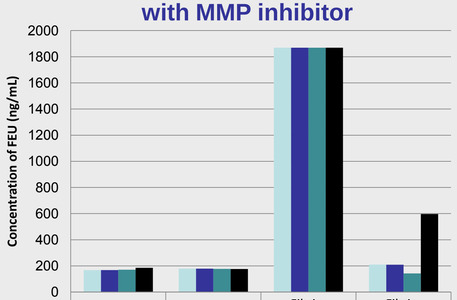
Concentration of Degraded Fibrin after 4h α2-AP treatment



	Fibrin	Fibrin, a2AP	Fibrin, Plasmin	Fibrin, a2AP,Plasmin
231	271.350777	-6.794749466	1057.936164	25.01191211
WTY6	1315.749	22.30002331	1046.477425	40.61867399
S5	1315.749	601.62	1103.815814	29.88811364
Media	-15.2746153		1025.102229	

Figure 3. After 4h treatment with α2-AP plasmin independent and dependent fibrin degradation was decreased. After 4h incubation in the absence of plasmin and in the presence WTY-6 or S5 fibrin degradation greatly increased. When treated with α2-AP The degradation was decreased. When 231 was treated with plasmin degradation increased (Concentration could not be calculated higher than 1315.749ng/mL)

Concentration of Degraded Fibrin with MMP inhibitor



	Fibrin	Fibrin, a2AP	Fibrin, Plasmin	Fibrin, a2AP,Plasmin
231	168.1519244	179.2070182	1869.11	209.9854506
WTY6	167.761166	179.4152192	1869.11	209.164519
S5	171.195548	176.9705485	1869.11	142.9004834
Media	185.8110757	176.268584	1869.11	596.312636

Figure 4. MMP inhibitors drastically decreased the plasmin independent fibrin degradation. Plasmin independent fibrin degradation greatly decrease when treated with BB94 (MMP inhibitor). Fibrin degradation still occurred in the presence of BB94 with treated with plasmin. The plasmin dependent was decreased when treated with α2-AP. (Concentrations could not be calculated higher than 1869.11ng/mL)

CONCLUSIONS

FAP may not play a significant role in the decrease of fibrin degradation in the presence or absence of plasmin, but BB94 significantly reduced the plasmin-independent degradation of fibrin suggesting that MMPs mediate the plasmin-independent proteolysis of fibrin. Surprisingly, the addition of α2-AP decreases fibrin degradation caused by MMPs secreted from the tumor cells. The high levels of fibrin degradation caused by plasmin were also decreased when treated with α2-AP. The mechanism of α2-AP-mediated inhibition of fibrinolysis by MMPs is not clear but may be related to the crosslinking of α2-AP to fibrin. MMP cleavage sites on fibrin may be masked when fibrin is crosslinked by α2-AP or the crosslinking itself may make the fibrin bundles denser and prevent access by MMPs.